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BIOLOGY OF INTERLEUKIN-7 VARIANT ISOFORMS

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ABSTRACT

The interleukin-7 (IL-7) gene produces nine alternatively spliced mRNA variants. Until now, the clinical and biological significance of alternatively spliced IL-7 products has not been well defined. In this thesis, we investigated IL-7 gene alternative splicing in association with chronic inflammation and infection. This thesis also investigated the biology of alternatively spliced IL-7 variants in T-cell subsets and CD14+ monocytes. We identified for the first time the alternatively spliced IL-7δ5 variant protein among IL-7 protein variants in healthy and diseased human tissue.

In paper I, we investigated the extent and impact of IL-7 pre-mRNA alternative splicing in Peripheral Blood Mononuclear Cells (PBMCs) from 16 patients with multiple sclerosis and 15 healthy individuals by transcript specific amplification of interleukin-7 and the interleukin-7 receptor gene. The relative abundance of the gene transcripts was correlated to health and disease (MS) status and also with the IL-7R SNP rs_6897932, which has been shown to affect alternative splicing of the IL-7R gene. Flow cytometric analysis of PBMC samples was carried out to enumerate abundance of immune cell subsets in healthy and diseased individuals along with the enumeration of IL-7R molecules on single cells to identify correlation with specific IL-7 alternative splicing patterns. This was the first study to i) investigate patterns of IL-7 and IL-7R in healthy human PBMCs, ii) show that the IL-7 alternatively spliced variant lacking exon 4 (IL-7δ4), and not the canonical IL-7, constitutes the major transcript of IL-7 in human PBMCs. This was also the first report to identify a novel IL-7R spliced variant lacking exon 5, 6 and 7. We observed changes in relative distribution of IL-7 and IL-7R spliced variants in healthy individuals and patients with MS.

In paper II, we investigated IL-7 and IL-7R alternative splicing in a rhesus macaque model. The study aimed to identify the extent of IL-7 and IL-7R pre mRNA alternative splicing and to gauge its impact in chronic inflammation i.e in *M. tuberculosis* (MTB) infection in a vaccination setting defined by clinical endpoints (improved survival after challenge with virulent MTB). This enabled to study the IL-7 protein directly in lung tissue. Fifteen rhesus macaques were divided in BCG vaccine groups and controls, followed by a challenge with a virulent MTB strain. PBMC samples before the vaccination, as well as before and after the virulent MTB challenge, along with tissues (lung with and without lesions, pulmonary hilus lymph nodes and spleen) from macaques which succumbed to MTB infection were analysed for IL-7 and IL-7R pre-mRNA alternative splicing. Measurement of IL-7, IL-17 and TGF-β at the site of infection (lung) was performed by immunohistochemistry and correlated with survival. The results showed that the spliced variants of IL-7 are similar in humans and rhesus macaque PBMCs; yet the relative distribution of IL-7 transcripts is different i.e. IL-7c and not the IL-7δ4 constituted the most frequent transcript in macaque PBMCs. The IL-7R gene exhibited extensive alternative splicing associated with soluble IL-7R in lung and lymph nodes. The study could correlate increased survival of rhesus macaques upon MTB challenge to increased local production of IL-7c and IL-17a as well as decreased TGF-β in lung lesions of non-human primates exhibiting increased survival.

In paper III, we elaborated on the data produced in the first 2 reports and investigated the effect of IL-7δ5 variant on T-cells. A mono-specific (affinity-purified) polyclonal antibody recognising exclusively IL-7δ5 (and no other IL-7 isoforms) was developed using a junction peptide forming the unique junction of the IL-7δ5 protein. The binding affinity and off-rate of

IL-7 and IL-7δ5 (using a soluble IL-7 receptor, sIL-7R) was examined by surface plasmon resonance on a biacore platform in order to study mechanistic properties of IL-7δ5 and IL-7R interaction. Sorted CD4+ and CD8+ T-cell subsets were stimulated with IL-7δ5 or IL-7c (along with unstimulated controls) and changes in the transcriptome were studied using a microarray gene expression platform. The effect of IL-7δ5 and IL-7c on T-cell subsets was investigated based on the phosphorylation of signalling transducers and BCL-2 induction using flow cytometry. This report identified IL-7δ5 production, for the first time, *in situ* using a monospecific anti IL-7δ5 antibody in granuloma and lymph node sections from patients with infections (M. tuberculosis and HIV). IL-7 and IL-7δ5 protein expression analysis indicated a different distribution *in situ*, both proteins induced similar gene expression pattern in CD4+ and CD8+ T-cell subsets. IL-7δ5 showed slightly higher affinity to IL-7R, based on the SPR assay and induced BCL-2 in both CD4+ and CD8+ subsets earlier (3 days versus 10 days as compared to IL-7c), suggesting a superior agonistic activity based on BCL-2 induction.

In paper IV, we investigated the effects of the IL-7c versus the IL-7δ5 spliced variant in regard to CD14 monocyte functions. Sorted CD14+ cells from healthy donors were stimulated with IL-7c or IL-7δ5 (along with unstimulated controls in triplicates) and changes in gene expression levels after 72 hours were measured by an RNA microarray platform; cell culture supernatants were examined for differential production of immune mediator cytokines and chemokines by a luminex assay. IL-7δ5 induced a pro-inflammatory profile in CD14+ monocytes, associated with upregulation of oxidised low density lipoprotein receptor 1 (OLR1) gene, confirmed by flow cytometry. In order to investigate IL-7δ5 protein expression, we elaborated on findings in paper III and performed immunohistochemistry to identify IL-7δ5 protein production in association with healthy and corresponding transformed tissues. We were able to show that IL-7δ5 can be identified in both, human healthy and diseased tissue; IL-7δ5 can be produced by epithelial cells as well as by CD68+ tissue residing macrophages.

LIST OF PUBLICATIONS

I.   Alternative splicing of interleukin-7 (IL-7) and interleukin-7 receptor alpha (IL-7Rα) in peripheral blood from patients with multiple sclerosis (MS).
Lalit Rane, Nalini Vudattu, Kasia Bourcier, Eva Graniar, Jan Hillert, Vicki Seyfert, M J Maeurer
Journal of Neuroimmunology 222 (2010) 82–86

II.   Increased (6 exon) interleukin-7 production after M. tuberculosis infection and soluble interleukin-7 receptor expression in lung tissue.
Lalit Rane, S Rahman, I Magalhaes, R Ahmed, M Spångberg, I Kondova, F Verreck, J Andersson, S Brighenti and M J Maeurer
Genes and Immunity (2011) 12, 513–522

III.   Alternatively spliced Interleukin 7:
IL-7delta 5 (IL-7δ5) induces a different Bcl-2 expression pattern in human T-cells.
Lalit Rane, Isabelle Magalhaes, Sayma Rahman, Chaniya Leepiyasakulchai, Jan Anderson, Susanna Brighenti and Markus J Maeurer
Manuscript

IV.   IL-7δ5 protein is expressed in human tissues and induces expression of the oxidised low density lipoprotein receptor 1 (OLR1) in CD14+ monocytes.
Lalit Rane, Sayma Rahman, Isabelle Magalhaes, Aditya Ambati, Jan Anderson, Susanne Brighenti and Markus J Maeurer
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CONTENTS

LIST OF ABBREVIATIONS

BCG	Bacillus Calmette-Guerin
BCL-2	B-cell lymphoma 2
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IP-10	Interferon gamma induced protein 10
JAK1	Janus kinase 1
JAK3	Janus kinase 3
KDa	Kilo Dalton
MCP-1	Monocyte chemoattractant protein -1
MIP	Macrophage inflammatory protein
NCBI	National center for biotechnology information
NHP	Non-human primate
PBMC	Peripheral blood mononuclear cell
PI3K	Phosphoinositide 3-kinase
Pyk	Pyruvate Kinase
SCID	Severe Combined Immunodeficiency
SNP	Single nucleotide polymorphism
Sn-RNP	Small nuclear ribonucleic particles
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TNF-α	Tumor Necrosis Factor alpha
TSLP	Thymic stromal lympho poietin

1 INTRODUCTION

1.1 INTERLEUKIN-7

Interleukin-7 is a non-redundant cytokine; it plays a central role in the immune system in particular in the development and maintenance of T-cell subsets. IL-7 was originally discovered as a pre-B-cell growth factor [1] but shortly afterwards identified as an essential cytokine in T-cell homeostasis [2]. IL-7 and its receptor, the Interleukin-7 receptor (IL-7R), are key mediators of T-cell homeostasis. The importance of IL-7 responses in T-cell biology is evident in knockout mice, as IL-7 gene-deleted mice fail to develop a functional immune system [2,3].

IL-7, due to its proinflammatory effects on the immune system, plays a double role. The potential of IL-7 to enhance and maintain immune response signifies its importance in infection, but IL-7 mediated effects can be detrimental in autoimmune responses. IL-7 stimulates immune responses, mainly through upregulation of IFN-γ production. This may be one of the factors mediating increased survival of *M. tuberculosis* infected mice treated with IL-7 [4]. IL-7 has also been suggested to serve as an early biomarker in sputum, which might help to predict mycobacterial clearance in TB patients [5].

Contrary to the role of IL-7 in infectious diseases, increased IL-7 levels in autoimmune diseases may lead to increased disease severity. Rheumatoid arthritis (RA) is an autoimmune disease which is characterized by increased levels of TNF-α, and IL-7 is a potent inducer of T-cell dependent TNF-α. It is also believed that IL-7 is capable of inducing inflammation, independent of TNF-α, contributing to autoimmune disease progression [6]. Chronic inflammatory conditions, like non-T non-B cell- mediated colitis, are reported to be prevented by IL-7 deficiency, hence intestinal mucosal cells expressing high levels of IL-7R are potential targets for therapeutic interventions in T-cell biology [7].

1.1.1 Sources of Interleukin-7

Interleukin-7 has been known for its minute presence in serum and the measurement of IL-7 has proved to be difficult. The low IL-7 levels might be attributed to local production. Bioactive IL-7 could also be bound to extra-cellular matrices, as in the case of mesenchymal fibroblasts during thymocyte development; IL-7 may therefore not be available in serum [8]. IL-7 is also known to dimerise with the hepatocyte growth factor beta, this chain can facilitate localized IL-7 interaction at stromal cell surfaces [9]. IL-7 expression is detected in stromal cells of bone marrow and thymus [10,11], intestinal epithelium cells [12], adult liver [13], dendritic and follicular dendritic cells [14,15], keratinocytes [16] as well as at the transcript level in various organs, including the brain [17,18].

1.2   INTERLEUKIN-7 RECEPTOR

The functional interleukin-7 receptor unit consists of the interleukin-7 receptor α (CD127, here referred as IL-7R) and the common γC cytokine receptor chain (CD132) [19]. The IL-7R is expressed on cells of lymphoid origin, particularly developing T-cells and B- cells, on mature T-cells, as well as on bone marrow derived macrophages. IL-7-R is also known to be expressed on non-immune cells including epithelial cells, bone marrow stromal cells as well as on transformed cells, e.g. cutaneous T-cell lymphoma cells, colorectal cancer cells, breast cancer cells and pancreatic cancer cells. The IL-7R molecule itself also acts as a co-receptor in the thymic stromal lymphopoietin receptor (TSLPR) complex which consists of the TSLPR and IL-7R heterodimer. The importance of IL-7R is evident in knockout studies, where IL-7R knockout results in a severe combined immunodeficiency (SCID) phenotype.
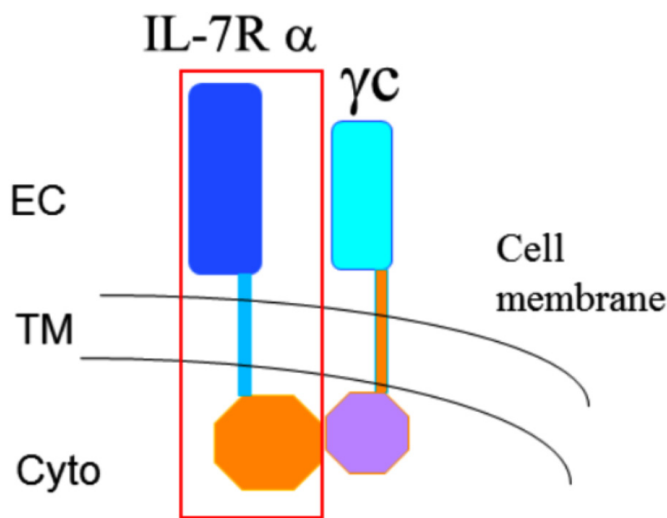

Fig1: The organization of the IL-7R complex. The IL-7R unit consists of a heterodimer of IL-7Rα and the common γc receptor chain. The IL-7Rα protein is divided in three domains, the extracellular domain (EC) for binding to the IL-7 molecule, the transmembrane domain (TM), and the cytoplasmic domain (Cyto) containing signaling elements.

The full length IL-7R protein consists of 459 amino acids, of which the first 20 amino acids code for the signal peptide. After peptide cleavage, the mature protein forms a 49.5 KDa functional unit. The IL-7R is a membrane anchored protein with 220 amino acids forming an extracellular domain for interaction with IL-7, and a 195 amino acid cytoplasmic domain, which recruits downstream signaling complexes upon stimulation [20].

