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ALTERNATIVE SPLICING OF INTERLEUKIN-7

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Dedicated to my Family, Gurus, Friends and Patients...

ABSTRACT

Alternative splicing of pre-mRNA is the primary source of creating proteome diversity in humans; it is therefore highly relevant to disease and therapy. Alternative splicing has been described for many cytokines and these splice variants appear to function as either agonists or as antagonists of the wild-type cytokine. Interleukin-7 (IL-7) plays a non-redundant role in early stages of normal B and T-cell development; IL-7-receptor signaling is required for the maintenance of T-cell homeostasis and memory T-cell formation. In the present study, we investigated IL-7 splice variant distribution in different human tissues and organs obtained from healthy individuals and patients suffering from cancer or chronic infections. Recombinant expression of an Interleukin-7 splice variant showed, as a paradigm, that IL-7 splice variants are biologically active defined by i) STAT5 phosphorylation, ii) prevention of apoptosis and iii) cellular differentiation.

In paper I, we have investigated the relative distribution of Interleukin-7 receptor (IL-7R) molecules on different T-cell subsets in peripheral blood by using 'high content' flow cytometry. Only IL-7R positive cells would be able to transmit an IL-7 or IL-7 isoform mediated signal. We analyzed peripheral blood mononuclear cells (PBMCs) from patients with MS in order to identify which immune cells express the IL-7R since Multiple Sclerosis (MS) is the first disease, in which differential IL-7R expression has been postulated due to variations in the IL-7R gene, defined by SNPs. The study was performed using 12 color flow cytometry including an anti-IL-7R monoclonal antibody to objectively enumerate IL-7R molecules on the single cell level. We were not able to identify any IL-7R positive immune cell subsets which showed statistically different composition in patients with MS as compared to healthy controls, but we were able to identify 19/189 immune cell subsets which were either increased or decreased in a significant fraction in patients with MS versus healthy controls. In contrast, we identified significant differences in IL-7R density, measured on a single cell level. 2/59 variables namely $TCR\alpha\beta+CD4+CD25-CD107a+$ and $TCR\alpha\beta+CD4+CD25$ intermediate T-cells showed increased numbers of IL-7R molecules in patients with neurological disease. We proposed that the $TCR\alpha\beta+CD4+CD25-CD107a+$ T-cell subset represents a differentiated, cytotoxic CD4+ T-cell population, which is associated with chronic antigen exposure in patients with MS.

In paper II, we asked whether each IL-7R positive cell is able to transmit an IL-7 signal. Surprisingly, PBMCs obtained from patients with breast cancer showed IL-7 mediated signaling

defects defined by STAT5 phosphorylation, despite the expression of IL-7R α on the cell surface. This was associated with reduced level of IL-7R α on CD8 $\alpha\alpha^+$ and CD4 $^+$ T-cells. Cells with IL-7R signaling defects were also impaired in cytokine production after PMA/Ionomycin stimulation. These data suggested that a detailed analysis of T-cell function, determined by IL-7 mediated signaling, may improve the design of biological therapy in patients with cancer and help to escort immunological strategies to improve anti-tumor T-cell reactivity.

In paper III, we described a map of IL-7 variant expression in different human organs and tissues. We demonstrated aberrant expression of IL-7 splice variants in different tissues obtained from patients with cervical cancer lesions and *Mycobacterium tuberculosis* positive granuloma lesions obtained from individuals with latent TB. These data supported the observation that IL-7 is differently spliced in transformed cells or in inflammatory processes associated with *M. tuberculosis* infection. The functional impact of IL-7 isoforms on immune cells was demonstrated by gauging anti-apoptotic activity, bcl2 protein levels and STAT5 phosphorylation in peripheral T-cells and thymocytes, induced by an IL-7 variant lacking exon5. This suggested that alternatively splice variants of IL-7 are indeed biologically active.

In paper IV, we demonstrated differentially spliced IL-7 in distinct anatomic areas of the brain. IL-7 and IL-7 splice variant proteins were able to shift differentiation of neural progenitor cells towards the glia cell lineage. Transcriptome analysis of IL-7 stimulated neural progenitor cells showed 58 differentially regulated genes; some of these genes were also involved in neural differentiation. Thus, IL-7 may play a significant role in neural development by participating in human brain architecture through glia cell formation.

In conclusion, the work described in this thesis provides new insights into the biology of differentially spliced IL-7 in health and disease.

LIST OF PUBLICATIONS

- I. Vudattu NK, Kuhlman-Berenzon S, Khademi M, Seyfert V, Olsson T and Maeurer MJ. Increased numbers of IL-7 receptor molecules on CD4+CD25-CD107a+ T-cells in patients with Multiple Sclerosis. *Manuscript submitted*
- II. Nalini kumar Vudattu, Isabelle Magalhaes, Marcus Schmidt, Vicki Seyfert-Margolis and Markus J. Maeurer. Reduced numbers of IL-7 receptor (CD127) expressing immune cells and IL-7 signaling defects in peripheral blood from patients with breast cancer. *International Journal of Cancer* 2007 Oct1; 121(7): 1512-9.
- III. NK Vudattu, I Magalhaes, H Hoehn, D Pan and MJ Maeurer. Expression analysis and functional activity of interleukin-7 splice variants. *Genes and Immunity* (2009) 10, 132-140.
- IV. Michaela Moors*, Nalini Kumar Vudattu*, Josef Abel, Ursula Kramer, Lalit Rane, Nobert Ulfing, Sandra Seccatelli, Ellen Fritsche and Markus J Maeurer Interleukin-7 and Interleukin-7 splice variants differentiate human neural progenitor cells. *Manuscript submitted*

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AS	Alternative splicing
BAD	Bcl-2 antagonist of cell death
Bcl-2	B-cell lymphoma 2
CFTR	Cystic fibrosis transmembrane conductance regulator
CIS	Cytokine inducible <i>Src</i> -homology
CLL	Chronic lymphoblastic leukemia
CMV	Cytomegalo virus
CNS	Central nervous system
EBV	Epstein-Barr virus
ENU	Ethyl nitrosourea
EST	Expressed sequence tag
GFAP	Glial fibrillary acidic protein
GFI	Growth factor independent
GLUT	Glucose transporter
HPV	Human papilloma virus
HTLV	Human T-cell leukemia virus
JAK	Janus kinase
LAMP	Lysosomal associated membrane protein
LPS	Lipopolysaccharide
MS	Multiple sclerosis
NHNP	Normal human neural progenitor
NMD	Nonsense mediated mRNA decay
OND	Other neurological disease
PBMC	Peripheral blood mononuclear cell
PI3	Phosphotidyl inositol 3
PPMS	Primary progressive multiple sclerosis
RAG	Recombinase activating gene
RRMS	Relapsing remitting multiple sclerosis
SCID	Severe combined immunodeficiency disease
SiRNA	Small interference RNA
SMA	Spinal muscular atrophy
SMN	Survivor of motor neuron
SNP	Single nucleotide polymorphism
snRNP	Small nuclear ribonuclear protein
SOCS	Suppressor of cytokine signaling
SPMS	Secondary progressive multiple sclerosis
STAM	Signal transducing adaptor molecule
STAT	Signal transducer and activator of transcription
TLO	Tertiary lymphoid organs
TSLP	Thymic stromal lympho poietin
TSLPR	Thymic stromal lympho poietin receptor

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1 INTRODUCTION

1.1 INTERLEUKIN-7 BIOLOGY

Interleukin-7 (IL-7) is a multifunctional and non-redundant cytokine that plays a pivotal role in normal development and maintenance of the human immune system. IL-7 was originally discovered as a growth factor for precursor B-cells (Namen, Lupton et al. 1988). Further studies demonstrated that IL-7 is an essential cytokine for T-cell survival and homeostasis (Morrissey, Goodwin et al. 1989). IL-7 and IL-7R knockout mice revealed the importance of this cytokine in T-cell development. IL-7 plays a critical role in the development of both T and B cells in mice and T-cells in man (Hofmeister, Khaled et al. 1999).

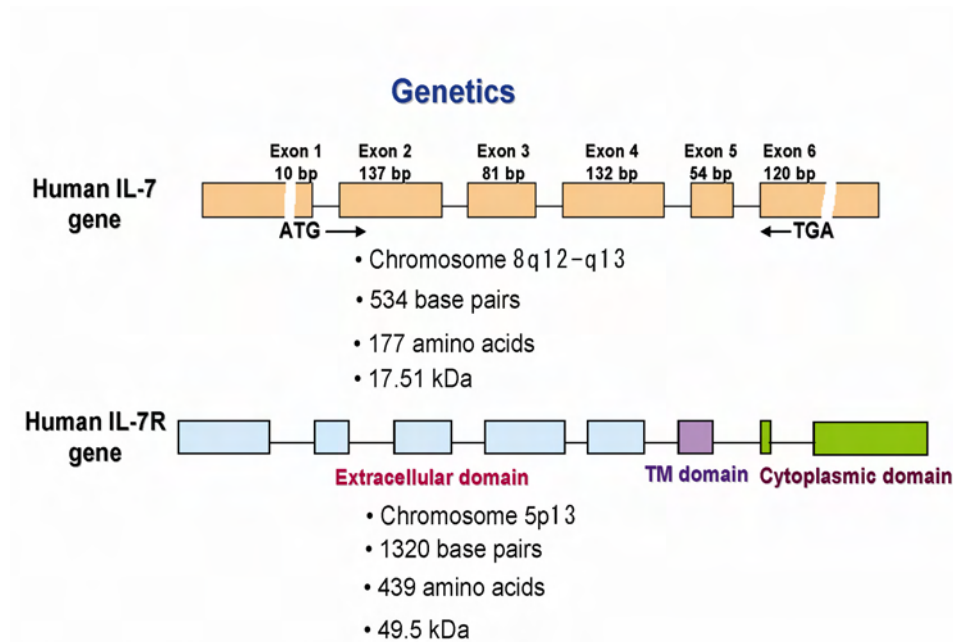
IL-7 is produced by non-lymphoid cells in lymphoid organs, primarily by fibroblastic reticular cells of the T-cell zone in lymphoid organs (Link, Vogt et al. 2007), in the thymus by thymic epithelial cells, intestinal epithelial cells (Madrigal-Estebas, McManus et al. 1997), keratinocytes (Heufler, Topar et al. 1993), hepatic tissue (Golden-Mason, Kelly et al. 2001), follicular dendritic cells endothelial cells and smooth muscle cells (Kroncke, Loppnow et al. 1996). IL-7 has been shown to bind to the extracellular matrix-associated glycosaminoglycan, heparin sulfate and fibronectin (Ariel, Hershkovich et al. 1997). The mechanism of constitutive production of IL-7 is yet to be studied in greater detail, more is known about the induction of IL-7 synthesis. Interferon- γ , IL-1 and Tumor Necrosis Factor- α (TNF- α) have been demonstrated to upregulate IL-7 mRNA (Ariizumi, Kitajima et al. 1995; Weitzmann, Cenci et al. 2000).

IL-7 shares an antagonistic relationship with Transforming Growth Factor- β (TGF- β), where TGF- β downregulates IL-7 production by stromal cells. IL-7 downregulates the production of TGF-beta from fibroblast cells (Dubinett, Huang et al. 1995). IL-7 can be also produced by tumor cells which may favor tumor development and progression (Al-Rawi, Mansel et al. 2003). Interactions between immuno-modulators and central nervous system demonstrated by trophic effects of IL-4, IL-7 and IL-8 on hippocampal neuronal cultures resulted in a marked increase in the number of astroglia and microglia cell formation (Araujo, Lapchak et al. 1993). Further studies suggested that the neurotrophic action of IL-7 may function as neural growth factor in the developing brain during Central Nervous System (CNS) ontogeny (Michaelson, Mehler et al. 1996). Functional cluster analysis, based on expression analysis of Normal Human Neural Progenitor (NHNP) neurospheres stimulated with IL-7, revealed that IL-7 modulates gene

expression of transcription factors, genes associated with protein metabolism and neural differentiation factors (Moors M et al under review).

1.1.1 Genetics and Structure

Figure1. Genetics of IL-7 and the IL-7R



The human IL-7 gene is located on chromosome 8q12-13 which spans 6 exons and has an open-reading frame of 534 base pairs, which yields a mature protein of 177 amino acids. (Figure 1) (Sutherland, Balaji et al. 1989). Homology between the human and murine IL-7 sequence is 81% in the coding regions and 60-70% in the non-coding regions. Human IL-7 show biological activity in murine cells, but mouse IL-7 is not active on human cells. The mature form of un-glycosylated human IL-7 is about 17.5kda and predicted to form a four- alpha helical structure with a hydrophobic core, which belongs to a type 1 cytokine of the hematopoietic family (Fry and Mackall 2002).

The major difference between the murine and the human protein is an insertion of 19 amino acids close to the C-terminal region in human IL-7, the N-terminal region of the protein contains a signal sequence which is conserved and shows 92% homology in the signal sequence (Jiang, Li et al. 2005). Both human and murine IL-7 promoters lack a TATA box and present a putative binding site for the E12 transcription factor which has been implicated in B and T- cell development (Bain and Murre 1998).

1.1.2 IL-7 receptor (IL-7R)

IL-7 mediates its actions via binding to the receptor nominal receptor, IL-7R. The IL-7 receptor consists of two components, the IL-7 receptor alpha chain (CD127) and a common gamma chain (γ c) that is shared by receptors for IL-2, IL-4, IL-9, IL-15 and IL-21 (Asao, Okuyama et al. 2001) and ubiquitously expressed on lymphoid cells. High affinity binding of IL-7 requires both IL-7R α and γ c (Kondo, Takeshita et al. 1994). IL-7R α is expressed on hematopoietic cells, particularly on cells of the lymphoid lineage, developing T and B cells, mature T cells and bone marrow-derived macrophages, normal human intestinal epithelial cells, cutaneous T cell lymphomas, colorectal cancer cells, renal cancer cells, human endothelial cells and human marrow stromal cells (Akashi K et al., 1997; (DiSanto, Muller et al. 1995; Hofmeister, Khaled et al. 1999; Iwata, Graf et al. 2002; Dus, Krawczenko et al. 2003; Al-Rawi, Rmali et al. 2004).

