IL-7-signalling and *IL7RA* genetic variants:
Impact on T-cell functions in human infectious and autoimmune diseases

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Summary

Interleukin-7 (IL-7) is a cytokine central for development and homeostasis of T cells. The cytokine signals through a receptor complex formed by the cognate IL-7 receptor α (IL-7Rα, CD127) and the common γ chain receptor (γ_c, CD132). Signalling of IL-7 is highly regulated by the levels of membrane-associated (m)IL-7Rα, and whereas IL-7 may promote the response of T cells against infectious agents, excess IL-7 can balance the immune response towards autoimmunity. In addition to mIL-7Rα, alternative splicing produces a soluble variant of the receptor, i.e. sIL-7Rα, which can bind circulating IL-7. Single nucleotide polymorphisms (SNPs) in the *IL7RA* gene have been associated with autoimmune and infectious diseases, including the rs6897932 SNP in exon 6 of *IL7RA*, which also affects alternative splicing and generation of sIL-7Rα. Own preliminary results indicated an impaired IL-7-induced response of T cells in a subgroup of tuberculosis patients. Here, the role of IL-7-signalling and *IL7RA* genetic variants is evaluated in patients with active tuberculosis and in patients with type 1 diabetes.

In paper I, factors involved in IL-7-signalling were evaluated in patients with active tuberculosis and healthy household contacts from Ghana. Reduced plasma levels of sIL-7Rα and increased concentrations of plasma IL-7 were detected in tuberculosis patients, and the levels normalised during treatment to reach similar concentration as healthy contacts. Although the exonic *IL7RA* SNP rs6897932 was associated with plasma levels of sIL-7Rα, the SNP could not explain the reduced levels of sIL-7Rα in tuberculosis patients on its own. Further, lower levels of mIL-7Rα was seen on T cells from tuberculosis patients with impaired STAT5-phosphorylation after *in vitro* stimulation with IL-7. Finally, the IL-7-promoted interferon (IFN)-γ-release from *Mycobacterium (M.) tuberculosis*-specific T cells was impaired in tuberculosis patients. Overall, it was shown for the first time that IL-7-dependent immunity was impaired in a chronic bacterial disease.

In paper II, serum levels of IL-7 and sIL-7Rα were analysed in patients with type 1 diabetes and subsequently compared to the *IL7RA* genotype of the patients. For a subgroup of patients, serum levels of IL-7 and sIL-7Rα were compared to the cytokine response after CD3/CD28 stimulation. Patients carrying the minor allele of rs6897932 – a SNP that is associated with protection against type 1 diabetes and multiple sclerosis – had reduced serum levels of both sIL-7Rα and IL-7. Patients carrying the minor allele of rs1494555, an exonic *IL7RA* SNP that has been associated with risk of multiple sclerosis, also had reduced serum concentration of sIL-7Rα, whereas no effect was seen on serum IL-7. No correlation was observed between serum sIL-7Rα and IL-7, and the two markers did not affect proportion of mIL-7Rα$^+$ T cells or production of cytokines from T cells after CD3/CD28 stimulation.

In paper III, six exonic *IL7RA* SNPs were genotyped in patients with active tuberculosis and healthy contacts from Ghana. For two SNPs, i.e. rs1494558 and rs11567764, the minor alleles were associated with protection against active tuberculosis. Functional analysis of the two SNPs showed that rs11567764 was associated with reduced expression of an IL-7Rα variant lacking exon 5-6, which presumably codes for a non-functional protein. rs1494558 is a non-synonymous SNP leading to a Thr66Ile substitution in exon 2 of IL-7Rα, which was associated with reduced secretion of sIL-7Rα and lower trafficking of mIL-

7Rα to the cell membrane of transfected HEK293T cells. Further, the importance of N-glycosylation for optimal secretion of sIL-7Rα and trafficking of mIL-7Rα to the cell surface was emphasised in this paper.