1.3   IL-7 MEDIATED SIGNALLING

IL-7 binding to IL-7R triggers the dimerization of the IL-7-IL-7R complex with the common γc cytokine chain in T-cells. This complex triggers downstream phosphorylation processes, with the γc chain phosphorylation of JAK3, and the IL-7R intracellular domain phosphorylatinγ JAK 1. The JAK3p facilitates JAK1 mediated

phosphorylation on the IL-7R cytoplasmic domain at the Tyrosine 449 (Y449) amino acid, as the IL-7R does not have tyrosine kinase activity of its own [21,22].
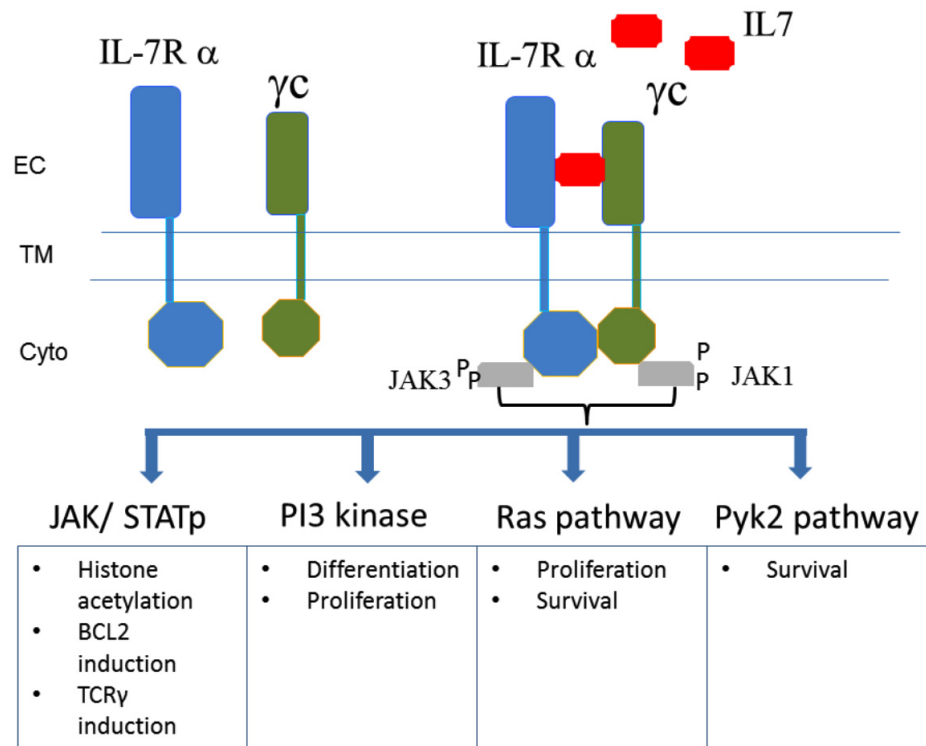

Fiγ 2: Schematic diagram of IL-7 signaling cascade events. The surface bound IL-7R, upon interaction with the IL-7 molecule, dimerises with common γc receptor chain to facilitate phosphorylation of downstream JAK1 and JAK3 kinases. The phosphorylated kinases phosphorylate in turn the STAT family proteins (STAT5A, STAT3) as major mediators of signaling, they could also facilitate the signaling event by a PI3 kinase, the Ras or Pyk2 pathway.

The phosphorylated tyrosine amino acid creates a docking site (SH2 domain) on the IL-7R for facilitation of signal transducer and activation of transcription proteins (STATs). The STAT family proteins are major transducers of IL-7 activity which is mainly carried out by STAT5 proteins mediating rapid trans locating of signals to the nucleus. STAT3 and STAT1 are also known to be phosphorylated by the IL-7-IL-7R signaling complex [23].

1.3.1 Effects of Interleukin-7

1.3.1.1 IL-7 and αβ T-cells

Although IL-7 was discovered as a B-cell growth factor in mice, IL-7 appears to influence almost every stage of T-cell development in both humans and mice. The thymic development of T-cells is defined by the presence of CD4+ and CD8+ markers on T-cell precursors. During thymic development, T-cells develop from the absence of both markers (double negative) stage, followed by co-expression of both CD4 and CD8 marker (double positive), and eventually to a single positive CD4+ or CD8+ T-cell. The IL-7R expression is associated with key cellular decisions. For instance, IL-7 promotes

TCR-γ chain rearrangement. Absence of IL-7 has been shown to block T cell development at the double negative stage in mice, as it is a non-redundant factor for early thymocyte survival [24]. The IL-7R expression is an absolute requirement for T-cell precursors to survive, except at the late double negative stage, where IL-7 facilitates expression of genes essential for differentiation and progression [25,26]. The expression of IL-7R on double positive T-cells is low, but rapidly regained after passing positive selection and associated with T-cell survival [27]. Also the upregulation of IL-7R at the end of the double positive cell stage is associated with lineage selection, it is a key event in CD8 T-cell line commitment [28]. IL-7 leads to increased thymic output and also to peripheral T-cell expansion and survival. This is particularly important in adulthood when thymic supply of naïve T-cells is reduced.

The effect of IL-7 on mature T-cells is equally important, since IL-7 mediates its survival, homeostasis and differentiation [29,30,31]. The peripheral availability of IL-7 is very low and until recently, the normal values of circulating IL-7 could not be reliably measured. The normal range of IL-7 in human blood is 8-12 pg/ml and appears to be a limiting factor in T-cell survival and proliferation. It has been proposed that the availability of IL-7 is regulated by its usage, mainly through T-cells [8,32]. The IL-7Rα is expressed by most resting T-cells and is downregulted during IL-7-mediated signaling and T-cell activation [33]. Also, it has been shown in Multiple Sclerosis (MS), that the soluble forms of the IL-7R gene sequester free IL-7 and could work as a sustained release `stockpile´ regulating the availability of free IL-7 molecules to surface bound receptors [33,34].

1.3.1.2   IL-7 and other immune cells

Not only TCRαβ T-cells, yet also other immune cells express the IL-7R. TCR γδ T-cells homeostasis is also partially controlled by IL-7 signaling [35]. IL-7 is essential in γδ T-cell development shown by the absence of γδ T-cells in IL-7R knockout mice [36]. Mature γδ T-cells are known to increase production of IL-17 upon IL-7 stimulation. A recent report also demonstrated that IL-7 stimulation selectively promotes IL-17 producing γδ T-cells, mediated by the STAT3 signaling pathway [37,38].

Natural Killer (NK) cells have been shown to express IL-7R in murine models as well as in humans; IL-7 has been shown to be essential for human NK cell development [39]. Mature CD56+ (bright) NK cells express the IL-7R, whereas few CD56+ (dim) NK cells stain positive for the IL-7R. IL-7 stimulation of NK cells leads to downregulation of surface IL-7R expression and induction of the BCL-2 protein, suggesting IL-7 mediated anti-apoptotic activity in NK cells [40].

The monocytes and monocyte derived cells also express IL-7R. During monocyte to macrophage differentiation, the IL-7R is expressed along the differentiation process and the IL-7R transcript is differentially expressed between M1 and M2 macrophages [41]. IL-7 acts also as a co-stimulus during monocyte differentiation towards dendritic

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cells and induces a distinct phenotype characterized by upregulation of the CD21 like molecule [42].

1.3.1.3   IL-7 and non-immune cells

IL-7´s potential to upregulate in general immune reactivity is being increasingly investigated with promising results, but there are limited studies investigating the effect of IL-7 on non-immune cells. The IL-7R is not only expressed by immune cells, but also by various non-immune cells, including transformed cells, such as lung cancer [43] and breast cancer cells, where IL-7 has been shown to signal via the IL-7R complex measured by STAT5 phosphorylation [44]. While it is undisputed that IL-7 would promote most T-cell malignancies [45]; recent data indicated that IL-7 stimulation of solid tumor cell lines, e.g. breast cancer cells, would lead to increased tumor cell proliferation [46]. IL-7 has also been shown to direct lineage decisions in the case of human neuronal progenitor cells [18].

1.3.2   Interleukin-7 in Immunotherapy

The property of IL-7 to act as a potent co-stimulatory factor in T-cell mediated responses, its role in promoting growth of cytotoxic T cell and its property to induce lymphokine activated killer cells (LAK), independent of Interleukin-2, makes it an attractive candidate cytokine for cancer treatment [47]. IL-7's capacity in NK cell restoration and upregulation of other cytokines like IL-1α, IL-6, TNF-α and IFN-γ signifies its potential in antitumor therapy [48]. Interleukin-7 therapy helps to restore thymopoiesis after chemotherapy. The advantage of using interleukin −7 in immune restoration is that activation of thymopoiesis may be achieved without activating T-cells [49], bearing an increased risk of developing autoimmune responses. Intratumoral administration of IL-7 has been shown to eradicate tumors in a murine lung cancer model; interleukin-7 increased CD4+ and CD8+ cell counts by up to 300% and 700% respectively [50,51,52]. In contrast, IL-7 may also drive expansion of IL-7R positive tumor cells [53], or lymphocytic leukaemia cells [54].

IL-7 may be used as a potent adjuvant in the treatment of HSV infections and be associated with a better clinical outcome [55]. A number of studies have been performed to investigate role of IL-7 in anti-retroviral (HIV) treatment. IL-7 has been shown to be critical in the induction and maintenance of cytotoxic T-cell responses (CTL) against HIV-infected cells. Downregulation of IL-7 receptor results in impaired CTL responses in HIV-infected individuals. IL-7 could also be a potent agent to treat patients, in whom T cell numbers have dropped below critical levels; IL-7 serum levels could be useful as a potential reference point to monitor patients undergoing antiretroviral therapy [56,57].

Despite the advantages of interleukin-7 in antiviral therapy, especially in treatment of HIV, it is speculated that IL-7 might increase viral load; fas-mediated human neuronal apoptosis might also occur [58]. A recent report, investigating IL-7 effect in HIV

infection, demonstrated an association of IL-7 treatment and viral transcripts during antiretroviral therapy [59].

The potential of IL-7 to activate immune responses qualifies IL-7 for anti-bacterial therapy. Studies in *Toxoplasma gondii* infection in a murine model suggested that IL-7 augments anti-toxoplasma responses mainly through increased IFN-γ production. In contrast, exogenously added IL-7 may also drive B cell lymphopoiesis via increase in T helper type 2 responses, which may counteract the effects of cellular immune responses [60].

1.3.3 IL-7 and IL-7R Genes

The human IL-7 gene is located on chromosome 8q 12-13 [61] and spans around 3kbp (NM_000880, NCBI) nucleotides. The region consists of 6 exons, pre-mRNA processing yields a 534 bp coding sequence corresponding to a 177 amino acids protein. The first 75 nucleotides (25 amino acids) form a signal peptide which is cleaved off to form a 152 amino acids protein. The IL-7 gene, based on cDNA alignment, is rather preserved in primates which suggests a similar 6 exonal structure. The murine IL-7 gene lacks nucleotides corresponding to exon 5 in the human IL-7 gene [62].

Fig 3: Structure of the IL-7 gene. Exons are in different colors in the 3D IL-7 model.

The human IL-7R gene is located on chromosome 5p13 constituted by approximately 23kbp of nucleotides on the DNA strand (NC_000005.9, NCBI). This region encodes a pre-mRNA consisting of 8 exons, exons 1-5 correspond to the extracellular domain and exon 7 and 8 form the intracellular domain of the IL-7R protein with amino acids at exon 6 corresponding to the transmembrane link between the extracellular and intracellular IL-7R domain [63].

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1.4 CYTOKINE PROTEIN ISOFORMS

Protein isoforms are abundantly observed in cytokines and their receptors. Protein isoforms lead to increased proteomic diversity over ´constant` genetic material. There are three known mechanisms which facilitate isoform formation:

- Single Nucleotide Polymorphism at the genome level
- Alternative splicing at the RNA level
- Proteolysis and proteomic modifications

These methods lead to formation of variant proteins from a single piece of genetic information [64,65,66]. It is known that proteins are highly specific in their action and that specificity is linked to the protein structure, dependent on protein size and amino acid sequence. As both of these parameters are highly variable in protein isoforms, it is essential to examine if cytokine isoforms have a functional role in immune responses.

1.4.1 Single Nucleotide Polymorphism

Single Nucleotide polymorphisms (SNPs) are DNA sequence variations that occur within a population when a single nucleotide in corresponding DNA location is different between the two DNA strands. This is a very common event occurring approximately once every 100 to 300 bases and appears to be random. These variations are diffused in the genome and may occur in the coding sequence where it could cause point mutations which could be synonymous i.e. not affecting corresponding amino acid of the protein product, or non-synonymous, causing a single amino acid variation in the final protein product [67].

These SNPs could also be part of non-coding sequences which are essential in determining DNA-protein or RNA-protein complex initiation and therefore altering gene regulation dynamics. Susceptibility of certain diseases, the course of disease and the response against a pathogen in an individual has been successfully linked to SNP patterns [66].

SNPs in the IL-7R are found to be associated with the clinical outcome and presentation of inflammatory diseases. The genomic region of IL-7R shows more than 15 SNPs [68]. Rs6897932 in exon 6 of the IL-7R is a C-T polymorphism, known to be a significant polymorphism in susceptibility to develop multiple sclerosis (MS): the single nucleotide variation leads to increased splicing of exon 6 in individuals with the C- allele whereas the T- allele decreases splicing. In contrast, the C- allele is the most common allele in rs6897932 IL-7R which is present in approximately 80 % of individuals and is linked with susceptibility to develop MS. IL-7R is the only known gene, except HLA genes, associated with increased risk to develop MS. SNPs, even in intronic sequences, may also impact on RNA expression and composition, due to predisposing pre mRNA for a particular mRNA splicing event or affecting binding sites of RNA regulatory elements [69,70].