The human IL-7R α gene is located on chromosome 5p13 which contains 8 exons (Venkitaraman and Cowling 1992). IL-7R α belongs to the type I cytokine receptor family and consists of 220-amino acid extracellular domain, 25- amino acid transmembrane region and a 195- amino acid cytoplasmic tail important in recruiting intracellular signaling molecules. The mature form of IL-7R α consists of 439- amino acids with a molecular weight of 49.5 KDa.

The extracellular region of IL-7R α contains a Trp-Ser-X-Trp-Ser (WSXWS) motif involved in proper folding of the protein. IL-7R α gene mutations are associated with Severe Combined Immunodeficiency Disease (SCID) (Giliani, Mori et al. 2005). The IL-7R α is also used by another cytokine, Thymic Stromal Lymphopoietin (TSLP). The second chain of the TSLP receptor is TSLPR but not γ c (Ziegler and Liu 2006).

Human TSLPR is restricted to monocytes and dendritic cells (Reche, Soumelis et al. 2001) and to regulatory T-cells (our unpublished data), while murine TSLPR is primarily expressed in immature T and B cells, lungs, testis and kidneys. TSLP binds TSLPR with low affinity; a high affinity receptor for TSLP is generated when IL-7R α is co-transfected (Isaksen, Baumann et al. 1999; Pandey, Ozaki et al. 2000; Park, Martin et al. 2000). TSLPR deficient mice develop severely attenuated (asthma) disease when immunized and challenged with antigen (Al-Shami, Spolski et al. 2005; Zhou, Comeau et al. 2005).

1.2 ALTERNATIVE SPLICING

Alternative Splicing (AS) is a post-transcriptional modification in which a single gene encodes for multiple proteins. AS enables a single gene to increase its coding capacity, allowing the synthesis of protein isoforms that are structurally and functionally distinct, an important source of protein diversity. The human genome sequencing project estimated the number of human genes to be around 21,000-24,000, which is much less than the previous estimation based on analysis of expressed sequence tags (ESTs). This increased diversity at the mRNA level can, in part, be accounted for by alternative RNA splicing. Understanding this diversity will be critical for future drug discovery and diagnostics efforts (Venter, Adams et al. 2001). About 40-60% of the genes are known to exhibit alternative splicing in humans. Alternative splicing is often regulated in a temporal or tissue-specific fashion. This gives rise to different protein isoforms in different tissues or developmental states; differential splicing is also regulated in response to external stimuli. Even a single cell may produce multiple proteins.

Alternative Splicing is regulated by 'quality control mechanisms' like the cytoplasmic degradation of mRNAs by the nonsense mediated mRNA decay (NMD) pathway which eliminates inappropriately spliced forms (Garcia-Blanco, Baraniak et al. 2004). Aberrantly spliced pre-mRNAs can be generated and might be neglected by NMD pathway in certain pathological conditions. Alternatively spliced proteins are the prime cause of some diseases e.g. cystic fibrosis, retinitis pigmentosa, spinal muscular atrophy, neurofibromatosis type 1, haemophilia A and Beta – thalassaemia (Pajares, Ezponda et al. 2007).

1.2.1 Alternative splicing mechanisms

Three different splicing mechanisms exist: 1. Single Nucleotide Polymorphisms (SNPs) at genome level 2) Alternative Splicing at RNA level and 3) Proteolysis at the Protein level

Splicing at RNA level

- a. Constitutive splicing: pre-mRNA (pre-mRNA) is processed in a way so that only one type of mRNA is produced and only one protein is generated from a given gene.
- b. Alternative splicing: one pre-mRNA can be processed in different ways to produce various mRNAs with different exon combinations which give rise to a variety of proteins.

A gene is first transcribed into a pre-mRNA, which is a copy of the genomic DNA containing intronic regions destined to be removed during pre-mRNA processing (RNA splicing), as well as

exonic sequences that are retained within the mature mRNA. During RNA splicing, exons can either be retained in the mature message or targeted for removal in different combinations to create a diverse array of mRNAs from a single pre-mRNA, a process referred to as alternative RNA splicing (Lopez 1998).

1.2.2 Alternative Pre-mRNA splicing

Pre-mRNA splicing is an essential, precisely regulated process that occurs after gene transcription and prior to mRNA translation. Pre-mRNA splicing begins with the ordered assembly and coordinated action of the particles U1, U2, U4, U5 and U6 snRNPs (small nuclear ribonucleoprotein particles) and non-snRNP proteins on the pre-mRNA. Each snRNP particle contains a small nuclear RNA molecule (snRNA) and several proteins. The complex of snRNPs and non-snRNPs is called the spliceosome. (Burge, Padgett et al. 1998).

The process of pre-mRNA splicing can be divided into three stages:

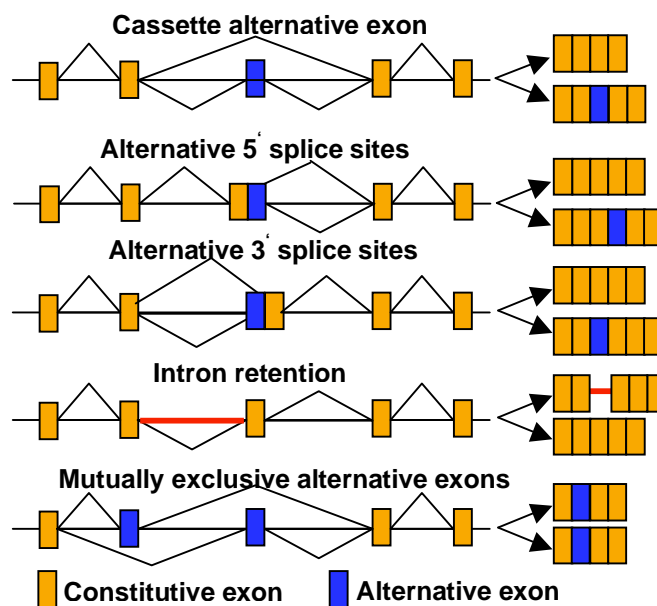
1. The consensus sequences recognized by the spliceosome are called splice sites. Initial recognition of the 5' and 3' splice sites is achieved by a correct pairing of 5' splice site with its cognate 3' splice site which contributes to the assembly of a highly stable complex called as spliceosome complex (5' splice sites, Branch point sequence followed by a polypyrimidine tract and a 3' splice site) (Hastings and Krainer 2001).
2. The interactions between spliceosome and pre-mRNA bring the reaction sites on the pre-mRNA together and create the catalytic sites for trans-esterification reactions.
3. The cleavage and ligation reaction required for intron removal and exon ligation proceeds via two trans-esterification reactions. In the first reaction the 5' exon is cleaved and the 5' end of the intron is joined to the branch point creating the intron lariat structure. The second reaction occurs when the free 3' end of the 5' exon is joined to the downstream exon resulting in exon ligation and release of the intron sequence.

Pre-mRNA splicing is a mechanism that removes intronic sequences and joins their surrounding exons. The mature mRNAs are exported from the nucleus to the cytoplasm where they can be translated into proteins.

There are 4 main types of Alternative splicing mechanisms which are described in Figure 2.

1. Exon skipping: This is the most common type of AS, it contributes for at least one-third of AS events which involve cassette – type alternative exons which are either skipped or included in the final message (exons are included or excised from the final gene transcript leading to extended or shortened mRNA variants).
2. Alternative 5 splice sites and 3 splice sites, which account for at least one-quarter of AS events. This type of AS is capable of introducing subtle changes into coding sequences which may arise as a consequence of stochastic binding of the spliceosome at neighboring splice sites. However, it is not yet clear how these subtle changes are functionally significant.
3. Intron retention can account for 3% of total AS events
4. Mutually Exclusive: This type of mechanism contribute for 30% of total AS events, either one or the other exons is included in the final mRNA and it is not possible to include both exons in the final transcript (Figure 2).

Figure 2. Different mechanisms of alternative splicing



1.2.3 Alternative splicing in diseases

Alterations in splicing may cause disease through several mechanisms.

cis-acting splicing mutations: mutations that inactivate function of *cis*-acting elements were first observed in beta-globin transcripts in Beta-thalassemic patients characterized by severe anemia which may be lethal. Another inherited disease is hemophilia B, caused by a G to T transversion at the +1 position downstream of exon f of factor IX which leads to skipping or altered splicing of

one exon in the human growth hormone gene resulting in an isolated growth hormone deficiency (Garcia-Blanco, Baraniak et al. 2004).

trans-acting mutations: mutations in genes that are involved in snRNP assembly and function. Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder affecting motor neurons which is caused by loss of the survival of motor neuron-1 (SMN1) gene product, required for the assembly of core snRNPs in the cytoplasm before final maturation and nuclear import (Garcia-Blanco, Baraniak et al. 2004). Retinitis Pigmentosa is one of the most common forms of blindness results from mutations in more than 30 genes. This disease occurs probably due to disruption of spliceosome function (Wang and Cooper 2007).

Alterations of splicing factors: cancer associated with chromosomal translocations resulting in the formation of fusion proteins, e.g. splicing factor, proline and glutamine rich (SFPQ) in renal carcinoma and FUS interacting protein 1(FUSP1) in leukemia's and sarcomas. It is difficult to predict the biological role of cancer-associated alternative splicing events discovered by genomic analysis. Functional studies are needed to understand relationships between alternative splicing of individual genes and the initiation and progression of certain types of tumors (Xing 2007).

1.2.4 Splicing-targeted treatment

The evaluation of splicing defects in human diseases suggests that splicing reactions can be potential therapeutic targets for treatment interventions.

There are 3 different approaches that are explored *in vitro* and *in vivo* to target aberrant splicing:

1. Antisense RNA
2. Trans-splicing
3. Targeting protein isoforms

1.2.4.1 Antisense RNA

Treatments based on oligonucleotides have been used to inhibit or to activate specific splicing events either by binding to an element and sterically blocking its activity or by binding an element and recruiting effectors to this site.

Human beta-thalassemias are caused by mutations that appear within the human beta-globin intron 2 which leads to the formation of alternative nonfunctional transcripts by activating cryptic splice sites. The cryptic splice sites were blocked by *in vitro* and *in vivo* by using antisense

oligonucleotides. Similar approaches were used by taking different formulations of antisense oligonucleotide- like molecules to correct aberrantly spliced Cystic fibrosis transmembrane conductance regulator (CFTR) transcripts, B-cell leukemia x (*Bcl-x*) transcripts and IL-5Ralpha transcripts (Garcia-Blanco, Baraniak et al. 2004).

Small interference RNA (siRNA) - This is a variation of the oligonucleotide antisense therapy which involves the use of exon-specific RNA interference (RNAi). This method can effectively knock down transcript levels that alter the ratios of alternative splicing isoforms.

1.2.4.2 Trans-splicing

A cellular process where two molecules of mRNA can exchange genetic information through recombination. Recombination of exogenous RNA sequences with the target mRNA is used to correct splicing aberrations. The advantage of this method is that small fragments of therapeutic DNA are needed.

1.2.4.3 Targeting protein isoforms

Aberrantly expressed isoforms contain unique epitopes which can be targeted by raising antibodies against these unique epitopes. For instance, Tenascin-C is an adhesion molecule involved in tumor growth. Generation of antibodies that specifically target a splice variant of Tenascin-C is currently being tested in animals. In-vivo administration of the anti-angiogenic Vascular endothelial growth factor (VEGF) isoform causes tumor shrinkage of prostate-cancer cells in xeno-transplanted to nude mice (Pajares, Ezponda et al. 2007).

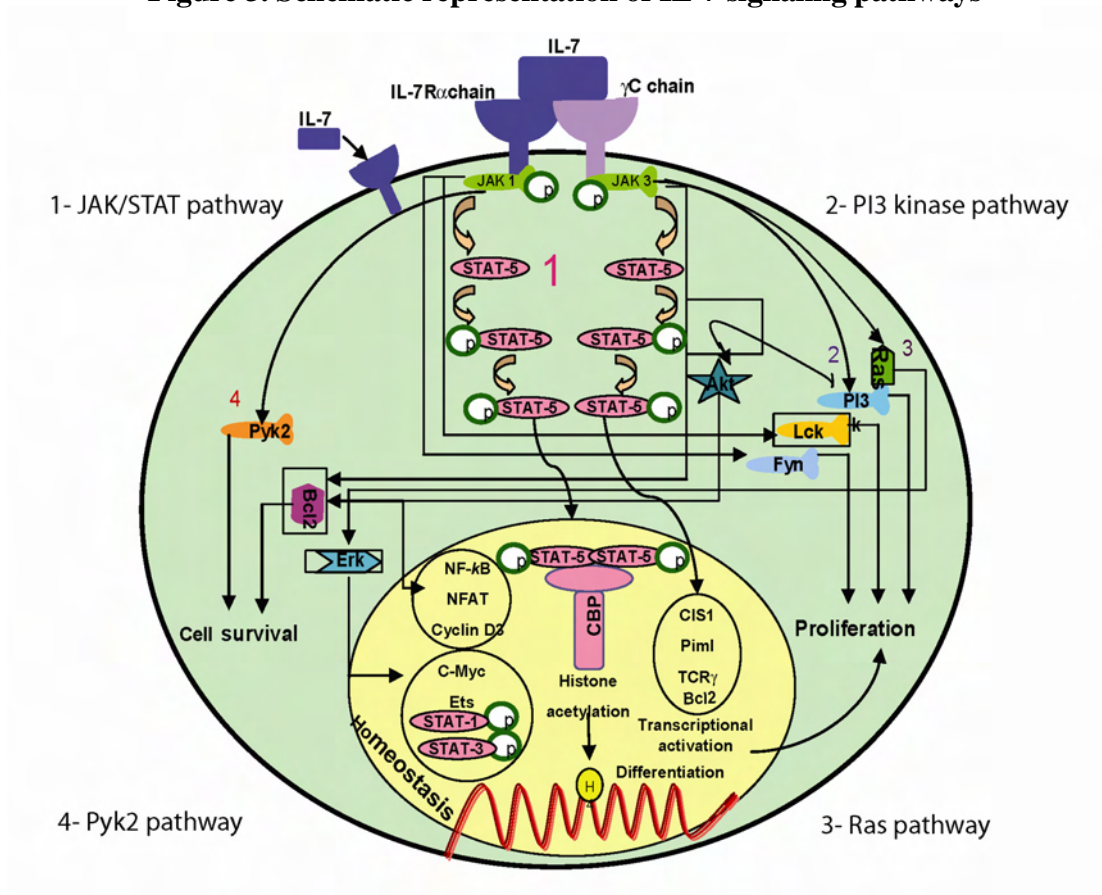
1.3 IL-7/IL-7R SIGNALING PATHWAY

IL-7 binds to the IL-7R α chain, leading to hetero-dimerization with the gamma chain (γ c) (Olosz and Malek 2000). IL-7 signaling involves interaction of intracellular signaling molecules, i.e. Janus kinase- 3 (Jak3) associated with γ c and Jak1, which are attached to the IL-7R α chain. The gamma chain plays an important role in mediating IL-7 effects, demonstrated by the fact that the IL-7R α chain lacks intrinsic tyrosine kinase activity and the need for Jak3 to trigger trans phosphorylation of IL-7R α associated Jak1 proteins. Jak1 proteins phosphorylate the tyrosine residue (Y449) present in the cytoplasmic portion of IL-7R α (Fry and Mackall 2002).