In paper IV, two cell line-based assays that allowed analysis of the *IL7RA* SNPs rs1494558 and rs6897932 were developed. In one assay, an IL-7Rα-construct was generated that allowed concurrent analysis of exon 6 splicing and its effect on the level of sIL-7Rα and mIL-7Rα. Both SNPs were found to affect the receptor levels. Further, an IL-7-dependent cell line was developed, which may prove useful for evaluating the effect of *IL7RA* SNPs on IL-7-signalling.

Overall, these results showed that IL-7-signalling was impaired in active tuberculosis, and new functional effects of disease-associated *IL7RA* SNPs were identified and described. Further, the association between molecules involved in IL-7-signalling was described in both tuberculosis and type 1 diabetes, and this may become useful for establishing biomarkers of the IL-7-response. Potentially, these findings on IL-7-signalling molecules and *IL7RA* SNPs can be generalised to other autoimmune and chronic infectious disorders, and the IL-7-signalling pathway may become a future target for prevention or treatment of disease.

Zusammenfassung

Das Zytokin Interleukin-7 (IL-7) ist für die Entwicklung und Homöostase von T-Zellen von zentraler Bedeutung. Es signalisiert durch einen Rezeptorkomplex, der durch den IL-7-Rezeptor α (IL-7Rα, CD127) und dem common γ-chain-Rezeptor (γc, CD132) gebildet wird. Die Signaltransduktion von IL-7 wird stark durch die Menge von membranassoziiertem (m)IL-7Rα reguliert, und während IL-7 die Antwort von T-Zellen gegen infektiöse Partikel fördern kann, kann überschüssiges IL-7 die Immunantwort in Richtung Autoimmunität verschieben. Zusätzlich zu mIL-7Rα erzeugt alternatives Spleißen eine lösliche Variante des Rezeptors, sIL-7Rα, die zirkulierendes IL-7 binden kann. Einzelnukleotid-Polymorphismen (SNPs) im *IL7RA*-Gen wurden mit Autoimmun- und Infektionskrankheiten in Verbindung gebracht, einschließlich des rs6897932-SNP im Exon 6 von *IL7RA*, welches auch das alternative Spleißen und die Bildung von sIL-7Rα beeinflusst. Eigene vorläufige Ergebnisse weisen auf eine gestörte IL-7-induzierte Antwort von T-Zellen in einer Subgruppe von Tuberkulosepatienten hin. Hier wird die Rolle von IL-7-Signalling und *IL7RA*-Genvarianten bei Patienten mit aktiver Tuberkulose und bei Patienten mit Typ-1-Diabetes untersucht.

In Manuskript I wurden Faktoren, die am IL-7-Signalling beteiligt sind, bei Patienten mit aktiver Tuberkulose und gesunden Haushaltskontakten aus Ghana untersucht. Reduzierte sIL-7Rα-Plasmakonzentrationen und erhöhte Konzentrationen von Plasma-IL-7 wurden bei Tuberkulosepatienten nachgewiesen; die Konzentrationen normalisierten sich während der Behandlung und erreichten ähnliche Konzentration wie gesunde Kontakte. Obwohl das exonische *IL7RA* SNP rs6897932 mit sIL-7Rα-Plasmaleveln assoziiert war, konnte das SNP die reduzierten sIL-7Rα Level bei Tuberkulose-Patienten nicht alleine erklären. Ferner wurden niedrigere mIL-7Rα-Titer bei T-Zellen von Tuberkulosepatienten mit gestörter STAT5-Phosphorylierung nach in-vitro-Stimulation mit IL-7 beobachtet. Zusätzlich war die IL-7-geförderte Interferon (IFN)-γ-Freisetzung aus *Mycobacterium* (*M.*) *tuberculosis*-spezifischen T-Zellen bei Tuberkulosepatienten beeinträchtigt. Insgesamt wurde erstmals gezeigt, dass die IL-7-abhängige Immunität bei einer chronischen bakteriellen Erkrankung beeinträchtigt ist.