1.4.2   Alternative splicing

Alternative splicing of pre RNA is a phenomenon extensively seen in eukaryotic cells. Estimated 95% of all human multi exonal genes undergo the process of pre- mRNA alternative splicing [71]. It is a highly regulated process controlled by regulatory motifs which interact with protein binding sites on pre-mRNA sequences. The splicing event of pre-mRNA is dependent on the facilitating mediators and the multi-exonal structure of the underling gene [72].

1.4.2.1   Mechanism of alternative splicing

The process of alternative pre-mRNA splicing is mediated via the spliceosome complex. The spliceosome complex recognizes the intron boundary at GU nucleotide at the 5´ intron site to the AG nucleotide at 3´ exon end [64]. The core spliceosome consist of U1 to U6 small nuclear ribonucleoprotein particles (snRNPs) along with accessary components.
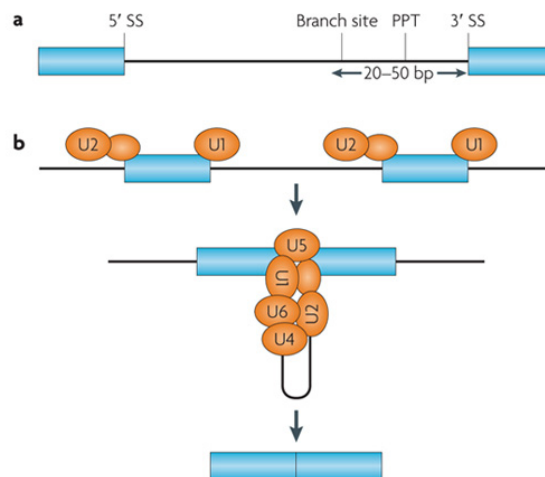

Fig 4: Schematic representation of the process of alternative splicing. (From Karen H. et al, 2010 [73]). The diagram (a) shows organization of exon-intron structure on a pre-mRNA. An intronic sequence (black line) is flanked by two exons (blue). The intronic sequence contains the nucleotides corresponding to the branch site and polypyrimidine repeats which take part in initiating the alternative splicing process. The figure (b) represents schematically three major events in the alternative splicing process i) snRNP U1 and U2 recognizing the 5´and 3´splicing site respectively, which upon recruitment of accessory spliceosome proteins forms a loop ii) the intron sequence bringing the adjoining splice sites together, finally connecting the 5´and 3´ends iii) to form a continuous mRNA sequence.

The initiation of splicing complex is mediated via the U1 splisosome protein identifying the GU nucleotide sequence which marks the end of an intron and the U2 snRNP binding to the branch site defined by polypyrimidine tract prior to the 3´AG nucleotides in the next exon in an ATP dependent manner. The bound U1 snRNP facilitates recruitment of the U4/U5/U6 trimer leading to formation of the U1/4/5/6 complex. The U5 snRNP binds with the 3´exon boundary and U6 and U2 bind to form a loop in the RNA structure. U1 is released from the spliceosome complex and U6 binds with 5´intron splice site with the U5 shifting from exon to intron sequence.

U6/U2 mediates trans-esterification of 5'end of the intron and ligates it to an adenine nucleotide on the intron. Similarly, U5 binds the exon at 3' splice site. The 5' site is cleaved, resulting in the formation of the lariat RNA-binding proteins. The open ends of exons are ligated by ATP hydrolysis and the intronal loop, containing spliceosomal proteins, is finally released [74].

1.4.2.2 Regulation of alternative splicing

The regulation of alternative splicing can be discussed at two distinct levels, firstly to understand the factors leading to a particular splicing pattern and secondly, regulation of alternatively spliced products.

Factors regulating alternative splicing involve interaction of protein-protein, protein-RNA, and RNA-RNA domains, organized in a tissue specific manner [72,75]. These sequences reside in introns to facilitate binding of spliceosome units to identify exons and eventually to process and connect two adjoining exons.

The first regulation facilitates the recognition of a splicing site, mediated via the SR protein family which contains conserved repeats of serine and arginine. These SR domain proteins facilitate binding of U1 snRNP to the 5´splice site and the U2 complex at the 3´splice site of the pre- mRNA in a phosphorylation dependant manner. There are more than 30 known SR family proteins, which are expressed in a tissue specific fashion [76].

The alternative splicing regulation is also influenced via the active inhibition of the splicing site recognition. It can be a direct effect of splicing silencers, blocking pre-mRNA splicing sites or the splicing enhancer, as seen in the polypyrimidine-tract binding protein (which interacts with pre-mRNA regions with pyrimidine repeats); it prevents the U2 complex elements from interacting with the exons. Similarly, there are proteins which actively block 3´splice site recognition of the U1 snRNP complex. Other regulator proteins act in a site-dependent manner either, promoting or inhibiting splicing events. The net alternative splicing therefore represents an outcome of competition between various splicing promoters and inhibitors [77].

A differential splicing event can induce frame shifts in mRNA sequences. These frame shifts could result in abrupt termination of protein synthesis, due to insertion of a premature stop codon, or disrupt the stop codon and give rise to a large coding sequence. After the alternative splicing has been completed, there are mechanisms which dictate stability of the resultant mRNA transcripts. ´Nonsense mediated decay` and ´sense or nonstop decay` are the most common phenomena. In `nonsense mediated decay´, abruptly terminating transcripts are stopped from undergoing translation as the premature stop codon is detected by regulatory proteins, which facilitates the degradation of the target mRNA. Alternatively, transcripts which lack the stop codon may be degraded by exosome complexes [78,79].

1.5 ALTERNATIVE SPLICING OF THE IL-7 AND IL-7R GENE

1.5.1 Alternative splicing of the IL-7 gene

Alternative IL-7 RNA products were first described by Goodwin and colleagues who identified the human IL-7 gene in a hepatocellular carcinoma cell line [80]. Other studies showed alternative splicing in small intestinal epithelial cells by identifying two distinct IL-7 RNA species. Both IL-7 isoforms were also found in the lamina propria, bone marrow, thymic tissue, in Caco-2 as well as T84 (human) epithelial colorectal adenocarcinoma cell lines. Interestingly, the amount of transcript found for both IL-7 isoforms was equal in bone marrow and intestinal epithelial cells [12].
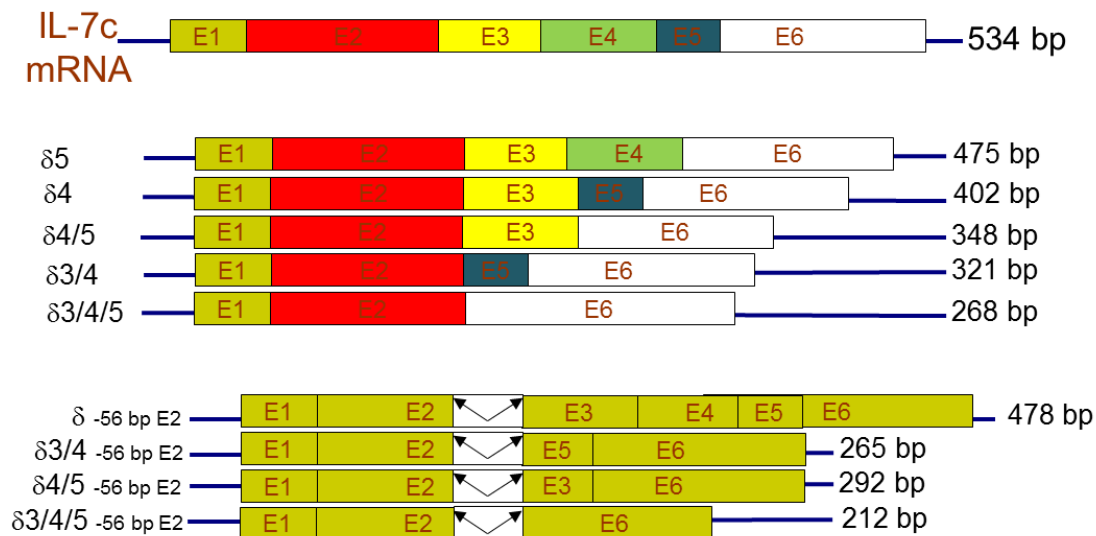

Fig 5: Observed alternatively spliced variants for the human IL-7 transcript

Mapping of IL-7 alternative splicing, at the transcript level, has been done previously from our group and suggested a tissue specific, alternative splicing pattern. Until now, nine IL-7 alternatively spliced isoforms have been described in humans; 7/9 IL-7 isoforms are also shown to be expressed in malignant hematopoietic cells. Some of these represent out of frame-spliced products of IL-7 pre-mRNA; these variants have spliced a 56 base pair fragment in the second exon. This results in a truncated translation sequence which has a stop codon shortly after the splicing junction. This might lead to degradation of the transcript by nonsense mediated decay (NMD) or production of a short peptide due to premature termination of protein synthesis.

1.5.2 Alternative splicing of the IL-7-R gene

The interleukin-7 receptor has originally been described to exhibit two alternatively spliced products: one membrane-bound and a soluble form [81]. The membrane bound form is devoid of any deletions in alternative splicing and corresponds to the complete coding sequence of IL-7R gene (IL-7Rc), whereas the soluble IL-7R lacks both the cytoplasmic and the trans-membrane domain as a consequence of exon 6 splicing, which induces a frame shift.
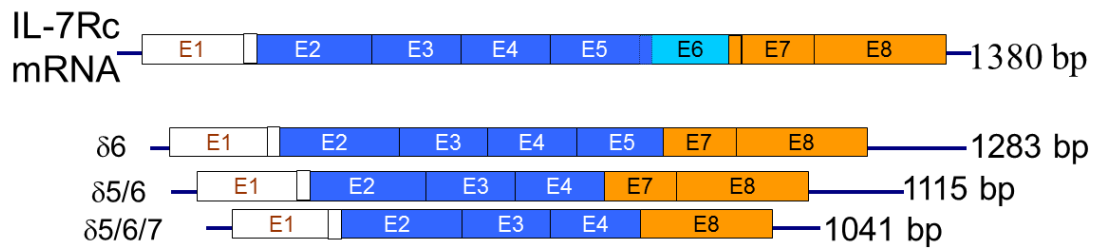
Fig 6: Alternative splicing of the IL-7R gene in humans.

Two additional, soluble IL-7Rs have recently been identified, one lacking exon 5 and 6, which also translates into a truncated protein sequence after the splicing event. The other lacks exons 5, 6 and 7 and uses the same stop codon as in the IL-7Rc [82].

1.6   FUNCTION OF ALTERNATIVE SPLICING IN THE IMMUNE SYSTEM

Alternative splicing in the immune system, and the complexity of resulting immune responses have recently been more appreciated. For instance, IL-2δ2 and IL-2δ3 are known to antagonize the activity of full length IL-2, defined by their ability to inhibit IL-2 mediated T-cell proliferation [83]. The cytokine isoform IL-4δ2 acts as an antagonist compared to IL-4 activity and has been observed to be elevated in individuals who control latent infection with *Mycobacterium tuberculosis;* it was also observed in individuals with *Helicobacter pylori* infection [84,85,86]. Alternatively spliced IL-15, lacking 21 amino acids corresponding to its signal peptide, represents an intracellular molecule which regulates the production of full length IL-15 [87]. IL-6δ6 is known to bind with the GP130 protein, suggesting it might be bio-active [88]. Some cytokine isoforms act as indicators for a disease as described for IL-10δ3, which represents a positive prognostic marker for acute lymphoblastic leukemia [89]; TNF-αR2δ7/8 levels are typically reduced in diabetes type 2 and the IL-13Rδ10 expression is associated with allergy [90,91].

The soluble cytokine receptors were thought to function as inhibitors of the membrane bound forms. Studies in the context of cytokine receptors suggest four different classes of soluble receptor functions:

(A) Receptors generated due to proteolysis of membrane bound receptor causing prevention of signal generation,
(B) Soluble receptors as binding proteins to stabilize ligands in extra-cellular space,
(C) Soluble receptors competing with membrane bound receptors to decrease the ligand signal and
(D) Soluble receptors associating with nonbinding receptor subunits and ligands to confer ligand sensitivity to cells that do not express specific membrane bound receptor for the respective ligand [92].

2 AIMS OF THE THESIS

We postulated that IL-7 splicing variants might have specific functions translating into the aims of the thesis:

- To define isoform expression patterns of human IL-7 and IL-7R in chronic inflammation (Paper I).
- To define isoform expression patterns of IL-7 and IL-7R associated with chronic inflammation in a *Rhesus macaque* model (Paper II).
- To identify IL-7 alternatively spliced products at the protein level (Paper III and Paper IV).
- Recombinant expression and biological characterization of the IL-7 isoform proteins (Paper III and Paper IV).

3 MATERIALS AND METHODS

Detailed description of material and methods used in this thesis are provided in the respective articles and manuscripts, a brief summary of the material and methods is presented here.

3.1 STUDY 1

We investigated the extent of alternative splicing of the human IL-7 and IL-7R gene in peripheral blood mononuclear cells (PBMCs) from healthy individuals (n = 15) and patients with multiple sclerosis (n = 16) to understand the extent of alternative splicing of the IL-7 and IL-7R gene and its impact on disease. The PBMCs were recovered using gradient separation and subjected to mRNA extraction and cDNA synthesis. The cDNA was amplified in a PCR reaction using sequence specific primers covering alternatively spliced products. Gene specific products were visualized using an agilent 2100 bioanalyser. To visualize the relative distribution of IL-7 and IL-7R spliced variants within the sample, the relative area under the curve values was plotted as percent abundance of the variant within the sample and correlated with the clinical endpoints of the study.