The receptor phosphotyrosines create docking sites for SH2 domain proteins including the Signal Transducer and Activators of Transcription (STAT) family of transcription factors mainly STAT5A/B (Foxwell, Beadling et al. 1995; Lin, Migone et al. 1995), to a lesser extent STAT3 (Pernis, Gupta et al. 1995) and STAT1 (van der Plas, Smiers et al. 1996). The phosphorylated

STAT molecules rapidly dissociate from the receptor and rapidly translocate to the nucleus where they are capable of activating target genes (Figure 3) (Al-Rawi, Mansel et al. 2003).

Figure 3. Schematic representation of IL-7 signaling pathways



1.3.1 Jak-STAT pathway

1.3.1.1 Jak3

The protein tyrosine kinase JAK3 is constitutively associated with the carboxy-terminal region of γ_c . Jak3 deficient mice exhibit a defect in lymphoid development and in humans, mutations in Jak3 gene results in a disease that is indistinguishable from XSCID (Macchi, Villa et al. 1995). Jak3 is highly expressed in T and B- cells (Kawamura, McVicar et al. 1994; Tortolani, Lal et al. 1995; Gurniak and Berg 1996) and at a lower level in monocytes, endothelial

cells and keratinocytes (Musso, Johnston et al. 1995; Verbsky, Bach et al. 1996). Jak3 mRNA expression has been found in lung cancer lines (Cosenza, Gorgun et al. 2002) and higher levels were identified in human breast cancer (Al-Rawi, Rmali et al. 2004). Jak3 activity is required to protect cells from apoptotic cell death (Eynon, Livak et al. 1999). There are other types of proteins that were reported to interact with Jak3 and mediate IL-7 signaling including the Signal Transducing Adaptor Molecule (STAM), Pyk2 and others (Hofmeister, Khaled et al. 1999). STAM is an adaptor molecule which contains a tyrosine moiety and is phosphorylated after stimulation with IL-7 (Takeshita, Arita et al. 1996).

1.3.1.2 *Jak1*

Jak1 is associated with the IL-7R α chain and phosphorylated through Jak3 upon binding of IL-7 to IL-7R α . Jak^{-/-} mice precedes the IL-7 response and very little is known about the signaling pathways dependent on Jak1. Pyk2, a member of focal adhesion kinase family is associated with Jak1 and has been shown to play a role in survival of a thymocyte cell line (Benbernou, Muegge et al. 2000). The downstream pathways for Pyk2 pathway are not yet clear. However, Jak1 activity is required for IL-7 mediated inhibition of TGF- β production by fibroblasts (Huang, Sharma et al. 2002). It was observed that Jak1 interacts with the p85 subunit of PI-3 kinase and cooperates with the IL-2 receptor β chain for recruitment and tyrosine phosphorylation of p85 in co-transfection experiments (Migone, Rodig et al. 1998).

IL-7 signaling can be interrupted through regulation of Jak1 activation (Jiang, Li et al. 2005). The inhibitor molecules, SOCS-1 and CIS-1 (cytokine inducible Src homology-2 protein) have been identified first (Yoshimura, Ohkubo et al. 1995), later other members of inhibitors (CIS2-CIS7 / SOCS2-SOCS7) have been identified (Hilton, Richardson et al. 1998). All Interleukin-7 signaling inhibitors contain a central SH2 domain, in which SOCS1 has been shown to bind and inhibit all Jak family members including Jak1. (Jiang, Li et al. 2005). CD45, a tyrosine phosphatase, plays an important role in inhibiting the activity of Src kinases and also dephosphorylates Jaks (Irie-Sasaki, Sasaki et al. 2001; Yamada, Zhu et al. 2002).

1.3.1.3 *STATs*

STATs, latent cytosolic transcription factors, bind to the phosphorylated cytokine receptors via their SH2 domains (Greenlund, Morales et al. 1995). There are 7 different types of STATs namely STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 (Hoey and Grusby

1999). The SH2 domain of STAT5 docks to Y449 on IL-7R α , after being itself phosphorylated on a specific tyrosine residue. It also mediates dimerization and interacts with other SH2 domains. The SH2 domain is followed by the linker, the DNA binding site, coiled coil and the amino terminal domains which are involved in transcriptional activity of STATs (Jiang, Li et al. 2005). IL-7 can also activate STAT1 and STAT3, but deficiencies in STAT1, 2 or 3 do not show defects in thymocyte development. There are two STAT5 isoforms, STAT5A and STAT5B that form homo- or heterodimers upon phosphorylation. Furthermore, tetramerization of STATs has been shown to occur in the case of STAT3, STAT4 and STAT5 mediated by N-terminal domains of STAT proteins. The STAT dimers and tetramers could have differences in their DNA binding specificities (Soldaini, John et al. 2000). STAT5A and STAT5B share about 96% identity at protein level (Liu, Robinson et al. 1995). IL-7 induces phosphorylation of both isoforms in T-cells (Rosenthal, Winestock et al. 1997; Yu, Young et al. 1998) and in B-lymphoblastic leukemia cells, also in pre-B cell lines (van der Plas, Smiers et al. 1996; Levin, Koelling et al. 1999). Different IL-7 concentrations have been shown to promote survival versus proliferation (0.1 and 1ng/ml), but the effect is not correlated with STAT5 phosphorylation (Moriggl, Sexl et al. 2005). STAT5A anti-apoptotic activity is well studied as it regulates the expression of several Bcl-2 family members and caspases (Debierre-Grockieo 2004).

Mutations in tyrosine Y449 residue of the IL-7R α chain abolished the activity of STAT5, but not STAT1 and STAT3 (Kim, Khaled et al. 2003) by inhibiting the induction of bcl-2 mRNA and induced translocation of Bax to mitochondria (Jiang, Li et al. 2005). STAT5A knockout mice develop impaired mammary gland development (Liu, Robinson et al. 1995). STAT5B dominant negative mice increased the double negative (DN) thymocytes and decreased the number of double positive (DP) thymocytes and $\gamma\delta$ thymocytes (Pallard, Stegmann et al. 1999). Mice deficient for both STAT5A and STAT5B developed a SCID- like phenotype (O'Shea, Husa et al. 2004; Yao, Cui et al. 2006). The lymphopenic conditions resulting from loss of these signaling molecules could be due to defects in T- cell proliferation which suggest that there may be additional routes for signaling pathways mediated by IL-7.

IL-7 signaling is required for $\gamma\delta$ T- cell development as it induces V(D)J recombination at the TCR γ locus by inducing histone acetylation which regulates chromatin accessibility by recombinase (RAG) mediated cleavage (Schlissel, Durum et al. 2000). STAT5 can also enhance DNA binding and transactivation of target genes by the transcription factor NF κ B (Nakamura, Ouchida et al. 2002).

1.3.2 PI3 kinase pathway

PI3 is one of the downstream pathways of IL-7 that regulates cell survival and proliferation of various cell types (Datta, Brunet et al. 1999). PI3K consists of the regulatory p85 subunit and a catalytic subunit which phosphorylate the 3-ring position of PI - 4,5 biphosphate to generate PIP3 (Toker and Cantley 1997). Conversion of PIP2 to PIP3 recruits AKT (Serine/Threonine kinase) to the cell membrane where it becomes activated. Mutations in PI3K/AKT pathway and the tumor suppressor PTEN, an inhibitor of PI3K/AKT signaling, are frequent causes of cancer in humans (Di Cristofano and Pandolfi 2000; Vivanco and Sawyers 2002). AKT targets are involved in initiating cell cycle progression which could be mediated by IL-7 induced proliferation. In human T-cells, Jak3 was found to be associated with p85. IL-7 induced phosphorylation of p85, which activates PI3K was essential for IL-7 mediated survival and proliferation of human T- cell precursors (Pallard, Stegmann et al. 1999).

Most of these studies were performed in transformed cell lines, like T-ALL or thymocytes which may not represent T-cells in the peripheral circulation. Recently, one study used untransformed mature T-cells and found that IL-7 did not activate the PI3K pathway, even though these cells proliferated in response to IL-7 (Lali, Crawley et al. 2004) which was also supported by another study suggesting that IL-7 did not induce phosphorylation of AKT in primary murine T-cells (Osborne, Dhanji et al. 2007).

Hence, the PI3/AKT pathway may not be directly required for T-cell proliferation. Kinetics of PI3K signaling studies suggested that IL-2 was able to induce two waves of PI3K activity: one rapid wave occurring within minutes and a second later wave occurs within hours favoring T- cell growth. IL-7 was also found to induce the second later wave of PI3K activity that may be responsible for cell cycling (Lali, Crawley et al. 2004), which is in contrast to the more rapid induction of STAT5 activity. Thus, the PI3K pathway may play a lesser role in the maintenance of peripheral T-cells by IL-7.

1.3.3 Src kinase pathway

Src family kinases (SFK) are membrane –associated non-receptor protein tyrosine kinases which include nine members (Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, Yrk). Stimulation of pre-B and myeloid cell lines with IL-7 leads to the activation of Src family kinases p59fyn and p53lyn

(Venkitaraman and Cowling 1992; Seckinger and Fougereau 1994). In human T-cells, both p59 fyn and p56lck were shown to associated with IL-7R α (Page, Lali et al. 1995).

The kinase activity of SFKs can be increased or decreased by tyrosine phosphorylation of specific residues which are regulated by other kinases and phosphatases. Lck and Fyn are the two abundantly expressed SFKs in T-cells (Mustelin 1994; Wange and Samelson 1996). They are associated with cellular membrane lipid rafts where they provide critical function in TCR-mediated signaling such as phosphorylation of TCR-CD3 subunits. Lck, via its unique domain, binds to CD4 and CD8 co-receptor molecules, it can also interact with a variety of surface receptor molecules including IL-7R α (Isakov and Biesinger 2000).

Lck^{-/-} mice show impaired development of CD4/CD8 double positive thymocytes (Molina, Kishihara et al. 1992; Lai, Molden et al. 1997). The requirement for Lck is necessary at the stage of pre-TCR signaling, but not at the IL-7 dependent stages of Pro-T1-T3 (Jiang, Li et al. 2005).

Fyn is expressed as two isoforms, Fyn T is expressed in T-cells and Fyn B is in B- cells differing in 50 amino acids in their composition. Fyn T- deficient mice showed signaling defects in thymocytes, but normal numbers of periphery T-cells were observed, suggesting Fyn is not uniquely required for IL-7R signaling in T-cells (Lowell and Soriano 1996). In B- cells Src kinases, Fyn and Lyn are associated with the B-cell receptor (BCR) and are involved in BCR-mediated signaling. Fyn deficient mice showed a moderate impairment of the response to BCR and CD38 ligation. Fyn/Lyn double deficient mice indicated that Fyn and Lyn activities are synergistic (Yasue, Nishizumi et al. 1997). Recently, proliferation of a Pre-B cell line in response to IL-7 has been found to be impaired by a Src family kinase inhibitor (Isaksen, Baumann et al. 2002).

1.3.4 IL-7 and Cell cycle

IL-7 dose responses will determine the survival function of this cytokine from its proliferative activity. At lower concentrations of IL-7 (<1ng/ml) sustains survival, while high doses of IL-7 (>1ng/ml) promote survival and cell cycling (Swainson, Kinet et al. 2005; Kittipatarin, Li et al. 2006). IL-4, IL-7 and IL-15 have the capacity to enhance proliferation of naïve T-cells in vitro, but only IL-7 was found to be essential for homeostatic proliferation of T-cells *in vivo* (Schluns, Kieper et al. 2000; Tan, Dudl et al. 2001). The complex nature of the IL-7 role in cell cycle has not been clearly described. IL-7 deficient triple negative (TN) thymocytes showed more cells in

G0/G1 and proportionally fewer cells in the S and G2/M phases of the cell cycle (von Freeden-Jeffry, Solvason et al. 1997) suggesting that IL-7 might regulate the transition from G1 to the S phase.