In Manuskript II wurden Serumlevel von IL-7 und sIL-7Rα bei Patienten mit Typ-1-Diabetes analysiert und anschließend mit dem *IL7RA*-Genotyp der Patienten verglichen. Für eine Untergruppe von Patienten wurden die Serumlevel von IL-7 und sIL-7Rα mit der Zytokinantwort nach CD3/CD28-Stimulation verglichen. Patienten, die das Minor-Allel rs6897932 trugen - ein SNP, das mit einem Schutz gegen Typ-1-Diabetes und Multiple Sklerose assoziiert ist - hatten reduzierte Serumlevel sowohl von sIL-7Rα als auch von IL-7. Patienten, die das Minor-Allel von rs1494555 trugen, ein exonischer *IL7RA*-SNP welches mit einem Risiko für multiple Sklerose in Verbindung gebracht wurde, hatten ebenfalls eine verringerte Serumkonzentration von sIL-7Rα, während für das Serum-IL-7 kein Effekt beobachtet wurde. Zwischen dem Serum sIL-7Rα und IL-7 wurde keine Korrelation beobachtet, und die zwei Marker beeinflussten nicht den Anteil von mIL-7Rα$^+$ T-Zellen oder die Produktion von Zytokinen aus T-Zellen nach CD3/CD28-Stimulation.

In Manuskript III wurden sechs exonische *IL7RA*-SNPs bei Patienten mit aktiver Tuberkulose und gesunden Kontakten aus Ghana genotypisiert. Für zwei SNPs, rs1494558 und rs11567764, waren die Minor-Allele mit einem Schutz gegen aktive Tuberkulose assoziiert. Die funktionelle Analyse der beiden SNPs zeigte, dass rs11567764 mit einer reduzierten Expression einer IL-7Rα-Variante assoziiert war, der das Exon 5-6 fehlte, das vermutlich ein nicht-funktionelles Protein kodiert. rs1494558 ist ein nicht-synonymes SNP, das zu einer Thr66Ile-Substitution in Exon 2 von IL-7Rα führt, die mit verringerter Sekretion von sIL-7Rα und geringerem Trafficking von mIL-7Rα an die Zellmembran von transfizierten HEK293T-Zellen assoziiert war. Des Weiteren wurde die Bedeutung der N-Glykosylierung für die optimale Sekretion von sIL-7Rα und den Transport von mIL-7Rα zur Zelloberfläche in diesem Artikel hervorgehoben.

In Manuskript IV wurden zwei zelllinienbasierte Assays entwickelt, die eine Analyse der *IL7RA*-SNPs rs1494558 und rs6897932 ermöglichen. In einem Test wurde ein IL-7Rα-Konstrukt erzeugt, das eine gleichzeitige Analyse des Exon 6-Spleißens und dessen Wirkung auf das Niveau von sIL-7Rα und mIL-7Rα erlaubt. Beide SNPs beeinflussten die Rezeptorlevel. Ferner wurde eine IL-7-abhängige Zelllinie entwickelt, die sich als nützlich für die Bewertung der Wirkung von *IL7RA*-SNPs auf die IL-7-Signalgebung erweisen könnte.

Insgesamt zeigten diese Ergebnisse, dass das IL-7-Signalling bei aktiver Tuberkulose einträchtig ist, und neue funktionelle Effekte von krankheitsassoziierten *IL7RA*-SNPs wurden identifiziert und beschrieben. Ferner wurde die Assoziation zwischen Molekülen, die an der IL-7-Signalgebung beteiligt sind, sowohl bei Tuberkulose als auch bei Typ-1-Diabetes beschrieben. Dies kann nützlich sein um Biomarker der IL-7-Antwort zu etablieren. Potenziell können diese Befunde zu IL-7-Signalmolekülen und *IL7RA*-SNPs auf andere Autoimmun- und chronische Infektionserkrankungen verallgemeinert werden, und der IL-7-Signalweg könnte ein zukünftiges Ziel für die Prävention oder Behandlung von Krankheiten werden.