3.2 STUDY 2

In the second study, we investigated the role of IL-7 and IL-7R alternative splicing in chronic inflammation (*Mycobacterium tuberculosis* (MTB) infection) using a non-human primate (*Rhesus macaque*) animal model. The animals were divided in three groups and were vaccinated with two different vaccine regimes using BCG (n=6) and recombinant BCG (n=6) followed with a protein boost along with unvaccinated controls (n=3). Subsequent challenge by a virulent MTB strain revealed increased survival in the group who received the recombinant BCG vaccine. We investigated the extent of IL-7 and IL-7R alternative splicing in PBMCs and secondary lymphoid organs from the study groups and also quantified IL-7, TGF-β and IL-17 production at the site of infection using immunohistochemistry, to understand the role of IL-7 in MTB infection and pulmonary pathology.

3.3 STUDY 3

In a third study we investigated differential effects of IL-7c and IL-7δ5 proteins on CD4+ and CD8+ T- cell subsets. In an explorative approach, we sorted CD4+ and CD8+ T cell subsets followed by stimulation with either IL-7c or IL-7δ5 along with unstimulated controls for 72 hours. Whole genome gene expression changes were identified using a microarray platform. Differences in IL-7 and IL-7δ5 effects were also examined using T-cell phenotype analysis and downregulation of the IL-7R expression upon stimulation with IL-7 and its isoforms. In order to identify mechanistic properties of IL-7 or IL-7δ5 binding to IL-7R molecule, we used the Biacore platform to identify differences in binding strength between the receptor and the ligand pairs. Monospecific antibodies detecting specifically IL-7δ5 protein were generated and used to measure

expression of IL-7δ5 protein in lymph nodes in chronic infection disease settings (HIV and tuberculosis).

3.4 STUDY 4

Differential effects of the IL-7c versus the IL-7δ5 splice variant were studied in regard to CD14 monocyte activation. Sorted CD14+ cells from healthy donors were isolated and stimulated with IL-7c and IL-7δ5 (along with unstimulated controls in triplicate); changes in gene expression levels after 72 hours were measured using an RNA microarray platform. Cell culture supernatants were examined for differential production of immune mediator cytokines and chemokines by a luminex assay. IL-7δ5 induced a pro- inflammatory profile in CD14+ monocytes, associated with oxidized low density lipoprotein metabolism and upregulation of the oxidized low density lipoprotein receptor 1 (OLR1) gene, confirmed by flow cytometry. In order to investigate IL-7δ5 protein expression, we performed immunohistochemistry to identify IL-7δ5 protein expression in association with health and disease [93]. We were able to show IL-7δ5 production in cells of healthy and diseased human tissues and proposed a new action of the IL-7/IL-7δ5 axis in monocytes affecting lipid metabolism.

4 RESULTS AND DISCUSSION

4.1 IL-7 AND IL-7R VARIANTS IN HUMAN PERIPHERAL BLOOD

4.1.1 IL-7 isoform expression in human PBMCs

Interleukin 7 is one of the driving factors for persistence of autoimmune responses. In multiple sclerosis (MS) increased IL-7/IL-7R polymorphism is correlated with disease pathogenesis [94]. The role of IL-7 alternatively spliced variants has not yet been examined. We investigated the extent of alternative splicing of IL-7 gene in PBMCs from patients with MS and compared it with the IL-7 pattern in blood healthy individuals.

Human PBMCs expressed five alternatively spliced IL-7 variants in measurable amounts. Interestingly, IL-7δ4 was the most abundant IL-7 transcript in patients with MS and in blood from healthy individuals followed by the full length IL-7c transcript.
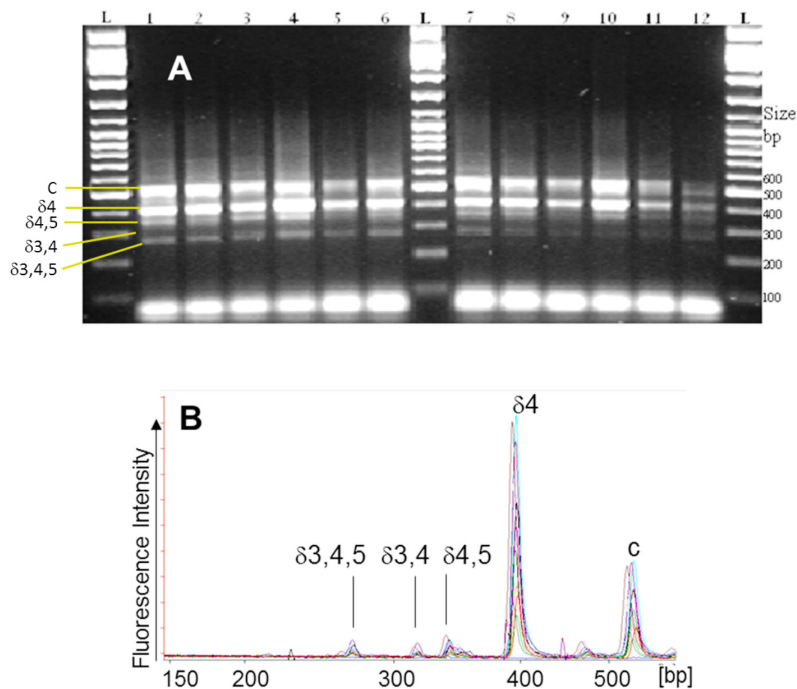

Fig 7: Expression of IL-7 alternatively spliced variants in human PBMCs. The PCR products were obtained by using primers covering full length of the IL-7 transcript. The gel image above (A) shows IL-7 PCR products after agar gel electrophoresis; lane L represents the DNA ladder and lane 1 to 12 are IL-7 amplification products in different samples. The band above 500 bp denotes full length IL-7 cDNA (541 bp PCR product) followed by IL-7δ4, IL-7δ4,5, IL-7δ3,4 & IL-7δ3,4,5 amplicons. Each PCR product was also analyzed using the Agilent 2100 bioanalyzer. In the figure (B) each color represents an individual sample, this enabled measurement of each product for calculating relative abundance of the respective IL-7 spliced variant.

4.1.2 Relative distribution of IL-7 isoforms

Agilent analysis enabled us to measure the amount of each band in the respective sample, defined by the area under the curve. After obtaining data from all study samples, the relative abundance of the respective spliced variant was examined in blood from healthy individuals compared to pattern in blood from patients with MS.
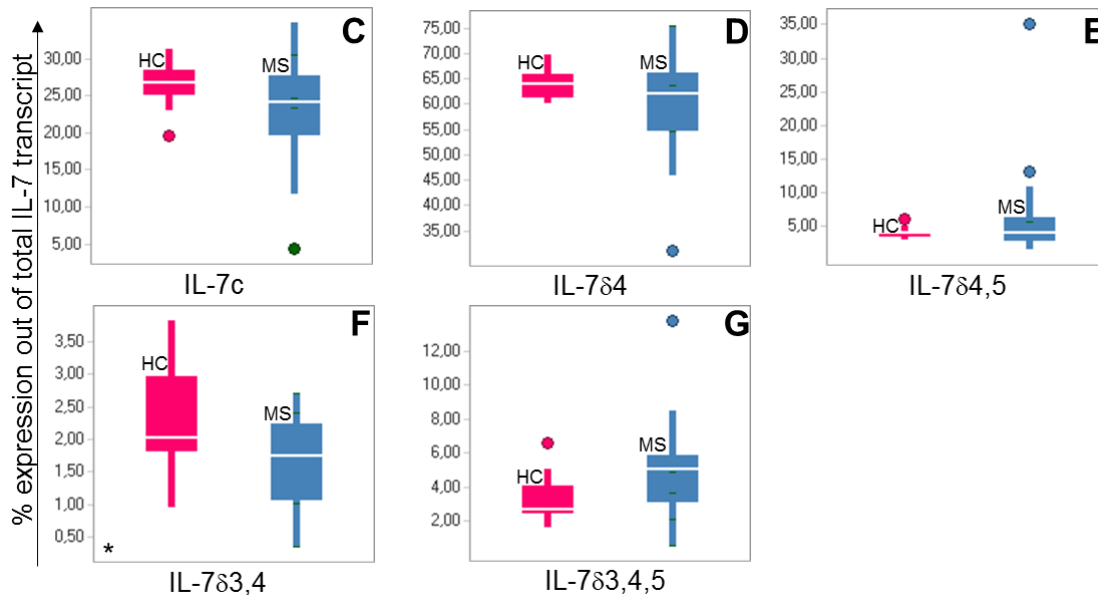

Fig 8: Relative distribution of IL-7 spliced variants in blood from healthy donors and patients with MS. In both study groups, IL-7δ4 constituted the most abundant IL-7 transcript comprising almost 60 percent of all IL-7 variants. The relative abundance of IL-7 spliced variant IL-7δ3,4 was statistically higher (p = 0.03) in healthy individuals when compared to expression in blood from patients with MS.

4.1.3 IL-7R isoforms in MS

Human IL-7R gene alternative splicing analysis revealed four distinct transcripts for the IL-7R gene. Interestingly, all alternative spliced products of the IL-7R were lacking exon 6, which represents amino acids corresponding to the transmembrane region of the protein, inducing a frame shift coding for a shorter protein. The resulting protein,
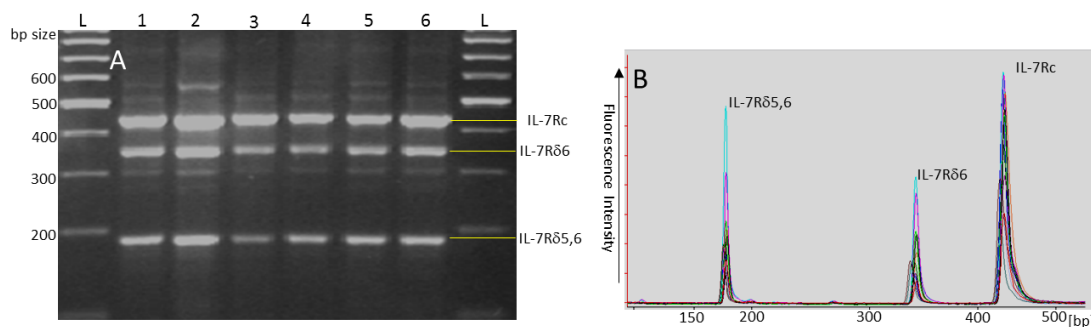

Fig 9: IL-7 receptor alternatively spliced variant analysis in peripheral blood mononuclear cells amplified with primer pair covering exon 4 – exon 8 region of IL-7R. The IL-7R transcript exhibited 3 distinct measurable spliced variants on gel (A) and by agilent capillary fragment analysis (B). The full length transcript (IL-7c) with a 446 basepairs (bp) product was the most abundant transcript followed by IL-7Rδ5,6 at 180 bp and IL-7δ6 at 350 bp.

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which lacks a transmembrane or intracellular component, would be secreted as a soluble IL-7R (sIL-7R). Yet it is still to be determined if viable IL-7Rδ6 and IL-7Rδ5,6 proteins exist in humans and contribute to the sIL-7R pool.

4.1.4 Conclusion Study 1

This study was the first attempt to identify IL-7 and IL-7R spliced variants in PBMCs from healthy human volunteers and allowed to compare isoform expression patterns in health and disease. Interestingly, IL-7δ4, and not the full length IL-7c transcript constituted the most abundant IL-7 transcripts in human PBMCs. The expression profile of IL-7 spliced variants exhibited a difference in relative expression in health and MS, which suggested a potential role of IL-7 variants in MS. The study also provided cDNA sequences which constituted the basis for further studies using recombinant proteins.

This report also investigated the extent of IL-7R gene alternative splicing in peripheral blood of healthy individuals for the first time and identified a novel IL-7R alternative splicing event (IL-7Rδ5,6,7). We could also confirm increased exon 6 skipping in patients with MS and its association with SNP rs_6897932.

4.2 IL-7 AND IL-7R ISOFORMS IN INFECTION

In the second study, we investigated the role of IL-7 and IL-7R alternative splicing in infection using rhesus macaques as an animal model. Human and rhesus macaque have a similar structure of the IL-7 gene which suggested that the genetic assembly of IL-7 and IL-7R gene may also be similar due to evolutionary conservation. The study animals were subjected to immunization regimes (Table 1) using BCG or a modified

Group	Prime	Boost	MTB challenge
A (n=6)	BCG	+	+
B (n=6)	recombinant BCG	+	+
C (control, n=3)	Saline	-	+

Table1: immunization strategy used to vaccinate NHPs, followed by an MTB challenge.

recombinant BCG strain, followed by a protein vaccine boost. After the immunization regime, the study group was challenged using a virulent MTB strain to investigate the immunization efficiency of the two vaccines. The challenge experiment showed increased survival in the recombinant BCG vaccinated group.

Peripheral blood samples were collected before and after immunization and MTB challenge; also lung tissue lymph node (pulmonary, hilus and axillary) and spleen tissues were obtained and investigated for measurement of IL-7 and IL-7R alternatively spliced variants.

4.2.1 IL-7 isoform expression in rhesus macaques

The IL-7 transcript analysis in the PBMCs revealed extensive alternative splicing. DNA sequencing confirmed the presence of seven alternatively spliced variants of IL-7 in rhesus macaques. The six exon covering IL-7c was identified as the most abundant transcript in tissues investigated, other IL-7 variants lacking either exon 3, exon 4, exon 4/5 or exon 3/4/5 were being observed in minute quantities. The sequencing also revealed out of frame spliced IL-7 variants lacking 56bp from exon 2.