Cyclin-dependent kinase 2 (cdk2) is required for G1 to S phase transition. One possible mechanism by which IL-7 could promote cell proliferation is through activation of substrates by the PI3k/AKT pathway. Furthermore, cyclin E and phosphorylated retinoblastoma (Rb) proteins were elevated after stimulation with IL-7 (Geiselhart, Humphries et al. 2001). IL-7 induced cell cycle might be controlled via two regulatory factors: the cdk inhibitor p27 kip1 and the cdk activating phosphatase, Cdc25A. p27Kip1 levels are highest in quiescent cells and must be down-regulated to allow cells to enter cell cycling (Kaldis 2007). In IL-2 dependent T-cell lines, the PI3K/AKT pathway has been shown to phosphorylate a transcription factor Foxo3, which induces p27Kip1 expression (Lali, Crawley et al. 2004). Foxo1 was required to maintain homeostasis of naïve T-cells through the regulation of several genes involved in T-cell trafficking (CCR7 and L-selectin) and survival (IL-7R α) (Kerdiles, Beisner et al. 2009).

1.3.5 IL-7 in metabolism

IL-7 maintains metabolic activity through the uptake of glucose from the extracellular milieu. In IL-7 dependent cell lines, deprivation of IL-7 resulted that cells rapidly stop glucose transport (Khaled and Durum 2003). The main glucose transporter in peripheral T-cells is Glut1, which is also the receptor for the human T-cell leukemia virus (HTLV) (Manel, Kim et al. 2003). Glut1 expression can be up-regulated either by cytokine signaling or through TCR engagement.

IL-7 induced proliferation in recent thymic emigrants is correlated with the up-regulation of Glut1 expression on the cell surface and glucose uptake. IL-7 can also induce expression of other nutrient transporters, e.g. the transferrin receptor (CD71); high levels of CD71 were correlated with elevated levels of Glut1 expression (Swainson, Kinet et al. 2005; Swainson, Kinet et al. 2007). Thus, both Glut1 and CD71 are associated with high metabolic activity which is a characteristic feature of actively cycling cells. Maintenance of glucose uptake and metabolism are basic requirements for active growing cells as well as in the prevention of apoptosis.

1.4 PLEIOTROPIC ACTIONS OF IL-7

Binding of IL-7 to the IL-7R α leads to a cascade of events. A central action is the survival of lymphoid cells by increasing expression of the survival proteins B-cell lymphoma 2 (Bcl-2), Myeloid cell leukemia sequence 1 (MCL1), by redistributing the cell- death proteins Bcl-2 associated X protein (BAX) and the Bcl-2 antagonist of cell death (BAD) (Khaled and Durum 2002). The importance of IL-7 in early thymic development was first shown by injecting mice with an anti-IL-7 specific antibody (Grabstein, Waldschmidt et al. 1993; Sudo, Nishikawa et al. 1993; Bhatia, Tygrett et al. 1995). The non-redundant role of IL-7 in murine B and T cell development is demonstrated by severe lymphopenia in IL-7 and IL-7R α deficient mice. Mutations in the IL-7R α chain of T-cells in patients with SCID showed complete absence of T-cells with normal B and NK cells. This demonstrates that IL-7 is essential for human T-cell lymphopoiesis, yet it is not absolutely required for B- cell development in humans (Puel, Ziegler et al. 1998). Under normal circumstances, IL-7 signaling is required for the efficient generation of the human B cell repertoire (Monroe and Allman 2004).

IL-7R α is required for the development of $\gamma\delta$ - T cells showed by IL-7R $^{-/-}$ mice knockout studies, but not for natural killer (NK) cells (He and Malek 1996; Maki, Sunaga et al. 1996). IL-7 has a non-redundant role in $\gamma\delta$ T-cells development in the thymus as well as in the intestine (Calabrese, Luczak et al. 2009). Recently, chronic exposure of dendritic cells (DCs) to IL-7 in lymphopenic mice revealed that fact that homeostatic proliferation of CD4 $^{+}$ naïve T- cells is, in part, controlled via the IL-7 mediated down-regulation of MHC class II molecules on DCs (Guimond, Veenstra et al. 2009). Spleens of IL-7R $^{-/-}$ mice were shown to have fewer plasmacytoid Dendritic Cells (pDCs) and conventional Dendritic Cells (cDCs): this underlines the importance of IL-7R in dendritic cell survival and development. (Vogt, Link et al. 2009). IL-7R α is an excellent marker of T reg cells in human peripheral blood cells. IL-7R (CD127) is expressed at lower levels on T reg cells as compared to other CD4 $^{+}$ T-cells (Seddiki, Santner-Nanan et al. 2006).

1.4.1 Interleukin-7 in health

Interleukin-7 has the capacity to co-stimulate for TCR activation in mature T-cells; IL-7 acts differently in lymphoreplete and lymphopenic hosts (Fry and Mackall 2005). TCR triggering in peripheral T-cells downregulates IL-7R α expression (Schluns, Kieper et al. 2000; Franchimont, Galon et al. 2002). Recent thymic emigrants obtained from human umbilical cord blood express

high levels of IL-7R α , their survival and proliferation are promoted by IL-7 (Hassan and Reen 2001).

1.4.1.1 Homeostasis

The homeostasis of the naive (precursor) T- cell pool in the peripheral circulation is maintained by signals through the IL-7R α . In resting T-cells, IL-7 suppresses transcription of the IL-7R α gene in both CD4⁺ and CD8⁺ T-cells. The suppression of IL-7R α in CD8⁺ T-cells is mediated by the transcriptional repressor growth factor independent 1 (GFI1), but not in CD4⁺ T-cells. The repression of IL-7R α is induced by other cytokines such as IL-2, IL-4 and IL-15, a situation which suggests a cross talk between pro-survival cytokines. IL-7 maintains T-cell homeostasis in the absence of antigen stimulation. When T-cells bind IL-7, they receive survival signals and down-regulate IL-7R α expression: as a result, the remaining IL-7 would bind only to T-cells with higher levels of IL-7R α expression (Park, Yu et al. 2004).

1.4.1.2 CD8⁺ memory T-cells

IL-7R α expression on CD8⁺ effector T-cells during an antiviral immune response is apparently reduced. If effector T-cells retain IL-7R α , they could compete with naïve and memory T-cells for the limiting supply of IL-7 and impair immune responses (Mazzucchelli and Durum 2007). However, virus specific T-cells retain high IL-7R-levels (Kaech, Tan et al. 2003); (Huster, Busch et al. 2004). The retention of IL-7R α on virus-specific T-cells may reflect the situation that they had been stimulated with ‘intermediate level’ of antigen. This data suggests that stronger stimulation leads to differentiation of T-cells to become effector cells, which includes down-regulation of IL-7R α expression, whereas weaker stimulation induces the T-cells to acquire a memory phenotype (Mazzucchelli and Durum 2007).

IL-7R α density was enumerated on different T- cell subsets during their maturation stages based on the expression of CD45RA and CCR7. It appears that IL-7R α is expressed at higher levels in central (CD45RA-CCR7⁺) and effector (CD45RA-CCR7⁻) memory T-cells as compared to precursor (CD45RA⁺CCR7⁺) or terminally differentiated (CD45RA⁺CCR7⁻) T-cells (Vudattu et al under review), consistent with previous observations (Huster, Busch et al. 2004). IL-7 has been found to be important for CD8⁺ memory T- cell generation and persistence (Schluns, Kieper et al. 2000; Kieper, Tan et al. 2002), it cannot be replaced by other cytokines like IL-15 (Lenz, Kurz et

al. 2004). Some chronic infections such as human immunodeficiency virus (HIV), cytomegalovirus (CMV) and Epstein-Barr viruses (EBV) are associated with CD8⁺ T- cell exhaustion. These ‘exhausted’ CD8⁺ T-cells have been shown to express reduced levels of IL-7R α , resulting in poor T-cell viability and function (Day, Kaufmann et al. 2006).

The decrease of IL-7R expression could be due to continuous TCR signaling, which occurs through persistence of viral antigens or continuous stimulation by cytokines. Virally encoded IL-7R α expressing myeloid cells in Rag^{-/-} mice showed lower recovery of peripheral T and B-cells and increased number of neutrophils, which might be due to myeloid cells consuming the limited amount of IL-7 available in the peripheral circulation: IL-7 mediated actions may therefore not only be restricted to lymphoid cells (Jiang, Li et al. 2005).

1.4.1.3 CD4⁺ memory T-cells

Initial reports suggested that CD4⁺ memory T-cells, expressing IL-7R α , are IL-7 independent (Tan, Ernst et al. 2002). However, recent studies concluded that CD4⁺ memory T-cells were shown to proliferate independently of IL-7, but did not survive without IL-7 (Seddon, Tomlinson et al. 2003). CD4⁺ memory T-cells, analyzed several months after viral infection, require IL-7 for survival and homeostatic proliferation (Lenz, Kurz et al. 2004).

1.4.2 Interleukin-7 in disease

1.4.2.1 *Haematopoietic malignancies and Solid Tumors*

IL-7 induces trophic and anti-apoptotic response in haematopoietic tissues, IL-7 is also capable of inducing the growth, development and differentiation of certain types of leukemias, lymphomas and solid tumors. IL-7 could stimulate the growth of B and T acute lymphoblastic leukaemia (ALL) cells *in vitro* (Touw, Pouwels et al. 1990; Eder, Ottmann et al. 1992) and chronic lymphoblastic leukaemia (CLL) (Frishman, Long et al. 1993). IL-7 is also able to stimulate proliferation of cutaneous T cell lymphoma cells (Dalloul, Laroche et al. 1992). IL-7 transgenic mice developed a lymphoproliferation that lead to malignancy (Rich, Campos-Torres et al. 1993).

IL-7 mRNA expression was observed in colorectal (Maeurer, Walter et al. 1997), renal (Trinder, Seitzer et al. 1999), head and neck squamous cell carcinoma (Paleri, Pulimood et al. 2001). IL-7 can stimulate the proliferation of epithelial cells in benign prostatic hyperplasia by increasing growth patterns of the fibromuscular tissues (Kramer, Steiner et al. 2001). IL-7 increases tumor-infiltrating lymphocytes in colorectal cancers, which might carry some prognostic implications (Maeurer, Walter et al. 1997). Elevated levels of IL-7R expression shown in breast cancer tissues and levels of IL-7R expression are associated with tumor size and nodal involvement in patients with breast cancer. IL-7 can also act as a lymphangiogenic factor in human endothelial cells (Al-Rawi, Watkins et al. 2005).

1.4.2.2 *Chronic Infections and autoimmune disease*

Inflammatory Bowel Disease (IBD) results from improper activation of mucosal immune responses. Intestinal epithelial cells produce Interleukin-7, which serves as regulatory factor for IL-7R positive mucosal lymphocytes. Transgenic mice expressing IL-7, developed acute and chronic colitis in the colonic mucosa, characterized by erosions and infiltration with neutrophils in the rectum (Watanabe, Ueno et al. 1998). IL-7 is strongly expressed on both the mRNA and protein level in patients with Spondylarthritis (Rihl, Kellner et al. 2008). In patients with Rheumatoid Arthritis (RA), IL-7 levels are elevated in synovial fluid (Hartgring, Bijlsma et al. 2006). Gene expression analysis of synovial fluid reveals increased levels of IL-7, IL-7R and IL-7R signaling molecules, associated with the presence of Tertiary Lymphoid Organs (TLO)

(Meier, Bornmann et al. 2007; Timmer, Baltus et al. 2007). Mice transgenic for IL-7, show progressive osteolysis and bone remodelling (Finke and Schmutz 2008).

IL-7 is able to induce the secretion of inflammatory cytokines namely IL-1 α , IL-1 β , IL-6, IL-8, Macrophage Inflammatory Protein (MIP)-1 β and TNF- α . (Alderson, Tough et al. 1991; Ziegler, Tough et al. 1991; Standiford, Strieter et al. 1992). A role of IL-7/IL-7R has also been proposed for other autoimmune diseases such as colitis (Watanabe, Ueno et al. 1998), multiple sclerosis (Lundmark, Duvefelt et al. 2007) diabetes (Calzascia, Pellegrini et al. 2008) and psoriasis (Bonifati, Trento et al. 1997). Elevated levels of IL-7 correlated with reduced levels of IL-7R expression and CD4⁺ T- cell depletion in patients with HIV (Rethi, Fluor et al. 2005).

IL-7 can induce Fas mediated apoptosis in both naive and memory T-cells, which involves translocation of Fas molecules from the intracellular compartment to the cell membrane suggesting the importance of IL-7 role in Fas-mediated regulation of T- cell homeostasis (Fluor, De Milito et al. 2007). Higher levels of IL-7 may act differently on non-activated and sub-optimally activated T-cells in lymphopenic hosts. Elevated levels of IL-7 induce Fas-mediated apoptosis in non-activated T-cells and in sub-optimally activated T-cells, IL-7 may lead to T-cell proliferation by increasing Fas-induced co-stimulatory signals in patients with HIV (Rethi, Vivar et al. 2008).

1.4.3 Interleukin-7 in Immunotherapy

Recombinant IL-7 therapy in humans may enhance cellular immune responses to weak antigens, increase the precursor T- cell pool and lower the proliferation of T reg cells particularly in individuals with limited number of precursor T-cells: a situation which is typical for older people or for lymphopenic hosts (Rosenberg, Sportes et al. 2006). Another study, which examined the therapeutic value of Interleukin-7 by injecting IL-7 into patients with refractory cancer, showed an increased TCR repertoire diversity of precursor T-cells (Sportes, Hakim et al. 2008). IL-7 in combination with highly active antiretro viral treatment (HAART) therapy in HIV patients can restore the precursor T- cell pool by enhancing thymopoiesis and peripheral proliferation (Beq, Nugeyre et al. 2006). Non-human primates treated with IL-7 showed better responses to vaccination against influenza and enhanced numbers of CD8 central memory T-cells (Aspinall, Pido-Lopez et al. 2007).