List of abbreviations

Akt	Protein kinase B
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
IL-7	Interleukin-7
IL-7Rα	Interleukin-7 receptor α-chain
JAK	Janus kinase
LCMV	Lymphocytic choriomeningitis virus
M.	*Mycobacterium*
mIL-7Rα	Membrane-associated interleukin-7 receptor α-chain
NOD	Non-obese diabetic
PD-1	Programmed cell death protein 1
PI3K	Phosphatidylinositol-3-kinase
SCID	Severe combined immunodeficiency
sIL-7Ra	Soluble interleukin-7 receptor α-chain
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TSLP	Thymic stromal lymphopoietin
TST	Tuberculin skin test

Table of Contents

1. Introduction

IL-7 is a cytokine vital for development and homeostasis of T cells. Lack of IL-7Rα, the cognate receptor for IL-7, leads to severe combined immunodeficiency (SCID) presenting with a $T^-B^+NK^+$ phenotype (Puel et al., 1998, Giliani et al., 2005). On the other hand, gain-of-function mutations in IL-7Rα cause T-cell acute lymphoblastic leukaemia (Zenatti et al., 2011). Together, this highlights the two extremes in the influence of IL-7-signalling on the fate of T cells. The relevance of IL-7-signalling in regulating the immune response is further supported by the association between *IL7RA* SNPs and autoimmune diseases such as multiple sclerosis (Liu et al., 2017, Tavakolpour, 2016, Wu et al., 2016) and type 1 diabetes (Todd et al., 2007). In addition, *IL7RA* SNPs have been associated to outcome of antiviral treatment of HIV-infected patients (Hartling et al., 2014).

IL-7 signals via a heterodimer composed of IL-7Rα and the γ_c receptor, the latter being a shared receptor subunit for IL-2, IL-4, IL-9, IL-7, IL-15 and IL-21 (Waickman et al., 2016). Among the functional effects described for IL-7, the cytokine has been shown to improve survival of T cells (Jiang et al., 2005) and to lower the activation threshold of T cells after T-cell receptor activation (Deshpande et al., 2013). Further, IL-7 has been shown to promote the immune response during chronic infectious diseases such as lymphocytic choriomeningitis virus (LCMV) infection (Pellegrini et al., 2011) and *M. tuberculosis* infection (Maeurer et al., 2000). Overall, IL-7 has a major role in shaping T cells and the T cell response in autoimmune and infectious diseases.

1.1 Structure of IL-7 and IL-7Rα

Human IL-7 was identified as a 152-aa cytokine with growth factor activity on bone marrow cells by Goodwin et al. (1989). Later, the cytokine was found to enhance growth of especially human T cells (Welch et al., 1989). Structurally, IL-7 is composed of a 4 helix-bundle with an up-up-down-down topology like other cytokines of the type I cytokine receptor family. IL-7 is produced by stromal cells in lymphoid organs and tissues, including thymus, bone marrow, lymph nodes, intestinal tissues and epidermis (Hara et al., 2012, Mazzucchelli et al., 2009, Shalapour et al., 2010). In general, IL-7 is believed to be produced at a constant rate without any negative feedback, meaning that the consumption of IL-7 – e.g. by lymphocytes – controls the available amount of the cytokine (Mazzucchelli and Durum, 2007, Martin et al., 2017). However, commensal bacteria have been shown to stimulate expression of IL-7 from intestinal epithelial cells with IFN-γ being an inducing factor, indicating that some regulation of IL-7-production may take place *in vivo* (Shalapour et al., 2010).