Clone	Insert	Exon structure*	Size (bp)
NHPIL-7-1	NHPIL-7c	Full length NHP IL-7	534
NHPIL-7-2	NHPIL-7δ3	Lacks Exon 3	453
NHPIL-7-3	NHPIL-7δ4	Lacks Exon 4	402
NHPIL-7-4	NHPIL-7δ4,5	Lacks Exon 4 & 5	348
NHPIL-7-5	NHPIL-7δ3,4,5	Lacks Exon 3,4 & 5	268
NHPIL-7-6	NHPIL-7δ4,5,-56E2	Lacks Exon 4,5 & 56 bp from exon 2	292
NHPIL-7-7	NHPIL-7δ3,4, -56E2	Lacks Exon 3,4 & 56 bp from exon 2.	266

Table 2: IL-7 isoforms observed in NHP PBMCs after sequencing. The size indicates the number of nucleotides from the start to the stop codon. (*Exonal structures are denoted after aligning respective sequence against the human IL-7 sequence).

4.2.2 IL-7R isoform expression in rhesus macaques

The IL-7R gene also exhibited similar splicing events as in humans, but represented a relatively large transcript corresponding to the surface bound form (IL-7Rc) in rhesus macaque peripheral blood mononuclear cells. Interestingly, the investigation of IL-7R alternative splicing at the site of infection i.e. in lung tissue from the study animals revealed that most of the IL-7R transcript produced was corresponding to the sIL-7R lacking exon 6. This increased sIL-7R was not differentially expressed in the study groups but was associated with the anatomic location as the increased exon 6 skipping could also be seen in healthy uninfected rhesus macaques and in human healthy lung tissue; the soluble IL-7R was found to be the most abundant IL-7R transcript in lung tissue.

4.2.3 Increased expression of IL-7c in infection

Next, we investigated IL-7 protein production by immunohistochemistry at the site of infection (lung). The animal group which received the recombinant BCG vaccine showed the highest amount of IL-7 protein, followed by the BCG vaccinated group and the control group.
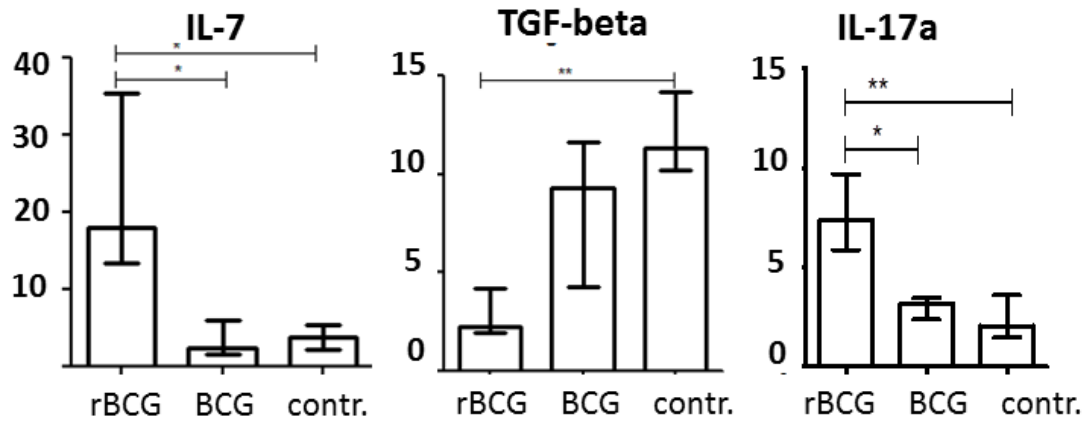
Fig 10: Quantification of IL-7, TGF-β and IL-17a protein was investigated by immunohistochemistry and analyzed by computerized image analysis. The vertical axis represents percent area expressing the protein in pulmonary sections, the recombinant BCG group expressed significantly higher amounts of IL-7 and IL17a and decreased TGF-β (indicated by the Kriskal-Walis test).

The same recombinant BCG group also exhibited the lowest amount of TGF-β and increased IL17a suggesting that the increase in pro-inflammatory cytokines (IL-7, IL-17) and decreased TGF-β at the site of infection could have resulted in improved survival in the recombinant BCG vaccinated group. This characteristic increase in IL17a was also evident in lymph node samples where the recombinant BCG group showed stronger transcripts expression as compared to controls or BCG-vaccinated animals.

4.2.4 Conclusion

This study investigated alternative splicing of IL-7 and IL-7R in a chronic infection setting using a non-human primate model. The analysis revealed that alternative splicing of IL-7 gene is similar in humans (study 1) and rhesus macaques suggesting an evolutionary conserved exon-intron structure. Though the study identified similar splicing events between humans (study 1) and rhesus macaques, the amount of IL-7 variants was low and could not be correlated with infection. Increased IL-7c in the recombinant BCG group, at the protein level, was associated with increased survival suggesting that higher local (pulmonary) IL-7 protein production could be beneficial in MTB infection. Also, as the splicing events between human and rhesus macaque are similar; the NHPs could be used as a model to investigate IL-7 spliced variant biology. We also found that the soluble IL-7R is the most abundant IL-7R transcript in pulmonary tissue. This lead the hypothesis that the soluble IL-7R may bind and neutralize IL-7 elaborated *in situ* thereby decreasing the risk of expanding autoreactive T-cells or cells responding to environmental antigens.

4.3 BIOLOGY OF IL-7δ5: T-CELLS

IL-7δ5 is the alternatively spliced variant of IL-7 gene that lacks exon 5 during the process of pre-mRNA splicing. The IL-7δ5 protein has been shown to induce STAT5 phosphorylation in T-cell subsets suggesting its role as an agonist of IL-7 activity. In this study, we investigated the biological role of the IL-7δ5 variant and compared it to IL-7c mediated activity. With an IL-7δ5 protein specific reagent, we were able to show for the first time IL-7δ5 protein expression *in situ*.

4.3.1 IL-7δ5 induced transcriptomic changes

Sorted immune T-cell subsets (CD4+, CD8+ T-cells) were investigated for comparative changes in gene expression profiles induced by exposure to recombinant IL-7δ5 or IL-7c along with unstimulated controls.

Microarray gene expression revealed similarities in gene expression induced by IL-7δ5 and IL-7c. Both proteins induced IL-7 mediated response genes in CD4+ as well as in CD8+ cells. The significance of microarray (SAM) analysis revealed more than 300 transcripts which differed in their expression upon IL-7 or IL-7δ5 stimulation against unstimulated controls at 72 hour after stimulation. There were no genes which were statistically differed between IL-7c and IL-7δ5 in the analysis at 0 false discovery rate (FDR). Both IL-7c and IL-7δ5 significantly upregulated more than 200 proteins coding genes and negatively impacted on the expression of 67 genes in sorted CD4+ T-cell subsets. 300 genes were upregulated and 35 for (IL-7c) and 53 for (IL-7δ5) genes were downregulated in CD8+ T-cell subsets.
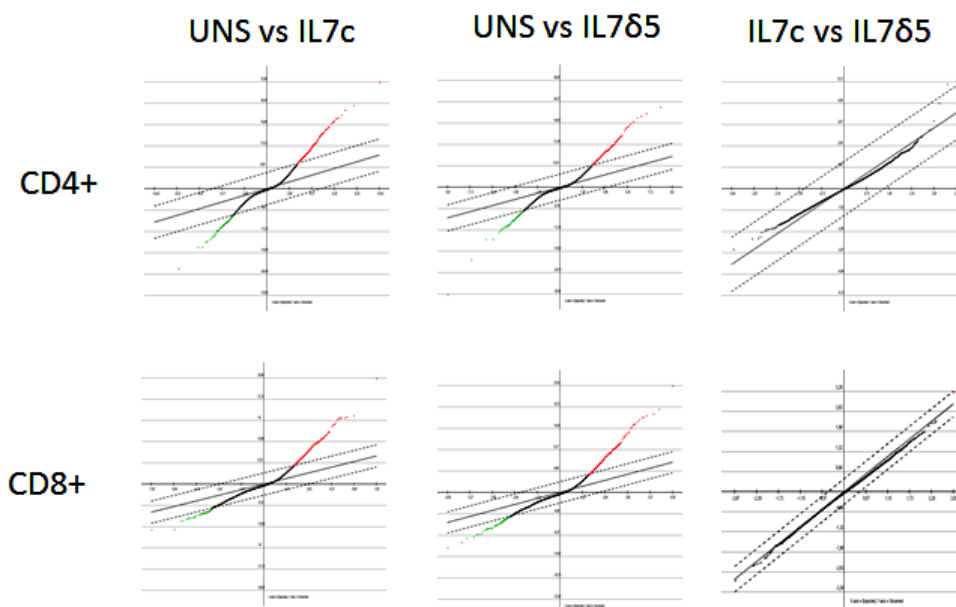

Fig 11: Dot plot analysis by significance of microarray analysis (SAM) results from CD4+ and CD8+ immune cells stimulated with IL-7c or IL-7δ5. Though large number of genes were significantly different (upregulated (red) and downregulated (green) as compared to unstimulated controls; there were no genes statistically different in their expression pattern between IL-7c and IL-7δ5.

4.3.2 Binding of IL-7 and IL-7δ5 to IL-7R

The mechanistic properties of interaction between IL-7c/IL-7δ5 and the IL-7R (CD127) are important to gauge the interaction dynamics of these three proteins. Surface Plasmon resonance analysis identified similar binding forces of receptor-ligand interaction between IL-7c/IL-7δ5 to that of IL-7R, with slightly better affinity of IL-7δ5 to its receptor.
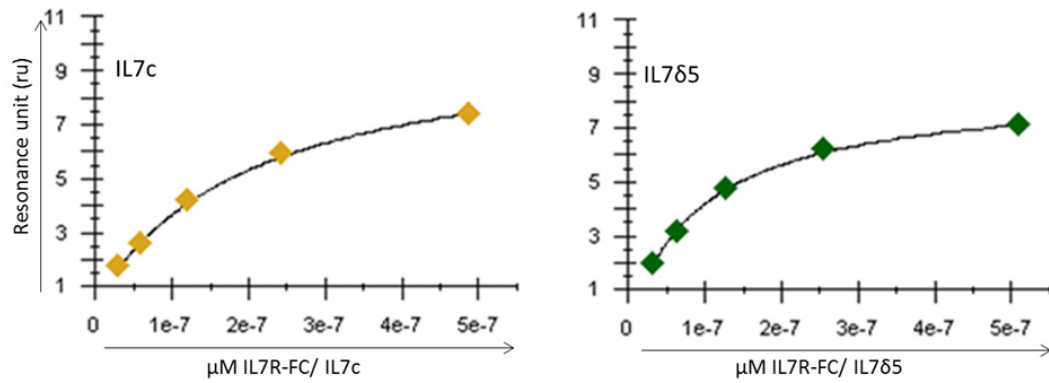

Fig 12: The IL-7 and IL-7δ5 have similar binding efficiencies i.e. $1.048 \times 10^6 \, M^{-1}S^{-1}$ and $0.9706 \times 10^6 \, M^{-1}S^{-1}$ respectively but the IL-7δ5 molecule has a lower off-rate at 0.1465 compared to 0.1939 of the IL-7c molecule. This implies a higher dissociation constant for IL-7δ5 at 0.9881 µM, suggesting that IL-7δ5 would have a slightly higher affinity towards the IL-7R molecule.

4.3.3 IL-7δ5 mediated signaling in T-cells

Both IL-7 and IL-7δ5 have been described to induce STAT5 phosphorylation in T-cells. IL-7 also phosphorylates STAT3, yet this has not been tested for IL-7δ5. IL-7δ5
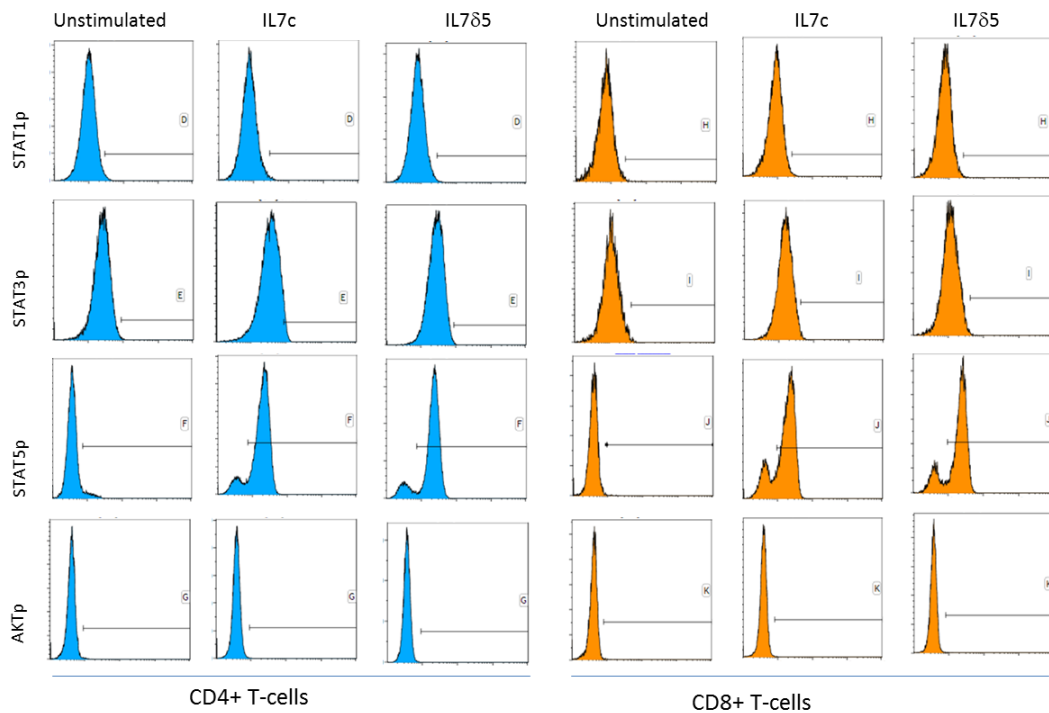

Fig 13: IL-7 and IL-7δ5 induced phosphorylation in CD4+ and CD8+ T-cells.

might also employ other signaling mediators e.g. STAT1 or AKT. We tested the difference in IL-7 or IL-7δ5 in inducing phosphorylation of STAT1, STAT3, STAT5 and AKT using flow cytometry.