Mammary adenocarcinoma tumor cells co-transfected with IL-7 resulted in high level of protective immunity (Cayeux, Beck et al. 1995). IL-7 transduced tumor cells administered into tumor lesions along with dendritic cells created potent anti-tumor responses to lung cancer (Sharma, Miller et al. 1997). CD8⁺T-cells may play an important role in IL-7 induced tumor rejection in murine models (Jicha, Mule et al. 1991). Administration of IL-7 after bone marrow transplantation in T-cell depleted hosts enhances T- cell repopulation by increasing antigen driven T-cell expansion. (Melchionda, Fry et al. 2005). Lymphopenia drives polyclonal proliferation of CD4⁺ and CD8⁺ T-cells associated with increased IL-7 levels in murine models (Bosco, Agenes et al. 2005). A recombinant fusion protein, DAB389-IL-7, composed of the catalytic and transmembrane domains of diphtheria toxin fused to IL-7 was created. Injection of DAB389 IL-7 into mice demonstrated that the fusion protein is selectively cytotoxic for cells positive for the IL-7R; a similar strategy may be useful as therapeutic approach against IL-7R-positive cancers or other disorders where IL-7R-positive cells mediate pathology (Sweeney, Foss et al. 1998);(Appasamy 1999).

Immunotherapies in oncology may be limited since patients often suffer from varying degrees of immunodepression. Current immunotherapies may not stimulate expansion of sufficient numbers of antigen specific T-cells to generate effective anti-tumor responses or T-cells are quickly anergized by TGF- β produced by tumors. IL-7 may play a critical role in overcoming the limitations of current immunotherapies, e.g. by counteracting TGF- β mediated effects.

1.5 BIOLOGY OF IL-7 AND IL-7R ISOFORMS

1.5.1 IL-7 isoforms

Alternative splice variants have been described for many cytokines which are either functional antagonists or functional agonists of the corresponding wild- type cytokine. Splice variants can be expressed as either soluble or membrane bound proteins and/or tissue specific variants. Alternatively spliced cytokines might function differently, associated with their localization, their function may also be altered at different degrees (Atamas 1997). Human IL-7 was first discovered by Goodwin et al in 1989, produced from a hepatocellular carcinoma cell line (SK-HEP-1). In addition to the 'canonical', 6-exon spanning IL-7 form, Goodwin was also able to characterize 3 different alternatively spliced variants which lacked either one or several exons. The significance of alternative transcripts at that time was unknown. Further studies revealed that alternative splicing exists for many cytokines, including IL-2, IL-4, IL-6, IL-12, IL-15 or IL-23.

3D models were used to study the interaction of different IL-7 isoforms with their specific receptor in comparison with the 'wild type' human IL-7. These 3D studies suggested that IL-7 isoforms bind with different affinities to the IL-7R and one of the isoform which lacks 19 aminoacids in the loop between helices C and D, seems to be an IL-7 agonist. (Kroemer, Kroncke et al. 1998). The secondary structure of human IL-7 was studied by using circular dichroism (CD) (35% alpha-helical, 31% random coil, 23% beta sheet and 11% beta turn), which supports that hIL-7 shows primarily an alpha-helical structure; three-dimensional studies predicted that human IL-7 consists of four alpha helices separated by connecting loops. Site-directed mutagenesis was also carried out in IL-7 by replacing 143 tryptophane (Trp) with alanine (Ala) in helix D. The biological activity of the mutant proteins was measured based on their ability to stimulate proliferation of 2E8 cells. The replacement of aminoacid in helix D altered the biological activity of the protein when compared to the canonical hIL-7 (Cosenza, Rosenbach et al. 2000). Further studies suggested that an aromatic acid residue is required at position 143 in helix D in order to bind to the IL-7R and to successfully transmit a signal (vanderSpek, Sutherland et al. 2002). Four alternatively spliced isoforms of hIL-7 mRNA have been detected in Follicular Dendritic Cells (FDC) (Kroncke, Loppnow et al. 1996).

Human IL-7 isoforms were later characterized in leukemic cell samples obtained from children with acute lymphoblastic leukemia (ALL) and in the human Burkitt lymphoma cell line Raji. Genome wide mutagenic studies in mice by N-ethyl-N-nitrosourea (ENU) identified a mutation G-to-A in splicing donor site of the third exon of IL-7 gene, resulted in skipping of exon3 (IL-7 δ 3). Recombinantly expressed murine IL-7 δ 3 showed an absence of anti-apoptotic activity when compared to wild type protein (Huang, Chiang et al. 2007). cDNA analysis obtained from equine adult lymphnode tissue revealed that three different IL-7 isoforms (δ 5, δ 3/5 and δ 3/4/5) exist in addition to the 6 exon-spanning IL-7. These isoforms appear to be biologically active demonstrated by their ability to enhance proliferation of T-cells after stimulation with supernatants collected from isoform transfected HEK cells. However, the IL-7 variants were not produced as recombinant proteins. Proliferation of T-cells was measured by thymidine incorporation experiments. IL-7 δ 5 and IL-7 δ 3/5 show a similar stimulation index as compared to the IL-7 wild type, but IL-7 δ 3/4/5 was 25% less active (Cook, Cook et al. 2008).

At present, the functional significance of IL-7 isoforms in human tumor cells is unknown. They might act as competitive inhibitors of the full length cytokine (Korte, Moricke et al. 1999), based on the observation that human IL-4 δ 2 inhibits IL-4 mediated T-cell proliferation (Atamas, Choi

et al. 1996; Arinobu, Atamas et al. 1999). The ratios of IL-4 and IL-4 δ 2 levels were significantly higher in patients with Systemic Sclerosis suggesting a pathogenic role of IL-4/IL-4 δ 2 (Sakkas, Tourtellotte et al. 1999) in autoimmunity. Splice variants of IL-2 (δ 2, δ 3) prevented IL-2 mediated T- cell expansion (Tsytsikov, Yurovsky et al. 1996). The IL-7 isoform ratio intensities were significantly higher in intestine and lamina propria as compared to their corresponding epithelial layer cultures (Madrigal-Estebas, McManus et al. 1997). Mutant human IL-6 protein variants showed significantly lower biological activity, caused by the weakening of gp130 binding without affecting affinity towards hIL-6R α (Savino, Lahm et al. 1994); different IL-6 isoforms were also characterized in murine models (Yatsenko, Filipenko et al. 2004).

IL-15 isoforms (δ 6, δ 7) were highly expressed in the duodenum and small intestine and appear to show antagonistic effects by inhibiting the activity of the canonical IL-15 (Tan and Lefrancois 2006). Non-secretable IL-15 generated by alternative splicing suppresses IL-15 gene transcription. This implies a novel autocrine regulatory mechanism for cytokine gene expression mediated by alternative splicing. (Nishimura, Fujimoto et al. 2005). Intracellular IL-15 levels in mast cells were elevated after stimulation with lipopolysaccharide (LPS) to limit recruitment of neutrophils to sites of infection. This suggests a pathogenic role of an IL-15 isoform in inflammation (Orinska, Maurer et al. 2007).

We mapped IL-7 isoforms in different organs and tissues. The data suggested that IL-7 isoforms are expressed in a organ/tissue specific fashion. The comparison of tumor free Human Papilloma Virus negative (HPV-) and HPV+ tumor lesions from the same patients (with cervical cancer) exhibited a shift of the IL-7 isoform profile. The changes in IL-7 isoform expression may be associated with the presence of pro-inflammatory cytokines. Remarkably, IL-7 δ 5, but not the canonical IL-7 RNA was present in granuloma lesions obtained from patients with latent tuberculosis (n=3/3). IL-7 δ 5 isoform acts as an agonist in function based on preventing T- cell apoptosis and phosphorylation of STAT5 and this may be advantageous in local immune-surveillance (Vudattu, Magalhaes et al. 2009).

Treatment of human neural progenitor (NHNP) cells either with IL-7 or IL-7 isoforms resulted in differentiation of NHNP towards the glia cell lineage (Moors M et al., under review). This provides a paradigm that IL-7 or IL-7 variants may not only be instrumental in differentiation of hematopoietic cells, but also in non-hematopoietic cells. The detailed analysis of IL-7 variants will aid to explore their use as therapeutic agents for targeted disease intervention such as in autoimmune disease or as biomarkers in the context of immune reconstitution.

1.5.2 IL-7R isoforms

IL-7R signaling is a key element for the maintenance of peripheral T-cell homeostasis. IL-7 and Thymic stromal –derived lymphopoietin (TSLP) share a common receptor chain, IL-7R α . IL-7 induces pro-inflammatory cytokines and TSLP signaling leads to expansion of T-cells producing Th2-type cytokines. Mutations in IL-7R α may impair the binding of IL-7 and result in a SCID phenotype in both mice and men (Giliani, Mori et al. 2005). Extensive alternative splicing of IL-7R α in leukaemic cells was observed in children suffering from ALL. In addition to the canonical IL-7R α , two IL-7R isoforms were observed, which lack either the cytoplasmic domain or both the cytoplasmic and the transmembrane domain ('soluble IL-7R'). The interactions between IL-7R isoforms and IL-7 studied by transfection of IL-7R variants on the cell surface demonstrated that all IL-7R isoforms are still able to bind IL-7 with different binding affinities. It is possible that the soluble IL-7R α may affect IL-7 mediated effects, for instance memory T- cell formation and generation of antigen specific T-cells. This could result in the impairment of anti-tumor responses. (Korte, Kochling et al. 2000). Interestingly, higher levels of IL-2 soluble receptor isoform have been associated with paediatric lymphomas (Pui, Ip et al. 1989). Similar pattern of soluble receptors isoforms were also observed for other cytokine receptors such as IL-4R α and IL-23R (Kruse, Forster et al. 1999; Kan, Mancini et al. 2008).

Multiple Sclerosis is a demyelinating neurodegenerative disease with a strong genetic correlation. Single Nucleotide Polymorphisms (SNPs) occur in exon 6 of the IL-7R α chain, this may be associated with a higher risk to develop MS (Gregory, Schmidt et al. 2007; Hafler, Compston et al. 2007; Lundmark, Duvefelt et al. 2007) or allergy (Shamim, Muller et al. 2007). The soluble form of IL-7R appears to be over-expressed in peripheral blood obtained from patients with Primary Progressive MS (PPMS) irrespective of the genetic haplotype (McKay, Swain et al. 2008), suggesting that IL-7R isoforms may play a more general role in the pathogenesis in autoimmune diseases. The canonical IL-7R α and the soluble IL-7R α are expressed in human developing brains, as well as in NHNP cells. This suggests that IL-7 and IL-7R α interactions are involved in neurodevelopment (Moors M et al under review).

2 AIMS OF THE THESIS

The main aim of this study was to understand the biology of alternatively spliced variants of Interleukin-7 (IL-7). IL-7 is a pleiotropic cytokine, which may show diagnostic or prognostic potential where immune-cell survival/differentiation plays a role. Until now, there are no studies which describe the biological function of alternatively spliced IL-7 variants in humans.

The specific aims of this thesis were

- To investigate the distribution of IL-7R on different T- cell subsets, since only these cells would be able to transmit an IL-7 or IL-7 isoform signal. (Paper I)
- Is each IL-7R positive cell functionally able to transmit a signal? (Paper II)
- Identification of IL-7 splice variants and their effects on T-cells and human thymocytes (Paper III)
- Characterization of IL-7 isoforms in the CNS- A paradigm that alternatively spliced IL-7 acts not only on immune cells (Paper IV)

3 RESULTS AND DISCUSSION

3.1 HIGH CONTENT IMMUNE CELLULAR PROFILING CLUSTER HEALTHY DONORS DIFFERENTLY FROM INDIVIDUALS WITH NEUROLOGICAL DISEASE BASED (PAPER I)

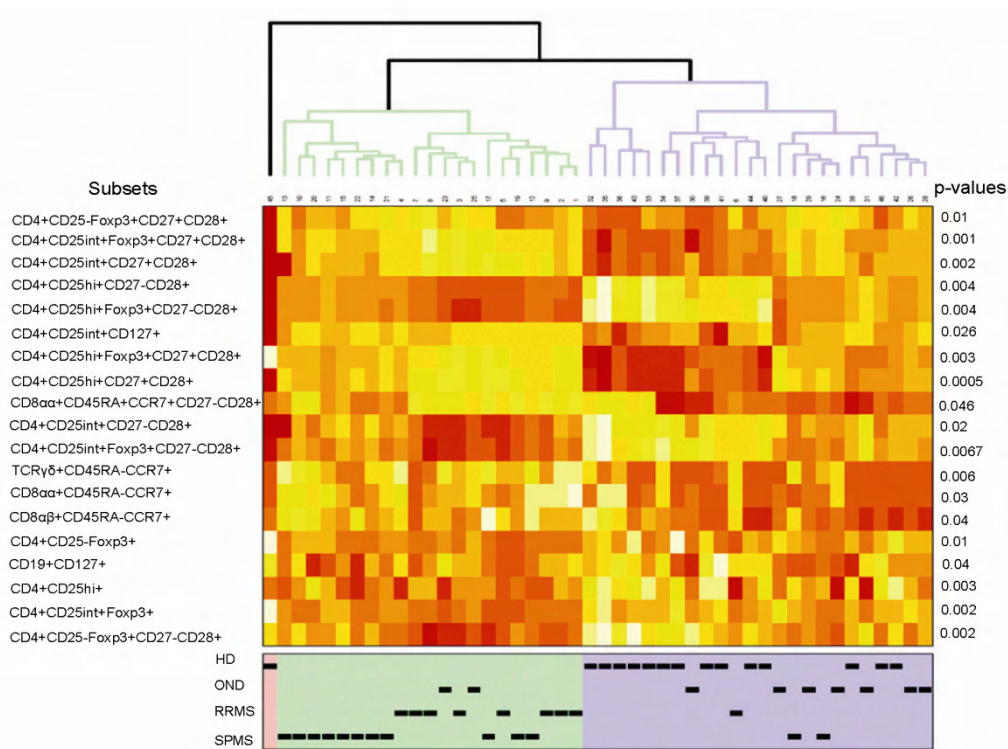
3.1.1 IL-7 and IL-7R in autoimmune disease

Interleukin-7 (IL-7) is an essential cytokine for lymphocyte development and survival, which was first discovered in 1988 as a growth factor that promoted the proliferation of murine B- cell precursors (Namen, Lupton et al. 1988). IL-7 has been postulated to play a pathogenic role in autoimmune diseases (Calzascia, Pellegrini et al. 2008; Churchman and Ponchel 2008). Genetic polymorphisms in IL-7R and other environmental factors may contribute to immune responses associated with MS. Recent studies addressed the question that alterations in immune cell subsets in the peripheral circulation are associated with CNS inflammation in patients with MS (Noseworthy, Lucchinetti et al. 2000; Sospedra and Martin 2005; Rinaldi, Gallo et al. 2006). Since MS was the first human disease which has been associated with differential expression of IL-7R, we attempted to enumerate IL-7R molecules on PBMCs from healthy individuals and compared the data with IL-7R expression analysis obtained in PBMCs from patients with MS. Many studies have been carried out to examine either the relative distribution or absolute numbers of immune cell subsets in PBMCs from patients with MS and none has addressed the number of biologically relevant molecules i.e IL-7R α on a single cell level.