IL-7Rα – part of the receptor complex for IL-7 and thymic stromal lymphopoietin (TSLP) – is a type I cytokine receptor composed of two fibronectin type III domains in the 219-aa extracellular chain followed by a 25-aa single transmembrane domain and a 195-aa cytoplasmic tail. The crystal structure of IL-7 bound to the IL-7Rα ectodomain expressed in S2 insect cells was determined by McElroy et al. (2009) (Figure 1). IL-7Rα encompass three disulphide bonds in the D_1 domain, while a WSXWS motif conserved in type I cytokine receptors is located in domain D_2. N-glycosylation of the receptor was

shown to enhance binding of IL-7, and of the 6 potential N-glycosylation sites, the crystal structure allowed identification of three glycosylated sites (Asn49, Asn65 and Asn151), while the glycosylation status of other potential sites (Asn182, Asn232 and Asn233) remains unknown (McElroy et al., 2009). Intracellularly, a Box 1 motif that serves as a docking site for JAK1 is found in close proximity to the membrane (Jiang et al., 2005), while three conserved tyrosine residues are located in the C-terminal end of the receptor.

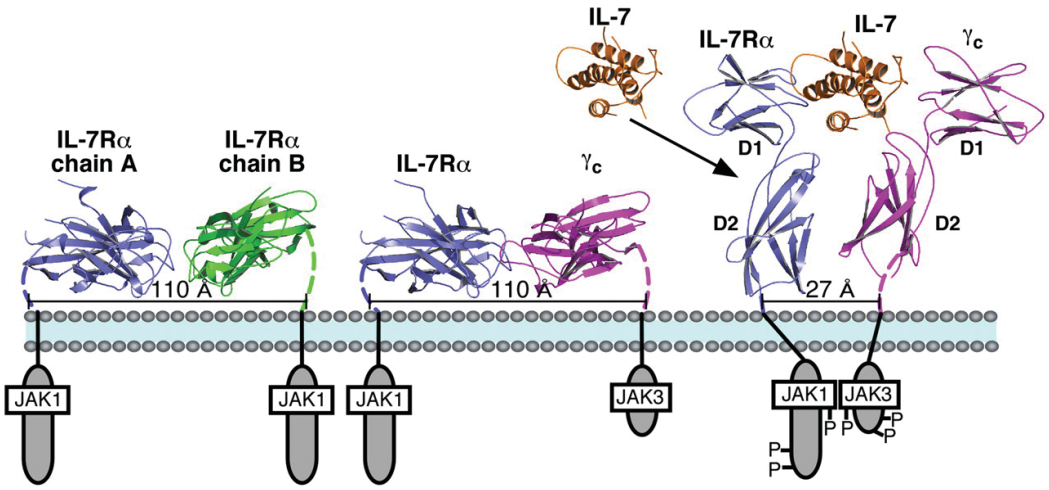

Figure 1: Proposed model of IL-7 signalling. In the resting state, IL-7Rα form homodimers or IL-7Rα:γ_c heterodimers, and the distance between the intracellular domains prevents signalling. In the presence of IL-7, IL-7Rα and γ_c rotate away from the cell membrane to bind IL-7, thereby allowing the intracellular domains to get into closer proximity to initiate phosphorylation and downstream signalling. Abbreviations: JAK: Janus kinase, D: domain. Figure adapted from McElroy et al. (2012) and reprinted by permission from *Proceedings of the National Academy of Sciences*. © 2012.

In the absence of IL-7, the crystal structure of IL-7Rα revealed that the receptor was found in the form of homodimers that are expected to lie flat on the cell surface (McElroy et al., 2012). This would keep the cytoplasmic tails distant from one another, thereby preventing activation of the associated JAK proteins (Figure 1) Due to a higher binding affinity for the IL-7Rα:γ_c complex than for the IL-7Rα:IL-7Rα complex (i.e. K_d ~ 3 µM for the IL-7Rα:γ_c heterodimer and K_d ~ 600 µM for the IL-7Rα:IL-7Rα homodimer), it was proposed that IL-7Rα:γ_c complexes would also be found on the cells in a similar inactive manner. IL-7 binds IL-7Rα with a K_d ~ 60 nM (McElroy et al., 2009), and upon binding, IL-7Rα is expected to rotate 90° away from the cell surface, and the IL-7Rα:IL-7 complex would subsequently associate with the γ_c chain, which would allow the cytoplasmic tails of the receptors to get into close proximity and initiate downstream signalling (Figure 1).