Both IL-7 and IL-7δ5 did not induce STAT1 and AKT phosphorylation in CD4+ and CD8+ T cell subsets. A minute cell population responded to IL-7c by STAT3 phosphorylation, but no STAT3p could be seen upon IL-7δ5 stimulation. As shown earlier, both IL-7 and IL-7δ5 induced strong STAT5p in CD4+ and CD8+ T-cell subsets.

4.3.4 IL-7δ5 mediated effects in T-cells

We concluded that IL-7c and IL-7δ5 are similar in their action, based on gene expression analysis and signaling transducer phosphorylation. The differences in their mechanistic properties of interaction with IL-7R might present a distinct response pattern. Therefore, we investigated comparative effects of IL-7 and IL-7δ5 in cellular changes associated with IL-7 signaling.

4.3.4.1 IL-7R downregulation

Cell surface IL-7R downregulation is a hallmark for defining IL-7 mediated activity. IL-7 signaling induced IL-7R downregulation was investigated by flow-cytometry.
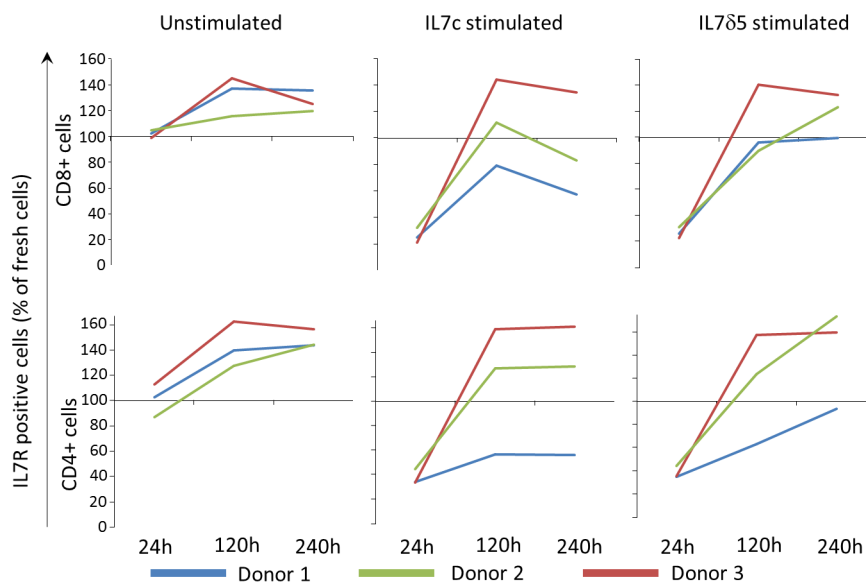

Fig 14: IL-7R downregulation in CD4+ and CD8+ T-cells upon stimulation by IL-7c or IL-7δ5.

CD4+ and CD8+ T-cells equally downregulated IL-7R cell surface expression at 24 hours after stimulation. This IL-7R downregulation was observed in both CD4+ and CD8+ T-cell populations.

4.3.4.2   BCL-2 induction

T-cell survival is one of the hallmark functions of IL-7 on T-cells mediated via upregulation of the BCL-2 protein. Both, IL-7 and IL-7δ5 induced BCL-2 expression in CD4+ and CD8+ T-cell subsets but with characteristic differences regarding the timeframe of BCL-2 induction. Thus, we tested the capacity of IL-7c and IL-7δ5 to induce BCL-2 expression in both CD4+ or CD8+ T-cell subsets. The analysis was performed for different time points at day 0, 3 and 10 by intracellular BCL-2 staining. The BCL-2 induction in CD3+CD8+ and CD3+CD4+ T-cell subsets was plotted on the X axis of the histogram denoting modal population of the cells for each condition.
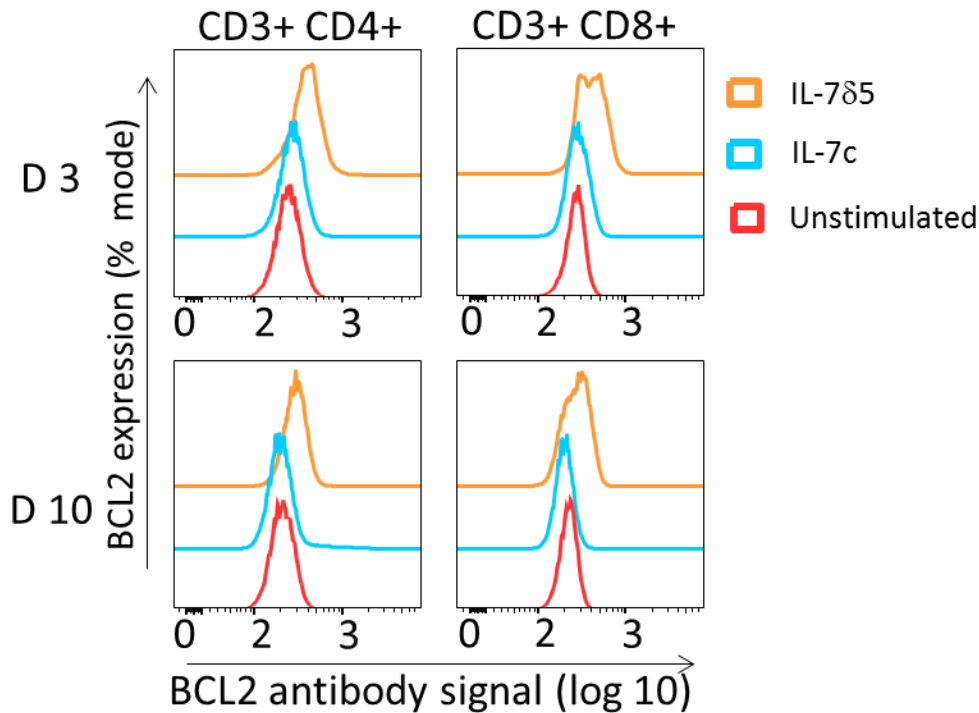

Fig 15: BCL-2 expression in T-cell subsets upon IL-7 or IL-7δ5 stimulation

T-cell populations were negative for BCL-2 protein expression at day 0. PBMCs stimulated with IL-7c for 3 days were negative for BCL-2 expression in both CD4+ and CD8+ T cells but at day 10, BCL-2 was detected in CD4+ (3.8 %) T-cells. In contrast, IL-7δ5 stimulated T-cells demonstrated expression of BCL-2 at day 3, in both CD4+ (4.41 %) and CD8+ (6.24 %) T-cells. Day 10 cultures of IL-7δ5 stimulation exhibited comparable induction of BCL-2 protein in CD4+ (4.11%) T-cell populations but along with increased BCL-2 induction in CD8+ T-cells (8.43%). IL-7δ5, elaborated *in situ*, may therefore be driving cellular immune response by BCL-2 induction. This is of clinical interest, since IL-7 RNA fragment analysis in samples from patients with latent TB showed IL-7δ5, yet less IL-7c production [17].

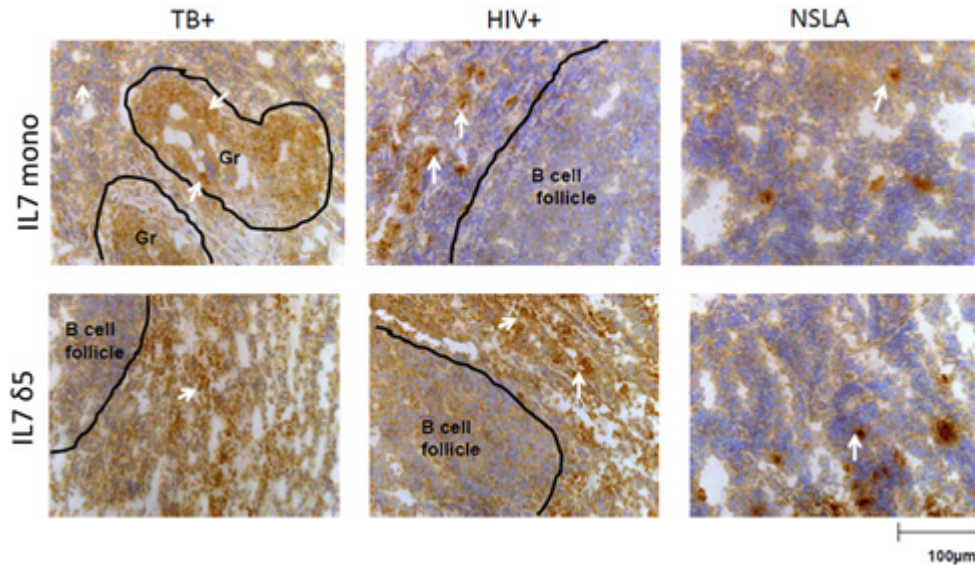### 4.3.5 *In situ* IL-7δ5 protein detection in infection

Fig 16: IL-7δ5 protein detection in lymph nodes from patients with TB+, HIV +ve and non-symptomatic lymphadenitis (NSLA). Marked differences in IL-7δ5 protein distribution can be observed in the lymph node structures, In TB+ve and HIV +ve lymph nodes, the IL-7δ5 protein expression was in cells diffused in the T-cell zone. NSLA lymph nodes showed islands of cell staining positive for IL-7δ5 protein.

We developed a monospecific antibody capable of recognizing IL-7δ5 among all IL-7 gene variants. This reagent provided, for the first time, an opportunity to visualize the distribution of IL-7δ5 protein in lymph nodes using immunohistochemistry. The IL-7δ5 protein was expressed in healthy as well as in lymph nodes from patients with tuberculosis or HIV infection and it was associated with the T-cell zone. The amount of IL-7δ5 positive staining material was investigated as percent positively stained area for the IL-7δ5 specific staining. We did not observe statistical differences in total amount of IL-7δ5 protein positive staining in association with infection, but the IL-7δ5 positive cell distribution was observed to be different in lymph nodes from NSLA and TB or HIV positive individuals.

4.3.6 Conclusion

This was the first study to report i) mechanistic properties of the interaction between IL-7δ5 - IL-7R (Biacore analysis), ii) as well as the identification if IL-7δ5 at the protein level using immunohistochemistry. The surface plasmon resonance analysis of IL-7δ5 – IL-7R interaction was slightly superior to the IL-7c – IL-7R interaction. The transcriptomic analysis using RNA microarray on IL-7δ5 stimulated cells indicated similarities in action between IL-7c and IL-7δ5. IL-7δ5 stimulation of CD4+ and CD8+ T-cells indicated a faster turnover of IL-7R surface expression and exhibited BCL-2 protein expression in both CD4+ and CD8+ cell subsets at day 3 as compared to day 10 in IL-7c stimulated cells.

4.4 DETECTION OF IL-7δ5 AT THE PROTEIN LEVEL AND ITS ROLE IN MONOCYTE STIMULATION

4.4.1 IL-7δ5 protein expression mapping

The monospecific reagent detecting IL-7δ5 at the protein level, provided an opportunity to investigate the presence of IL-7δ5 *ex vivo* and to shed light on its expression in tissue structures. The expression pattern for the IL-7 spliced variants were previously
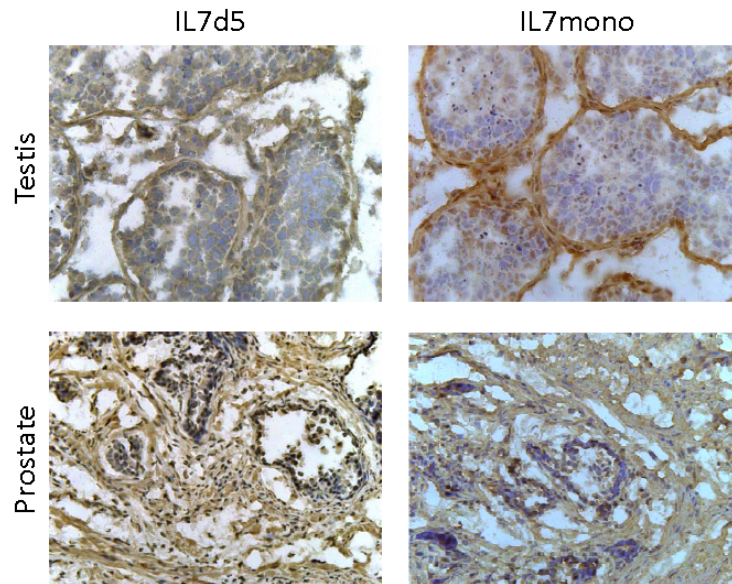

Fig 17: IL-7δ5 and IL-7mono (detecting all spliced variants of the IL-7 protein) antibody staining healthy human testis and prostate tissue.

examined on the transcript level from our group, but its presence in histological sections was yet to be determined.
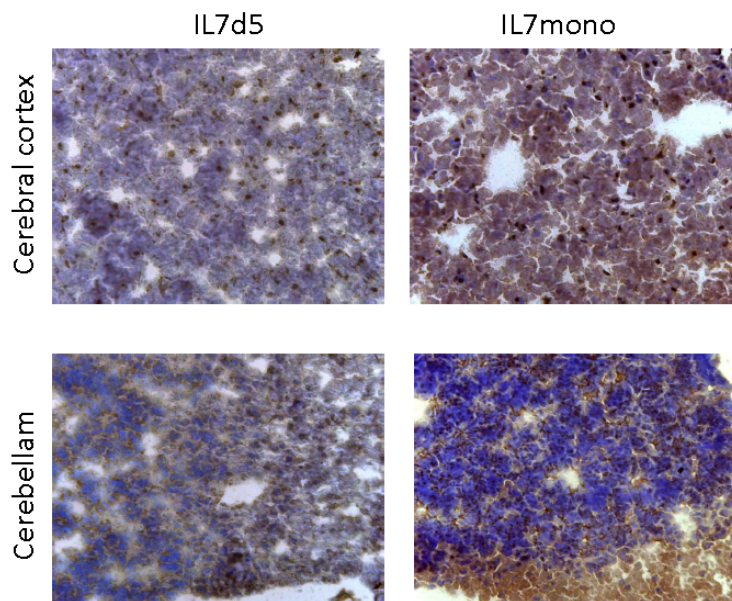

Fig 18: IL-7δ5 and IL-7mono (detecting all spliced variants of IL-7 protein) antibody staining healthy human cerebral and cerebellum tissue.