Our study examined the percentages of 189 individual T-cell subsets along with the density of IL-7R α (CD127) on 59 individual immune T-cell subsets by using molecules of equivalent soluble flouochrome units per cell in peripheral blood mononuclear cells obtained from patients with MS to identify cellular markers associated with MS. The statistical analysis of 189 T-cell subsets between healthy donors and patients with MS resulted in 19 significant T-cell subsets which clustered differently in individuals with neurological disorders as compared to healthy controls. The heatmap plot analysis showed up that one healthy individual had a different pattern of measurements from the rest of the healthy individuals. When excluding that individual, the clustering of both groups resulted in 18 significant variables which gave a slightly different profile.

We identified lower numbers of TCR $\alpha\beta$ +CD4+CD25^{high} T-cells (p=0.0008), which had been reported to be decreased in patients with MS (Viglietta, Baecher-Allan et al. 2004) and in other autoimmune diseases like Rheumatoid Arthritis (Ehrenstein, Evans et al. 2004). We observed an increase in the percentage of central memory cells in TCR $\alpha\beta$ +CD8 $\alpha\beta$ + cells and TCR $\alpha\beta$ +CD8 $\alpha\alpha$ + T-cell subsets. The latter immune cell population presents a distinct memory T-cell subset, which down-regulated the CD8 beta chain, presumably associated with long-term antigen exposure (Figure 4). This T-cell subsets may show detrimental immune effector functions and contribute to chronic inflammation in MS (McDole, Johnson et al. 2006), other reports suggested that these cells may also show suppressive functions (Johnson, Suidan et al. 2007).

Figure 4. Heatmap visualization of immune cell frequencies clustered in PBMCs from patients with MS



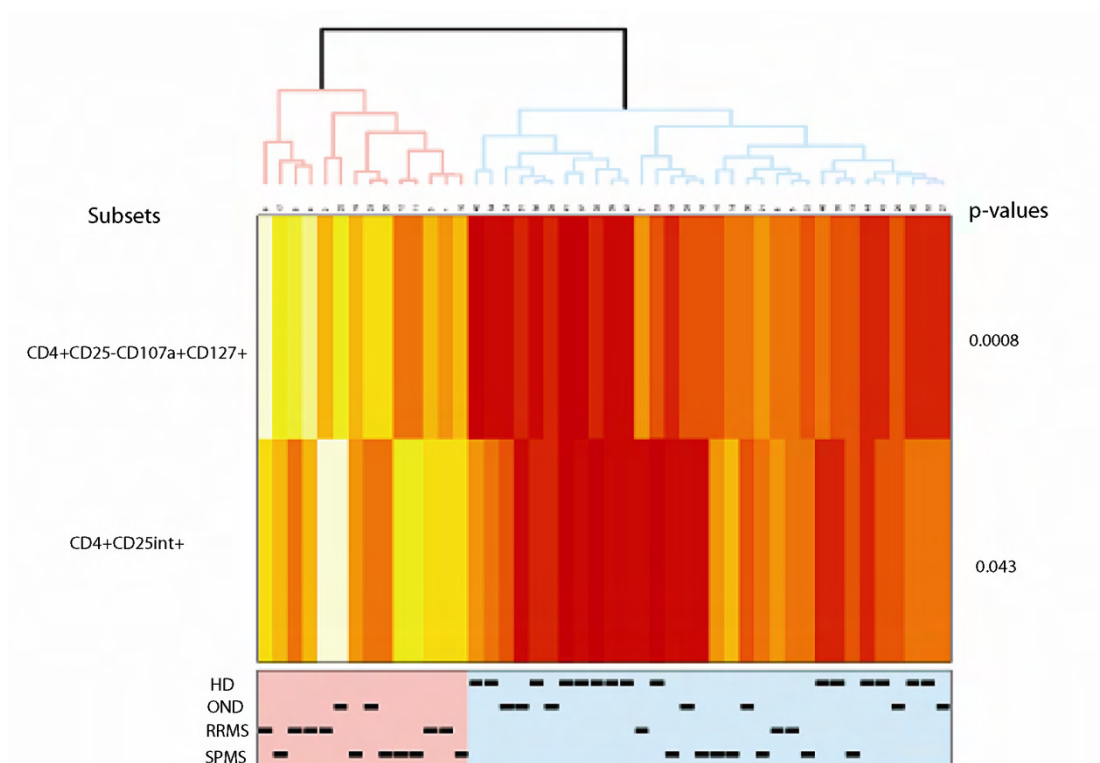
Among 189 immune cell subsets, only 19 turned out to be statistically significant in patients with MS as compared to healthy controls. Recently, a population of CD8^{low}+CD4+ cells was identified in untreated RRMS patients, as well as individuals with an isolated demyelination syndrome (De Jager, Rossin et al. 2008). A more detailed study in twenty patients showed significant differences in ten lymphocyte subsets associated with active MS and these markers included the innate and adaptive arm of the immune system (Rinaldi, Gallo et al. 2006). Based on these

studies, it appears that only a combination of cell surface markers allows us to define differences in distinct immune cell subsets between healthy controls and individuals with neurological diseases.

3.1.2 Variation in IL-7R α density on immune cell subsets segregates patients with MS from healthy individuals

Enumeration of IL-7R density was examined at a single cell level on 59 immune cell subsets. We could demonstrate an association of IL-7R density with the maturation/homing phenotype in T-cell subsets. The total number of IL-7R α molecules on individual cells may range from 10,000 to 500,000 per cell (Armitage, Ziegler et al. 1992). Central/peripheral memory T-cells (CD45RA-CCR7+ or CD45RA-CCR7-) exhibited the highest IL-7R density and differentiated T-cells defined by CD45RA+CCR7- phenotype exhibited the lowest number of IL-7R molecules in our study.

Figure 5. Higher levels of IL-7R density on cytotoxic CD4+ T-cells obtained from patients with Multiple sclerosis



There were no differences between T-cells subsets that would segregate healthy individuals and individuals with neurological diseases. The Kruska-Wallis test performed on 59 immune cell

subsets after FDR correction showed two significant T-cell subsets ($\text{TCR}\alpha\beta+\text{CD4}+\text{CD25-CD107a}+\text{CD127}+$ and $\text{TCR}\alpha\beta+\text{CD4}+\text{CD25int}+\text{CD127}+$) which were clustered differently among healthy donors versus individuals with neurological disorders (Figure 5). CD107a is a (degranulation) marker for vesicles which contain preformed perforin and granzyme molecules. CD107a can be expressed on both $\text{CD4}+$, $\text{CD8}+$ T-cells, particularly on antigen-specific T-cells with cytotoxic potential.

A similar phenotype was reported in human CMV-specific effector $\text{CD4}+$ T-cells which produce pro-inflammatory cytokines in the absence of IL-2 and exhibit lytic activity based on CD107a expression. These ($\text{CD4}+$) T-cells may be important for CMV containment (Casazza, Betts et al. 2006). We propose that the $\text{TCR}\alpha\beta+\text{CD4}+\text{CD25-CD107a}+$ T-cell subset represents a long-lived, differentiated and cytotoxic $\text{CD4}+$ T-cell population associated with chronic antigen exposure in patients with MS.

3.2 ALTERED LEVELS OF INTERLEUKIN-7 RECEPTOR (CD127) AND IL-7 SIGNALING DEFECTS IN IMMUNE CELLS OBTAINED FROM PATIENTS WITH BREAST CANCER (PAPER II)

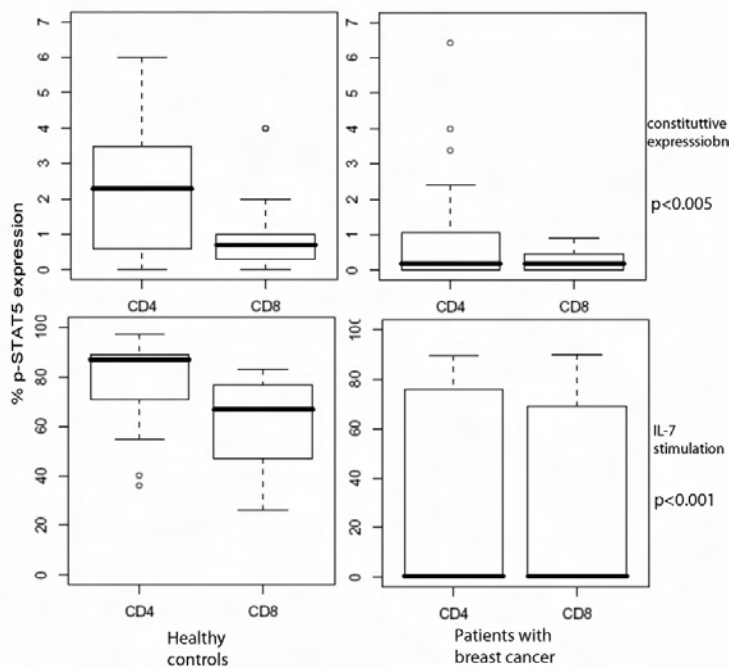
IL-7R α is constitutively expressed on non-stimulated T-cells, a central pre-requisite for T-cell homeostasis and maintenance of T-cell memory. The functional impact of decreased IL-7R α expression on immune cells show decreased proliferative capacity as compared to IL-7R α positive CMV-reactive T-cells (van Leeuwen, de Bree et al. 2005). Altered levels of IL-7R α on effector immune cells showed significant differences between young adults and older people which leads to impaired STAT5 signalling and survival response to IL-7 (Kim, Hong et al. 2006). IL-7 mediated signals are essential to maintain effective immune functions in animals, as well as humans. Mutations in IL-7R α affects humoral and cellular immune responses, it predisposes to opportunistic infections (Giliani, Mori et al. 2005). IL-7R α mediated signals are carried out by Jak1 and Jak3, Src kinases and STATs (signal transducer and activators of transcription) mainly STAT5A/b. STAT5A knockout mice develop impaired mammary gland development (Liu, Robinson et al. 1997). In the current study, we observed lower levels of IL-7R α expression on immune cells, dysregulated T-cell responses characterized by IL-7 mediated signaling defects and loss of effector functions demonstrated by reduced cytokine secretion in PBMCs obtained from patients with breast cancer.

3.2.1 IL-7R α analysis in T-cells obtained from patients with breast cancer

PBMCs obtained from age-matched healthy volunteers and patients with breast cancer were analyzed for IL-7R α and CD107a+ (LAMP-1, Lysosomal associated membrane protein-1) expression. The majority of CD8 $\alpha\alpha$ +, CD8 $\alpha\beta$ + and CD4+ T-cells stained positive for IL-7R α in healthy donors, but a reduced number of IL-7R α positive CD4+ T-cells were observed in PBMCs obtained from patients with breast cancer. Co-expression of CD107a+ and CD127+ on CD8 $\alpha\alpha$ + T-cells and CD4+ T-cells was found in PBMCs from patients with breast cancer. At this time, we did not gauge the density of IL-7R or CD107a expression on the single cell level. Therefore, we measured mean channel fluorescence intensity (MFI) values for CD107a+ and quantified IL-7R molecules on T-cell subsets using calibrated beads.

In general, T-cells which co-express both CD107a and CD127 show higher MFI values for CD107a expression as compared to T-cells negative for IL-7R α , suggesting that CD127+ T-cells may show a stronger cytotoxic capacity than T-cells which express CD107a alone. Lower MFI values were observed for CD107a and CD107a+CD127+ expression on T-cell subsets from patients with breast cancer. Thus, a higher percentage of CD107a/CD127+ T-cells is not necessarily associated with increased IL-7R α in these T-cell subsets, reflected by reduced MFI values.

Figure 6. IL-7 signal impairment in PBMCs obtained from patients with breast cancer



3.2.2 IL-7 signaling defects and cytokine production is impaired in PBMCs from patients with breast cancer

STAT5 plays an important role in transmitting IL-7 signals. STAT5 mediated signaling sustains TCR induced gene expression via IL-2 and stabilizes gene expression program in CD8+ T-cell effector cells in murine models (Verdeil, Puthier et al. 2006).