For signalling of IL-7, most focus has been on phosphorylation of STAT5 through the JAK/STAT pathway. After binding of IL-7, JAK1 and JAK3 associated to IL-7Rα and y_c, respectively, are cross-phosphorylated and activates Tyr449 on IL-7Rα that acts as a docking site for STAT5 (Jiang et al., 2005). Subsequently, STAT5 is phosphorylated leading to activated STAT-dimers that translocate to the nucleus

and initiate signalling. Still, while Tyr449Phe mutants show no STAT5-phosphorylation and a ~90% reduction in T cell development (Jiang et al., 2004, Osborne et al., 2007), the incomplete lack of T cells for Tyr449Phe mutants as compared to IL-7Rα$^{-/-}$ hematopoietic stem cells indicates that additional signalling pathways are initiated by IL-7, e.g. the PI3K/Akt pathway (Jiang et al., 2005, Carrette and Surh, 2012).

1.2 Role of IL-7-signalling in T cells

The effects of IL-7-signalling in T cells include improved cellular survival by induction of the anti-apoptotic protein Bcl-2 and inhibition of the pro-apoptotic proteins Bad and Bax (Jiang et al., 2004). Further, IL-7 induces the development of TCRγδ cells (Ye et al., 1999, Ye et al., 2001, Shitara et al., 2013), and decrease of threshold for TCR-activation in T cells (Deshpande et al., 2013). Interestingly, after IL-7-signalling, the IL-7Rα receptor is internalised and targeted for proteasomal degradation with a concurrent decrease in *IL7RA* mRNA expression, i.e. a negative feedback mechanism on both mRNA and protein level (Ghazawi et al., 2013, Henriques et al., 2010, Faller et al., 2015).

T helper cells express various levels of IL-7Rα on the cell surface based on their phenotype. Memory cells were found to have higher IL-7Rα surface expression than naïve T cells, while lowest levels of IL-7Rα was found on regulatory T (Treg) cells (Heninger et al., 2012). Further, lower induction of STAT5-phosphorylation was seen for Treg cells after stimulation with IL-7 when compared to conventional CD4^{+} T cells, indicating the importance of the surface expression level of IL-7Rα for downstream signalling of IL-7 (Heninger et al., 2012). Despite the differential surface level of IL-7Rα on T helper cells, all cell subsets benefit from IL-7 by increased viability and increased cell number (Arbelaez et al., 2015, Simonetta et al., 2012). Still, Th1 cells appear to benefit the most from IL-7 stimulation, both due to an improved cellular response of Th1 cells, and also since IL-7 affects the plasticity of Th17 cells that to some extent differentiate into Th1 cells (Arbelaez et al., 2015, Lee et al., 2011). Further, IL-7 was shown to impair the suppressive activity of Treg cells, which also altered the balance towards a more pro-inflammatory response (Heninger et al., 2012). This was also indicated in the murine model for experimental autoimmune encephalomyelitis (Arbelaez et al., 2015, Lawson et al., 2015).

1.3 Alternative IL-7Rα variants

As the cDNA clone encoding the human IL-7Rα was identified, a putative soluble variant was also found (Goodwin et al., 1990). The soluble variant, which was generated by alternative splicing, lacked 94 nucleotides found in exon 6 (Δ6, sIL-7Rα) that codes for the transmembrane region of the receptor, and the soluble receptor could be detected in plasma (Rose et al., 2009, Pleiman et al., 1991). Due to a change in reading frame, an alternative 26-aa C-terminal tail is generated followed by a premature stop codon (Figure 2). In addition to the IL-7Rα Δ6 variant, mRNA for a variant lacking exon 5-6 (Δ5-6) was identified (Rose et al., 2009, Rane et al., 2010). However, since this variant lacks the WSXWS motif in

exon 5 central for folding of type I cytokine receptors (Olsen and Kragelund, 2014), the Δ5-6 variant is likely not functional, and the variant was not found in plasma (Rose et al., 2009). Further, due to a premature stop codon located upstream of an exon-exon junction (Figure 2), the mRNA transcript is presumably a target for nonsense-mediated mRNA decay (Maquat, 2004).