One of the possible modes of IL-7 activity is its local production and consumption, inferred from the low presence of IL-7 in serum. We mapped IL-7δ5 in human tissues using immunohistochemistry and found that the expression of IL-7δ5 is largely associated with epithelial cells along with sporadic expression in brain tissue that seem to coincide with cells that produce other IL-7 transcripts defined by a monoclonal antibody capable of detecting all IL-7 spliced variants (IL7mono). The skin epithelial cells are a known source of IL-7 in cutaneous tissue; we observed that IL-7δ5 is also expressed in epithelial cells as well as in cancer cells (squamous cell cancer).
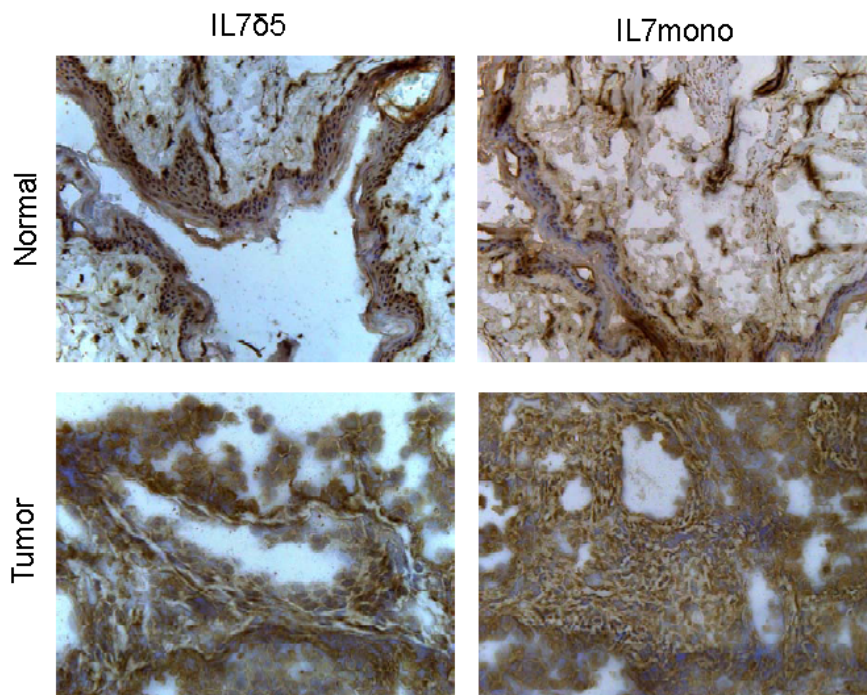

Fig 19: IL-7δ5 and IL-7mono (detecting all spliced variants of IL-7 protein) antibody staining of healthy human skin tissue and in skin cancer.

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The characteristic IL-7δ5 expression in various normal and tumor tissues is summarized in table 3.

No	Tissue	Healthy	Tumor
1	Skin	subcutaneous epithelial cells	diffused expression throughout the tumor mass
2	Breast	epithelial cells of the mammary gland	associated with tumor cells
3	Pancreas	positive staining in few cells scattered in pancreatic tissue	sporadic and does not seem to be associated with the tumor
4	Prostate	present in epithelial layer and few cells positive in surrounding tissue	glandular structures are stained positive but sporadic expression in surrounding tissue
5	Brain	appear to be scattered in the brain tissue	mostly associated with tumor mass
6	Small Intestine	low / sporadic expression diffused in the section	low but consistent expression diffused in the section
7	Colon	the tissue architecture is poorly defined; yet positive staining reaction for IL-7δ5 protein	mostly associated with epithelial tumor cell mass.
8	Uterus	associated with smooth muscle cell lining.	low level of expression throughout non-apoptotic tumor mass.
9	Skeletal muscle	muscle fiber as well as vasculature is positively stained.	seems absent

Table 3: characteristics of IL-7δ5 expression in normal and matched tumor tissue section.

The diffused expression in tissue microenvironment suggested that tissue resident monocytic cells could be one of the contributors in IL-7δ5 expression, as it has been shown that these cells contribute to the peripheral blood IL-7 pool [95]. We investigated co-expression of IL-7δ5 and the CD68 marker, which defines cells of monocyte linage, and could confirm that indeed the IL-7δ5 positive signals are associated with CD68 positive cells.
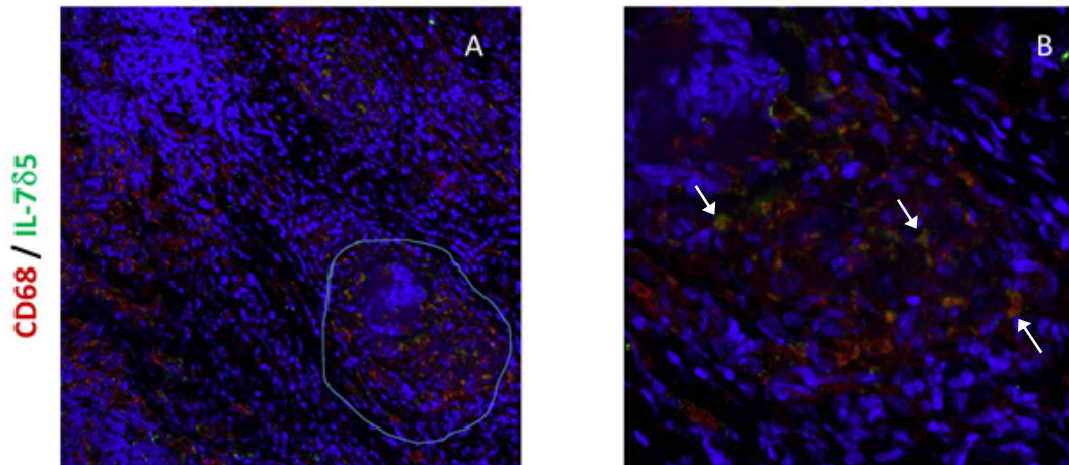
Fig 20: Monocytes or monocyte-derived cells could be a relevant source of IL-7δ5 protein. we examined co-expression of IL-7δ5 with monocytes in lymph nodes from human subjects with TB and stained the IL-7δ5 protein (green) together with the macrophage marker CD68 (red). The IL-7δ5 protein was identified in close association with CD68 in the granulomatous region of *M. tuberculosis*-infected lymph nodes, which suggests that macrophages produce IL-7δ5 protein within the lymph node structure.

The monocytic cells are one of the major contributors in the development of arterial atherosclerosis. We investigated whether the IL-7δ5 molecule in atherosclerosis lesions is differentially expressed and found indeed strong IL-7δ5 expression in the resident

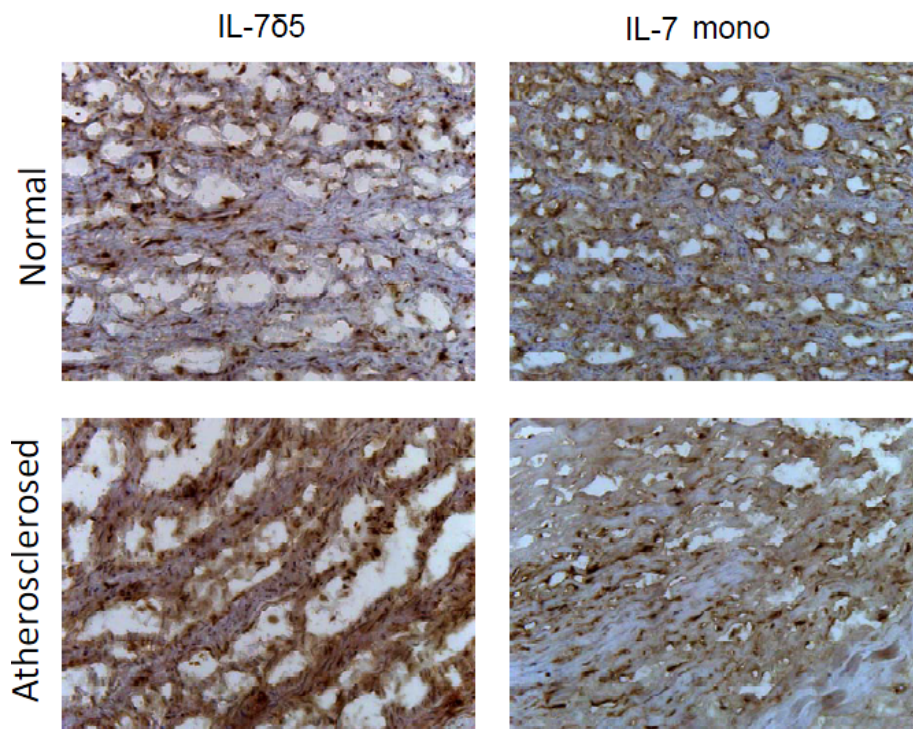Fig 21: Tissue sections from human atherosclerotic aorta tissue and matched, normal aorta were investigated for IL-7δ5 expression by immunohistochemistry. The IL-7 monoclonal antibody, recognizing all IL-7 variant proteins, was used as a positive control. The staining showed presence of IL-7δ5 positive material in both normal and atherosclerotic aorta and stronger IL-7δ5 staining in atherosclerotic aorta tissue.

cells in atherosclerotic aorta. These results posed an interesting conjecture because peripheral blood monocytes are known to harbor IL-7R on its cell surface and could respond to IL-7 and IL-7δ5 signaling differentially.

4.4.2 Cytokine and chemokine profile upon IL-7δ5 stimulation.

The potential differential signaling of IL-7 and IL-7δ5 could result in differential secretory protein production by monocytes. The chemokine and cytokine secretion profiling in IL-7δ5 stimulated cells (PBMCs and sorted CD14+ cells) indicated differences. The chemokine production pattern in supernatants from IL-7δ5 stimulated PBMCs showed more MCP1 and IP-10 production than in IL-7c stimulated cells.
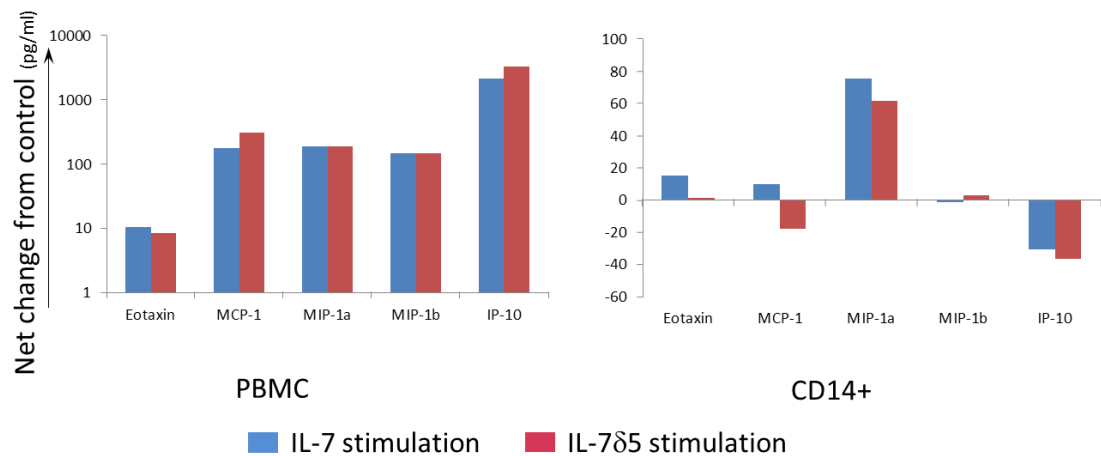

Fig 22: Chemokine response in IL-7 and IL-7δ5 stimulated monocytes. The PBMCs and CD14+ sorted monocytes were stimulated by IL-7c or IL-7δ5 along with unstimulated controls for 3 days. The supernatants were collected and analyzed for chemokine levels by a Luminex assay. The values obtained are subtracted with the respective chemokine amount identified in unstimulated controls and plotted as net change (in pg/ml) from unstimulated PBMC and CD14+ supernatants.