We evaluated the 'fitness of T-cells' defined by IL-7R α mediated signaling using a technique, which measures phosphorylated STAT5 (p-STAT5) upon stimulation with recombinant human IL-7. PBMCs obtained from patients with breast cancer showed lower proportions of p-STAT5 constitutively and after stimulation with rhIL-7 in both CD4+ and CD8+ T-cells as compared to healthy donors with a significant p-value <0.001 (Figure 6). Next, we tested if other immune effectors functions are impaired in CD4+ and CD8+ T-cells in patients with breast cancer, demonstrated by secretion of IFN- γ and IL-2 after stimulating with PMA/Ionomycin. PBMCs obtained from 9/19 patients with breast cancer were not able to respond to either PMA/Ionomycin or to OKT-3 stimulation and the same patients also showed defects in IL-7 signaling

LAMP-1 or CD107a has been described as a cytotoxic T-cell marker; enhanced CD107a+ expression was observed in T-cells obtained from seminoma lesions as compared to peripheral T-cells, which suggested enhanced cytotoxic capacity of T-cells infiltrating into human tumors (Hadrup, Braendstrup et al. 2006). Enhanced CD107a expression from patients with breast cancer coincides with unresponsiveness to IL-7 defined by STAT5 phosphorylation except in a single patient. Profound IL-7-signaling defects were observed in patients with breast cancer, despite the surface expression of IL-R α . Stimulation of PBMCs obtained from healthy donors either with OKT-3 or Daudi cells for 6 hours, resulted in decreased levels of p-STAT5 in response to IL-7 stimulation, which suggests that T-cell activation may lead to decreased STAT5 phosphorylation.

Dysregulated T-cell responses were observed in PBMCs obtained from patients with breast cancer (Campbell, Scott et al. 2005). It is noteworthy that PBMCs from 9/13 patients showed impairment both in response to IL-7 stimulation or PMA/Ionomycin stimulation. A detailed analysis of T-cell function, including the analysis of IL-7R mediated signaling may improve the design of biological therapy in patients with cancer.

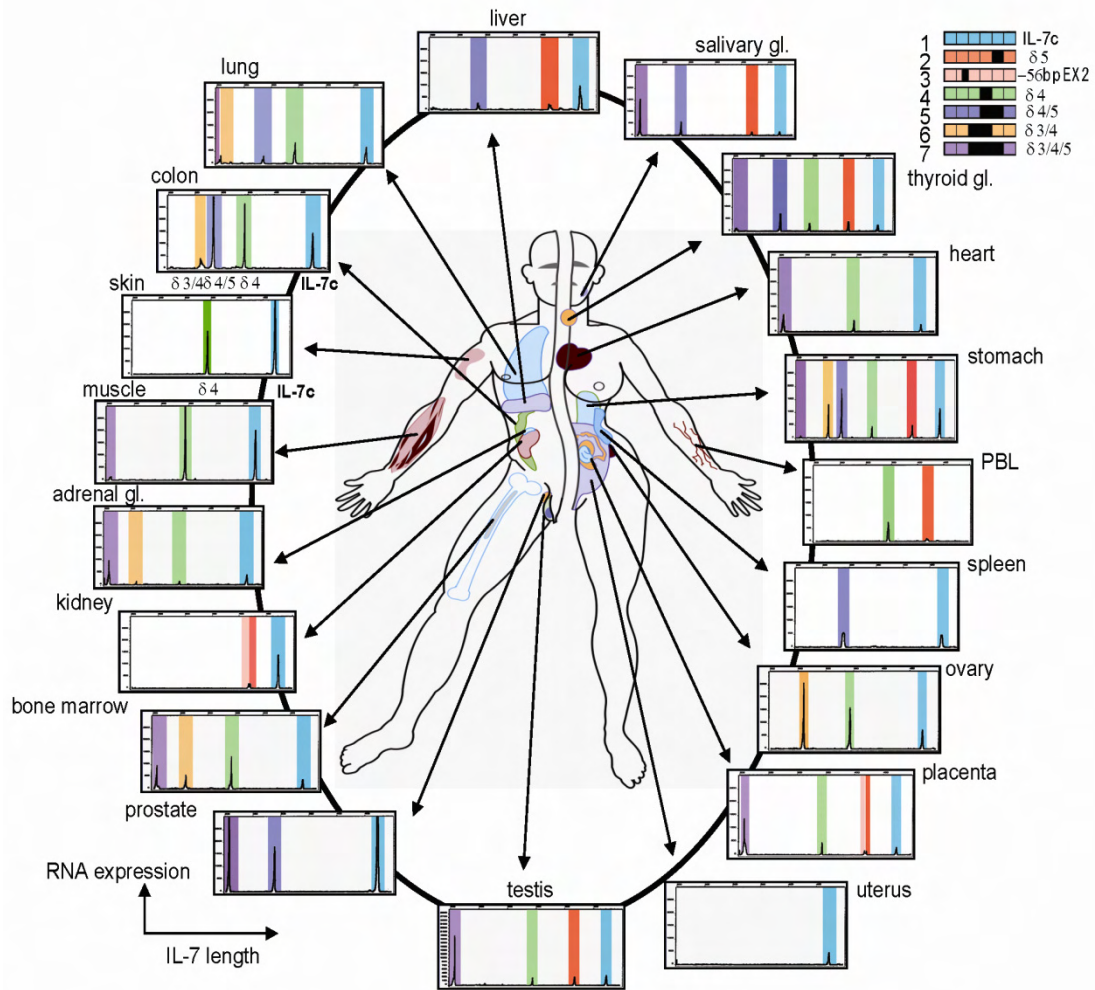
3.3 ALTERNATIVE SPLICE VARIANTS OF IL-7 AND THEIR EFFECTS ON HUMAN T-CELLS AND THYMOCYTES (PAPER III)

Alternative splicing results in multiple protein isoforms derived from a single gene. Alternatively spliced proteins may act as agonists or antagonists as compared to the wild type protein. For instance, the alternative splice variant of IL-4, IL-4 δ 2, a potent IL-4 inhibitor, is expressed in thymic tissue and in the airway system (Atamas, Choi et al. 1996). Similarly, IL-2 δ 2 and IL-2 δ 3 inhibit binding of the full-length IL-2 to the high affinity IL-2 receptor (Tsytikov, Yurovsky et al. 1996). A non-secretable IL-15 isoform which co-localizes with the IL-15 receptor in the nucleus was observed in the intestinal epithelium (Nishimura, Fujimoto et al. 2005). Although IL-7 isoforms were described in the original paper of the human IL-7 gene discovery (Goodwin, Lupton et al. 1989), the functional significance of these variants was still unclear up to this point.

Recently, an IL-7 isoform which lacks exon3 has been found in a mutagenized mouse strain. It showed no detectable anti-apoptotic activity and this mouse strain is severely affected by reduced cell numbers of 10 to 20 fold in lymphnodes and in the thymus (Huang, Chiang et al. 2007). Three IL-7 variants were described in horses (Cook, Cook et al. 2008). Alternative spliced IL-7 transcripts are present in both transformed (Frishman, Long et al. 1993; Korte, Moricke et al. 1999) and non-transformed cells (Madrigal-Estebas, McManus et al. 1997) and the physiology of these IL-7 splice variants have not been addressed. We mapped therefore IL-7 isoform expression in human tissues, in freshly harvested tumor, non-tumor lesions as well as in tissues with *M. tuberculosis* infection using RNA fragment analysis. IL-7 variants were expressed as recombinant proteins and tested for biological activity using the STAT5 phosphorylation assay, Bcl-2 expression and prevention of apoptosis.

3.3.1 Map of IL-7 isoform expression in human tissues

Figure 7. Human map of IL-7 isoform distribution in different organs / tissues

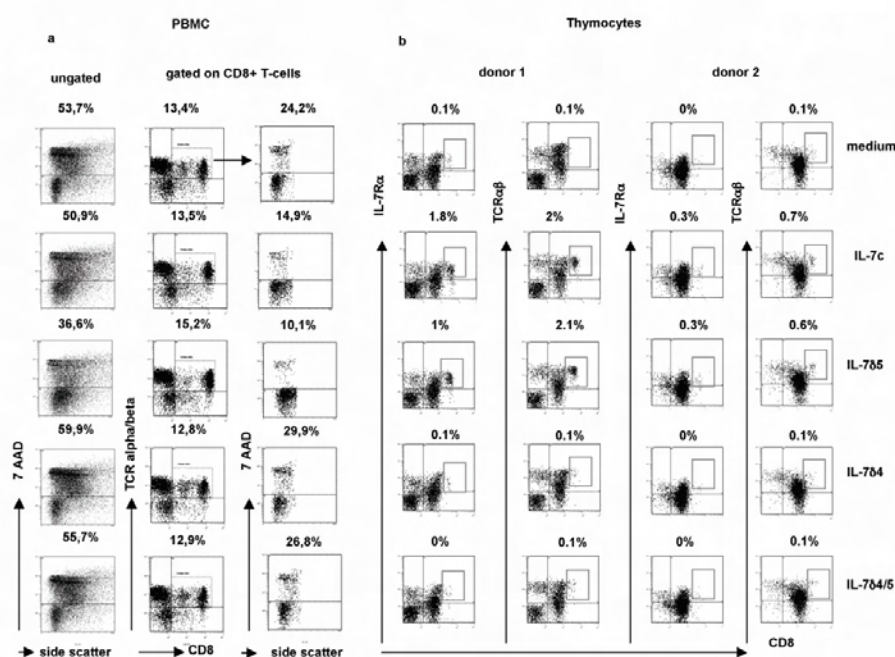


The IL-7 transcript is present in most organs. Some tissues and organs express different IL-7 isoforms, e.g. ovary (IL-7c, IL-7 δ 4 and IL-7 δ 4/5), skin and muscle (IL-7c and IL-7 δ 4) (Figure 7). The IL-7 δ 5 was detectable in tissues from the kidney, spleen, liver, stomach, salivary gland, placenta and testis. Thymic tissue showed abundance for the IL-7c and IL-7 δ 3/4/5 isoforms and transcripts for IL-7 δ 4 were detected in the thymic cortex, intermediate regions and medulla; the IL-7 δ 3/4 transcripts were detectable in the intermediate region. Non-transformed cervical tissue showed a uniform expression pattern (IL-7c, IL-7 δ 4 and IL-7 δ 4/5) as compared to corresponding HPV+ tumor lesions (IL-7c or the IL-7 δ 4/5). The differences in IL-7 mRNA expression pattern may be due to the presence of many different cell types in the tumor or the presence of pro-inflammatory cytokines like IFN- γ , which may switch the IL-7 transcription profile.

To address this question, we examined IL-7 isoforms expression in both HPV-positive (ME180) and HPV-negative (C41) cervical cancer cell lines, which showed expression of IL-7c. Stimulation with IFN- γ resulted to a shift in expression levels of IL-7 splice variants, noticed by lower expression of IL-7c in ME180 and higher levels of IL-7 δ 4/5 and IL-7 δ 5 isoforms in the C41 cell line. We were further interested to see whether chronic infections can lead to any shift or unique expression profiles of IL-7 isoforms. Interestingly, *Mycobacterium tuberculosis*-positive granuloma tissue from individuals with latent tuberculosis exhibited a uniform pattern of IL-7 δ 5 and IL-7 δ 4/5 isoforms but not the IL-7c. Profiling of tumor cell lines of neuronal origin also revealed a distinct IL-7 isoform expression pattern (IL-7c, IL-7 δ 4 and IL-7 δ 4/5), suggesting that IL-7 isoforms may play an important role in different disease conditions.

3.3.2 IL-7 δ 5 mediates T-cell survival and STAT-5 phosphorylation

Figure 8. Functional impact of IL-7 isoforms on T-cells and Thymocytes



There are no antibody reagents available that can distinguish IL-7 isoforms from IL-7c. To study the function of IL-7 splice variants, we cloned and expressed the IL-7c, IL-7 δ 5, IL-7 δ 4 and IL-7 δ 4/5 in Hi-Five insect cells and tested their anti-apoptotic effect on peripheral blood mononuclear cells as well as on human thymocytes. Incubation of PBMCs with different IL-7 splice variants showed that IL-7 δ 5 lead to enhanced survival of T-cell and thymocytes (Figure 8). The bioactivity of IL-7 δ 5 is further characterized by increased expression of the survival factor

Bcl-2 (determined by ELISA) and phosphorylation of STAT5 in PBMCs and thymocytes. Expression of IL-7 isoforms in *Escherichia coli* yielded identical results, which suggests glycosylation may not be crucial for IL-7 functions in the experimental readouts tested in the current report. IL-7 and IL-7 δ 5 mediated STAT5 phosphorylation could be blocked using a monoclonal antibody against the IL-7R α , but not with an isotype-matched control. IL-7 δ 4 or IL-7 δ 4/5 did not show any biological effect determined either by STAT5 phosphorylation or T-cell survival assays.

Pre-incubation of thymocytes or peripheral blood cells with either IL-7 δ 4 or IL-7 δ 4/5 followed by incubation with IL-c or IL-7 δ 4/5 did not suggest that IL-7 δ 4 or IL-7 δ 4/5 may act as antagonist defined by STAT5 phosphorylation. To summarize, IL-7 isoforms are present in human tissues, detectable in freshly harvested human tumor specimens or granuloma lesions. IL-7c and IL-7 δ 5 showed bioactivity based on T-cell survival and cellular downstream effects determined by the STAT5 phosphorylation. Aberrant IL-7 isoform expression may represent a biologically relevant tumor escape mechanism as only IL-7c or IL-7 δ 5, but no other IL-7 protein isoform, have been shown to induce T-cell survival. Since IL-7c is used as a therapeutic agent in patients with cancer to enhance anti-tumor responses (Maeurer, Walter et al. 1997; Gattinoni, Finkelstein et al. 2005), the therapeutic role of IL-7 δ 5 is yet to be studied.

Antibodies that are commercially available to detect IL-7c also crossreacted with IL-7 δ 5 (data not shown). The pro-inflammatory cytokine IFN- γ , may in part, shape the IL-7 variant expression profile. The detailed analysis of IL-7 variants will aid to effectively address these topics in a molecularly defined manner.