4.4.3 IL-7δ5 induced transcriptomic changes in CD14+ cells.

The transcriptomic profile after IL-7c or IL-7δ5 stimulation was investigated to identify a group of genes which are potentially differentially regulated by the effect of IL-7δ5. Peripheral blood CD14+ beads sorted PBMCs were examined using the affymetrix gene expression analysis platform and analyzed by the significance of microarray (SAM) method to compare changes in gene expression profile with respect to the unstimulated controls. The SAM analysis (at 20% false discovery rate) revealed that there were 96 transcripts which were significantly overexpressed in IL-7c stimulated samples as compared to unstimulated controls. In contrast IL-7δ5 stimulation upregulated 40 transcripts, as compared to the unstimulated control. There were no

transcripts which were statistically downregulated between IL-7c *vs* unstimulated or IL-7δ5 *vs* unstimulated CD14+ cells.
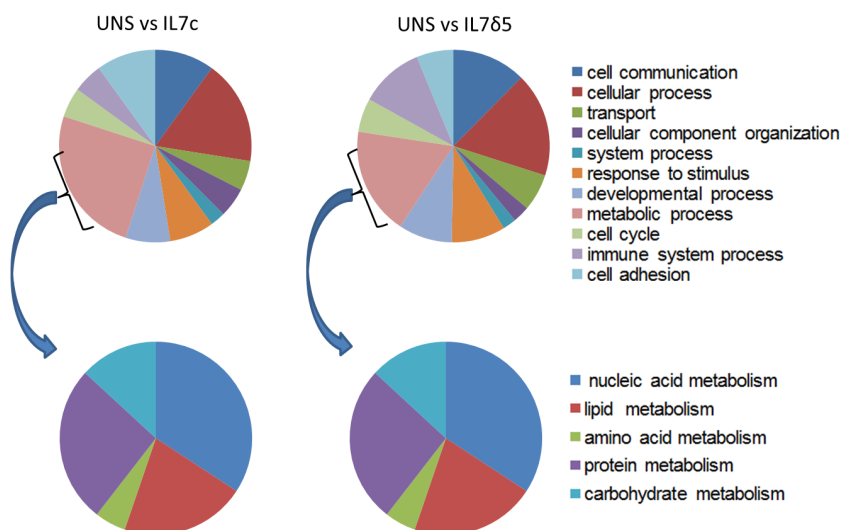

Fig 23: Gene ontology analysis of significant genes in CD14+ cells stimulated with either IL-7c or IL-7δ5 compared to unstimulated controls. In the figure above, the top line of the pi chart denotes the number of genes in the respective gene ontology class color coded according to the top right legend. Upon dissecting the gene ontology group for metabolic processes, the next level clustering is shown (bottom part).

Differentially expressed genes were clusterd based on the pathway analysis and ontology tool to visualize physiological processes. Both IL-7c and IL-7δ5 treatment resulted in upregulation of genes involved in metabolic processes, cell communication and cellular response elements to external stimuli. The surface receptor transcripts among the upregulated genes indicated a pro- inflammatory phenotype, particularly through the upregulation of the Oxidised Low density Lipoprotein Receptor-1 (OLR-1) gene.

4.4.4 IL-7δ5 and Monocyte mediated lipid metabolism.

Next, to confirm the observation of OLR1 gene expression upon Il-7δ5 stimulation, we tested cell surface OLR1 protein expression in IL-7 or IL-7δ5-stimulated CD14+ cells. PBMCs obtained from (n=6) healthy subjects were cultured in IL-7c or IL-δ5 for 72 hours and exhibited an increase in CD14+ cells expressing the OLR1 protein. The frequency of CD14+ OLR1+ cells in unstimulated controls was 0.32% (STDV 0.104) of the total CD14+ population which increased to 3.16% (STDV 3.15) with IL-7c stimulation and 4.34 % (STDV 2.87) with IL-7δ5. The change in frequency of CD14+ cells expressing OLR1 (p= 0.035) was significant among the treatment groups with the one way ANNOVA test. Statistical analysis between the groups using the paired T- test revealed that the frequency of CD14+ cells expressing OLR1 is significantly different

between unstimulated cells and the IL-7δ5 treatment group (p = 0.02). The same was found to be true for the comparison of IL-7c *vs* IL-7δ5 (p = 0.05).
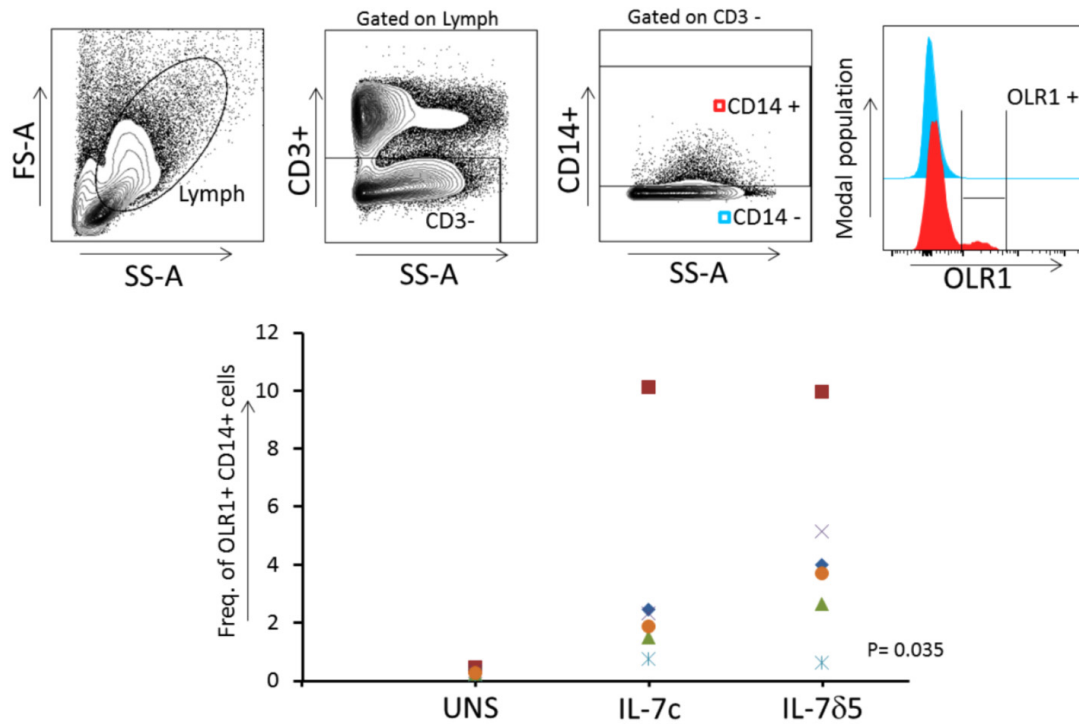

Fig 24: Expression of the oxidized low density lipoprotein receptor was investigated by flow cytometry. The scatterplot shows the gating strategy. Cells were visualized using side and forward scatter within the parental lymphogate. CD3+ cells were identified and the CD3 neg cell population was used to identify CD14+ cells. OLR1 expression on CD14+ cells was investigated for each donor and the frequency of OLR1 expressing CD14+ cells was used to compare the effect of IL-7δ5 treatment. In the scatter plot, each shape and color denotes a donor, the X axis represents the frequency of CD14+ OLR1+ cells in CD14+ cell subsets; the stimulus is indicated on the Y axis (UNS (unstimulated), IL-7c and IL-7δ5). The frequency of CD14+ OLR1+ cells was obtained for each treatment group and compared (one way ANNOVA) to investigate differences of expression between the respective stimuli.

4.4.5 Conclusion

In this report, we mapped the IL-7δ5 protein expression in greater detail and assessed its impact on CD14+ cells. IL-7δ5 expression was observed in both healthy and tumor tissues and was largely associated with cells of epithelial origin. The report also identified the proinflammatory role of the IL-7δ5 stimulus on CD14+ monocytes based on transcriptomic changes and secretory chemokines through upregulation of the OLR1 receptor.

4.5 BIOLOGICAL ROLE OF OTHER IL-7 VARIANTS.

The current thesis project also investigated the activity of four other IL-7 alternatively spliced variants (IL-7δ4 / IL-7δ3,4 / IL-7δ4,5 / IL-7δ3,4,5) based on known parameters of IL-7c stimulation. These alternatively spliced variants did not mediate the changes known for IL-7c activity in T-cells. The results from the analyses are summarized in the following table 4.

Assay	*IL-7c*	*IL-7δ5*	*IL-7δ4*	*IL-7δ34*	*IL-7δ45*	*IL-7δ345*
STAT5 p	+	++	-	-	-	-
STAT3 p	-	-	-	-	-	-
BCL-2 induction	Day 7	Day 3	-	-	-	-
ICP (IL-2, IFN-γ, TNF-α, IL-17)	+	+	-	-	-	-
T- cell expansion (CFSE)	+	+	-	-	-	-
STAT1 p	-	-	-	-	-	-
Akt p	-	-	-	-	-	-

Table 4: Analysis of IL-7 variant activity in T-cells.

Based on the results in table 4 above, IL-7 spliced variants can be classified in two groups 1) IL-7 isoforms which are similar in their IL-7R mediated function (IL-7c and IL-7δ5) and 2) IL-7 isoforms which do not mediate IL-7R mediated functions (IL-7δ4 / IL-7δ3,4 / IL-7δ4,5 / IL-7δ3,4,5). The size and composition of the IL-7 alternatively spliced products changes considerably; it could also be possible that the IL-7 alternatively spliced variants have unique functions which are independent of known IL-7 roles in IL-7 physiology.

5 CONCLUDING REMARKS

Alternative splicing of pre-mRNA is a mechanism to generate proteomic diversity from (relatively) constant genetic material. The process of alternative splicing is a common attribute for many cytokines and cytokine receptors. The alternatively spliced variants, in a simplistic view, can act as agonists or antagonists to the activity of the ´canonical` protein; yet, IL-7 isoforms may also represent novel functions. IL-7 is a unique molecule due to its non- redundant role in T- and B- cell biology and known to generate alternatively spliced variants in humans. The thesis investigated the extent of IL-7 gene alternative splicing in association with infection and inflammation and addressed the biology of IL-7 variant proteins in line with known functions of IL-7c.

In paper I, we investigated the role of IL-7 and IL-R alternative splicing in association with multiple sclerosis; based on relative abundance of IL-7 and IL-7R alternatively spliced variant transcripts from PBMCs of healthy individuals and patients with MS. We could show that the pattern of alternative splicing in PBMCs was identical among the donors and surprisingly, the IL-7δ4 transcript variant, and not the IL-7c, represented the most abundant IL-7 transcript. The IL-7R gene also showed extensive alternative splicing. We could show increased sIL-7R in association with the IL-7R gene polymorphism and described a novel soluble IL-7R candidate.

In paper II, we investigated IL-7 and IL-7R alternative splicing in a non-human primate animal model and its impact on the outcome of MTB infection. We observed that the alternative splicing events of IL-7 gene in rhesus macaque and human PBMCs are similar, yet the relative distribution of IL7 variants is quite different. IL-7c represents the most frequent transcript in rhesus macaque´s PBMCs, and its production is increased at the site of infection (lungs) correlated with increased survival in the MTB challenge group; most likely a function of TGFβ downregulation.

In paper III, we investigated the IL-7 driven biology of the IL-7δ5 alternatively spliced variant by studying its mechanistic properties concerning the interaction with the IL-7R. The surface plasmon resonance analysis of IL-7δ5 − IL-7R interaction is slightly stronger than the IL-7c − IL-7R interaction; perhaps this minute change in ligand − receptor interaction contributes to differential IL-7R expression dynamics on the T-cell surface and faster IL-7δ5 mediated BCL-2 protein induction in CD4+ and CD8+ T-cells. In paper III, we identified, for the first time, IL-7δ5 protein production in tissue and accessed its impact in infection using an IL-7δ5 specific antibody.

In paper IV, we elaborated on the IL-7δ5 specific reagent and mapped IL-7δ5 productions in human healthy and tumor tissue. We also investigated the impact of IL-7δ5 proteins on CD14+ monocytes in a explorative approach and could identify that IL-7c and IL-7δ5 favor an inflammatory phenotype based on transcriptomic changes and chemokines. This study also identified a link of IL-7c and IL-7δ5 protein in lipid metabolism, through upregulation of OLR1 expression on CD14+ monocytes.

The investigation of biology associated with other IL-7 alternatively spliced variants (IL-7δ4, δ3,4, δ4,5, δ3,4,5), at least measured by known IL-7 functions on T-cell populations, indicated no activity.

6 FUTURE PERSPECTIVE

The work presented in this thesis shows that IL-7 alternatively spliced variants are abundantly expressed in humans and that these variant proteins are differentially expressed in health and disease. Our data from T cell assays, investigated for known effects of IL-7c activity, suggests that the IL-7 spliced variants can be divided in two groups i) variants with similar functions as IL-7c (IL-7c and IL-7δ5) and ii) variants which do not exhibit functions attributed to IL-7c activity (IL-7δ4, IL-7δ3,4, IL-7δ4,5 and IL-7δ3,4,5).

IL-7δ5 exhibited a ´super-agonistic` role as compared to IL-7c activity based on its ability in faster BCL-2 induction in T-cells. It would be important to evaluate its role in T-cell activation models to address if faster induction of the BCL-2 protein may impact on T-cell activity and / or expansion of antigen specific T-cells upon re-encounter of the nominal target antigen. It would also be important to identify the biological significance of the OLR1 gene upregulation observed in CD14+ cells in response to IL-7δ5; specifically by addressing if the upregulation of OLR1 correlates with increased uptake of oxidized low density lipoprotein and increased scavenging activity by monocyte derived cells. This could play a role in chronic inflammation associated with malignant transformations; it could also lead to decreased immune effector mechanisms of monocytes in intracellular infections. Other IL-7 variant isoforms did not exhibit biological activity associated with known functions of IL-7c; it could very well be the case that these IL-7 variant proteins would interact with novel proteins and cellular compartments which are not representative of IL-7c mediated activity. This could be addressed by identifying:

1) Proteins interacting with IL-7 gene variants.
2) Localization of IL-7 variants in subcellular compartments.
3) IL-7 variant knock-out / knock-in in models.

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