3.4 CHARACTERIZATION OF IL-7 ALTERNATIVE SPLICE VARIANTS IN THE CENTRAL NERVOUS SYSTEM AND THE EFFECT OF IL-7C OR DIFFERENT IL-7 ISOFORMS ON NORMAL HUMAN NEURAL PROGENITOR (NHNP) CELLS (PAPER IV)

Alternative splicing is a crucial mechanism for expanding the repertoire of gene functions in neuronal identity. The first tissue-specific regulation of RNA processing pathways in central nervous and endocrine systems were described by characterization of calcitonin gene expression (Rosenfeld, Mermoud et al. 1983). The large number of tissue specific isoforms was found in brain, which represented 18% of all tissue specific alternative splicing (Xu, Modrek et al. 2002). It is suggested that 10 up to 30% of alternatively spliced genes have tissue specific isoforms (Mortazavi, Williams et al. 2008) and 73% of all genes display differences in expression levels

between tissues (Xu, Modrek et al. 2002). Alternative splicing has been described for cytokine genes and their receptors. Recently, alternative splicing of IL-23 receptor alpha chain represents most likely the cytokine receptor with the highest number of potential transcripts, 18 individual proteins are predicted (Kan, Mancini et al. 2008). Genome-wide SNP search analysis for IL-7 and IL-7R genes revealed that a single nucleotide polymorphism in exon 6 of IL-7R gene resulted in an alternative splice variant of IL-7R, i.e. soluble IL-7R, associated with increased risk to develop Multiple Sclerosis (Gregory, Schmidt et al. 2007; Hafler, Compston et al. 2007; Lundmark, Duvefelt et al. 2007).

We examined differentially spliced IL-7 in distinct anatomic brain areas, IL-7 and IL-7R isoforms in developing brains as well as in human neural progenitor cells. Next, we investigated the impact of IL-7 or IL-7 splice variants in differentiation of neural progenitor cells. Finally, transcriptome analysis of IL-7 stimulated neural progenitor cells showed that 58 differentially expressed genes, some of which are involved in neural differentiation, are differentially regulated by IL-7: IL-7 may influence neural development.

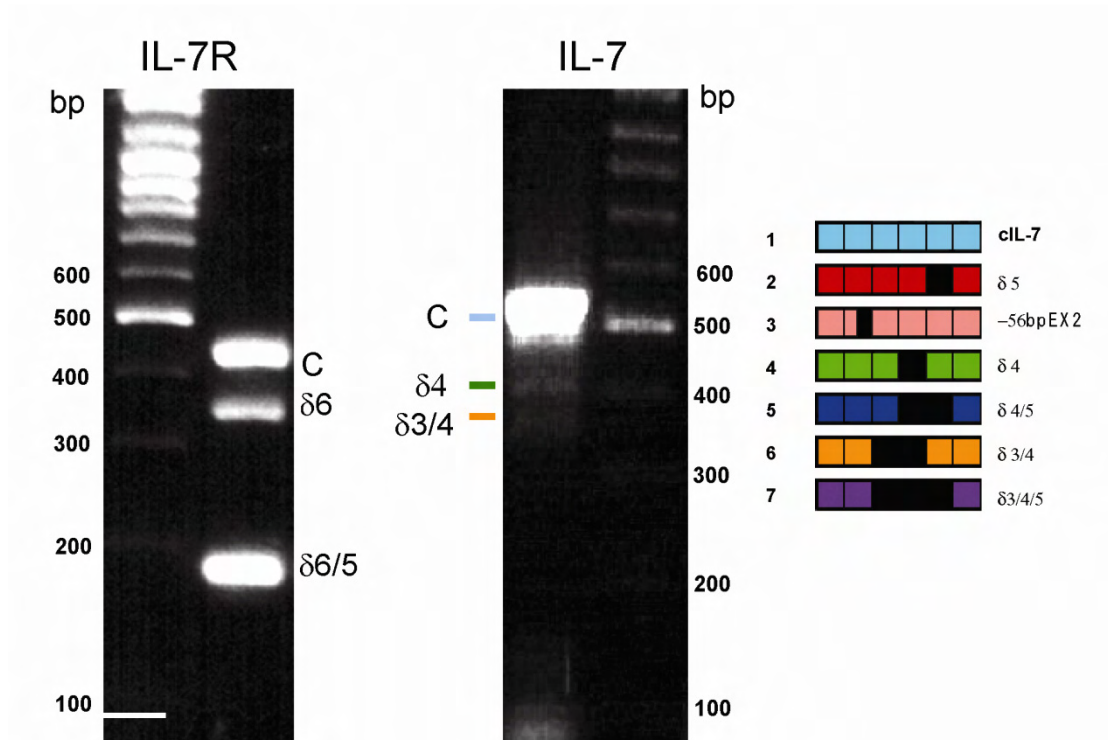
3.4.1 Mapping of IL-7 isoforms in adult and developing brains

A cDNA library obtained from human brains showed exclusive expression of IL-7c in the cerebellum and pons, the IL-7 δ 4 is exclusively expressed in the caudate nucleus. Tissue from spinal cord showed expression of IL-7c and IL-7 δ 4/5. Human embryonic brains revealed expression of IL-7c and IL-7 isoforms such as IL-7 δ 5, IL-7 δ 4, IL-7 δ 4/5 or IL-7 δ 3/4. All IL-7 splice variants were confirmed by sequence analysis and expressed as recombinant proteins in insect cells.

3.4.2 IL-7c or IL-7 isoforms induces neural cell differentiation

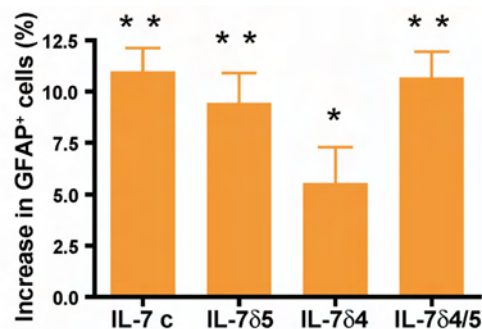
Normal human neural progenitor (NHNP) cells represent homogenous, self-regulating cell populations of human neural precursor and neural stem cells defined by expression of nestin and other neural early markers (Moors, Cline et al. 2007).

Figure 9. Expression profiles of IL-7 and IL-7R splice variants in human neural progenitor cells



We characterized the presence of either IL-7 or IL-7R splice variants in NHNP cells by RNA expression analysis, showing that NHNP cells express IL-7c, IL-7 δ 4 and IL-7 δ 3/4. NHNP cells also express full length IL-7R and the splice variants of IL-7R IL-7R δ 6 and IL-7R δ 5/6 (Figure 9). NHNP cells cultured for four days in the presence or absence of IL-7 or IL-7 splice variants revealed a distribution of 15-30% of beta (III) tubulin positive neurons and 70-85% GFAP positive glia cells. IL-7c, IL-7 δ 5 and IL-7 δ 4/5 were the most potent isoforms based on their capacity to differentiate NHNP cells towards glia cells, whereas IL-7 δ 4 exhibited a significantly weaker potency compared to IL-7c and IL-7 δ 5 ($p < 0.05$) (Figure 10).

Figure 10. IL-7 splice variants differentiate neural progenitor cells



IL-7 has been shown to induce neuronal differentiation of immortalized neural cells (Mehler, Rozental et al. 1993); IL-7 trophic effects were demonstrated in primary neural cells in a dose dependent manner (Michaelson, Mehler et al. 1996). We reported for the first time the functional impact of IL-7 splice variants on neural progenitor cells. Further, bioactivity of IL-7c and IL-7δ5 was confirmed by phosphorylation of STAT5 in NHNP cells (data not shown).

3.4.3 IL-7 effects on gene expression in neural progenitor cells

NHNP neurospheres stimulated with IL-7c for 24 hours, and whole transcriptome expression analysis demonstrated that 58 genes were differentially regulated in control versus IL-7c stimulated NHNP neurospheres. 50/58 genes were down-regulated, whereas 8/58 genes were up-regulated. Functional cluster analysis revealed that most of the genes are involved in transcriptional regulation (40% of clustered genes), protein metabolism (34%) and 17% of the genes were related to neural differentiation. A summary of genes associated with neuronal development and regulation further validated by quantitative real time PCR are listed in Table 1

Table 1. List of IL-7 regulated genes which participate in neuronal differentiation demonstrated by transcriptome analysis

Sequence ID	Protein	Function	References
<i>NM_015061</i>	JMJD2C	stem cell differentiation	Loh YH et al., 2007
<i>NM_138962</i>	MSI2	neural stem cell maintenance	Okano H et al., 2002
<i>NM_138957</i>	MAPK1	neurogenesis	Samuels IS et al., 2008
<i>NM_030752</i>	TCP1	tubulin- biogenesis	Schuller E et al., 2001
<i>NM_002902</i>	RCN2	neural synapsis	Helbig I et al., 2008
<i>NM_007249</i>	KLF12	neuronal development	Wang Y et al., 2006
<i>NM_178169</i>	SOS2	CNS injuries	Marciano PG et al., 2004
<i>NM_000313</i>	PROS1	preventing brain injury	Liu D et al., 2003
<i>NM_004462</i>	FDFT1	cholesterol biosynthesis	Yoshida E et al., 2004
<i>NM_002130</i>	HMGCS1	cholesterol biosynthesis	Yoshida E et al., 2004

To summarize, our data suggest that IL-7 plays an important role in human brain development; it is able to shift differentiation of human neural progenitor cells towards glia cell formation. We hypothesize that cytokines may not only play a physiological role in immune function, but also participate in tissue differentiation. This could be of importance for patients suffering from Multiple Sclerosis, since several studies showed independently that IL-7R contributes to the increased risk to develop MS.

4 CONCLUDING REMARKS

Alternative splicing greatly expands the level of transcription and creates versatility of the transcriptome through expression of different protein isoforms from an individual gene. Cytokines are small protein molecules, which display broad range of effects on various cell types. Alternative splicing of cytokine and cytokine receptors has been revealed for many cytokines and these variants are either functional agonists or antagonists of the corresponding wild-type cytokines. Interleukin-7 is an exceptional cytokine, it plays a central role in B- cell and T-cell development in mice and T-cell development in humans. Interleukin-7 splice variants have been reported in mice, humans and horses, but their biological activity was only described in mice and horses.

In paper I we analyzed the expression of IL-7R on different T-cell subsets along with IL-7R density values. We could show differences in IL-7R density on 2/59 T-cell subsets (TCR $\alpha\beta$ +CD4+CD25-CD107a and TCR $\alpha\beta$ +CD4+CD25 intermediate) on a single cell level, but not in the percentage of IL-7R positive immune cell subsets. In addition, there were 19/189 significant T-cell subsets which were clustered differently in patients with MS as compared to healthy individuals. These markers may be useful for prognosis/diagnostic or treatment of autoimmune diseases.

In paper II, we evaluated the expression and function of IL-7R in PBMCs from patients with breast cancer: Increased expression of cytotoxic markers (CD107a) and reduced levels of IL-7R along with impairment of IL-7 signal or cytokine secretion may represent an early disease state, it may also aid to monitor cellular immune responses in patients with breast cancer.

In paper III, we mapped IL-7 variants that are specific for different organs/tissues and also in disease. Aberrant IL-7 isoforms expression in (Mtb+) granuloma lesions may greatly impact on expansion and survival immune cell subsets *in situ*. Recombinant expression of IL-7 and IL-7 splice variants demonstrated that IL-7 δ 5 seems to be (super) agonistic in function as compared to the canonical IL-7.

In Paper IV, we described IL-7 splice variants in different anatomic areas of the brain, human embryonic brains and human neural progenitor cells. This study also addresses the question of

tissue specific isoforms. Functional impact of IL-7 splice variants on neural progenitor cell differentiation unveils the role of IL-7 splice variant activity in cellular differentiation.

Since the discovery of IL-7 or IL-7 isoforms, which was about 20 years ago, we have witnessed the importance of hIL-7 in the immune system. Little was known about the significant role of IL-7 isoforms in humans and the association of IL-7 isoforms with disease, or actions of IL-7 isoforms on non-immune (i.e. neuronal) cells. We hope that the work presented in this thesis will contribute to the understanding of the biology of IL-7 isoforms. We hope that the increased knowledge in IL-7 biology may be useful to develop diagnostic markers or to target IL-7 isoforms, as a therapeutic approach, in autoimmune disease.

5 FUTURE PERSPECTIVES

Our data from Paper III and Paper IV suggests that alternative splice variants of IL-7 are biologically active determined by the anti-apoptotic activity in T-cells and thymocytes and their ability to differentiate neural progenitor cells. Further characterization of IL-7 splice variants is still needed to see the distribution of these isoforms *in situ*, which can only be possible by producing IL-7 isoform-specific monoclonal antibodies. We addressed the bioactivity of IL-7 isoforms in *in vitro* conditions, but it would be important to evaluate their function in appropriate models such as non human primates (IL-7 isoforms have not yet been described in mice). The binding affinities of IL-7 isoforms (IL-7c and IL-7 δ 5) to the IL-7R appeared to be different based on blocking experiments of the IL-7R with an anti-IL-7R antibody, but a more detailed analysis is needed to address the affinities of IL-7 variants to IL-7R. The single nucleotide polymorphisms in the IL-7R gene increases susceptibility to Multiple Sclerosis (MS) demonstrated by three independent studies, but the role of IL-7 isoforms in MS is enigmatic. The relative ratios of IL-7 isoforms will be studied in PBMCs obtained from patients with MS and healthy donors in our laboratory. This may enable us to understand the biology of IL-7 isoforms in Multiple Sclerosis, a paradigm of a chronic, inflammatory disease with expansion of auto-reactive T-cells. Ultimately, we need to evaluate whether soluble IL-7R-IL-7 variant complexes show any biological activity.

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