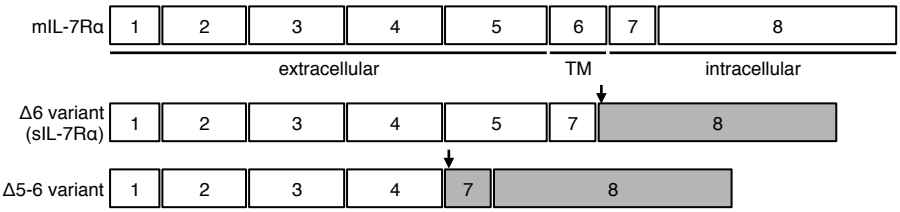

Figure 2: Exon structure of IL-7Rα. The membrane-associated (m)IL-7Rα is composed of eight exons. Alternative splicing generates two variants lacking exon 6 (soluble (s)IL-7Rα, Δ6) or exon 5-6 (Δ5-6). Arrows indicate premature stop codons due to changes in reading frames, and untranslated regions are shaded in grey. Abbreviations: TM: Transmembrane. Figure modified from Lundtoft et al. (In review).

The exact role of sIL-7Rα is still a matter of discussion, and both inhibitory and promoting functions on IL-7-signalling have been described. On the short term, sIL-7Rα inhibits STAT5 phosphorylation and IL-7-induced proliferation (Monti et al., 2013) . On a longer term, however, sIL-7Rα was shown to act as a reservoir for IL-7, thereby promoting the IL-7-response and increasing disease severity in a murine experimental autoimmune encephalomyelitis model (Lundström et al., 2013). Remarkably, the rs6897932 SNP located in exon 6 of *IL7RA* was found to play a role in splicing of the exon, thereby affecting the level of Δ6 *IL7RA* transcript and plasma level of sIL-7Rα (Gregory et al., 2007, Jäger et al., 2013, Lundström et al., 2013). This SNP has also been associated with a range of diseases, including multiple sclerosis (Tavakolpour, 2016, Wu et al., 2016), type 1 diabetes (Todd et al., 2007), rheumatic arthritis (O'Doherty et al., 2009), inhalation allergy (Shamim et al., 2007), graft-versus-host disease (Kielsen et al., 2018), and treatment-outcome for HIV patients (Hartling et al., 2014). Therefore, sIL-7Rα may add an additional layer of complexity in IL-7-signalling.

1.4 IL-7 in tuberculosis

Approximately 1.7 billion individuals are estimated to be infected with *M. tuberculosis* worldwide (Houben and Dodd, 2016). The bacterial infection was estimated to cause active tuberculosis in 10.4 million individuals in 2016, leading to 1.7 million deaths (WHO, 2017). Although being used for a century, the current bacille Calmette-Guérin vaccine used against tuberculosis demonstrates moderate and varying protection against progression to active disease after *M. tuberculosis* infection, and mainly in children (Kaufmann et al., 2014, Roy et al., 2014, Mangtani et al., 2014).

Diagnosis of active tuberculosis can be based on multiple signs and symptoms that include sputum smear-staining and culture, chest X-ray and immunodiagnostic tests, together with patient history of *M. tuberculosis*-exposure and immunosuppression (Maartens and Wilkinson, 2007). Immunodiagnostic tests make use of the ‘memory‘ of the adaptive immune system due to previous exposure to *M. tuberculosis* and include the tuberculin skin test and the IFN-γ release assay. However, the incapability of the tests to distinguish between latent and active tuberculosis together with a suboptimal sensitivity and specificity especially in endemic areas may limit the applicability of the tests (Campion et al., 2015).

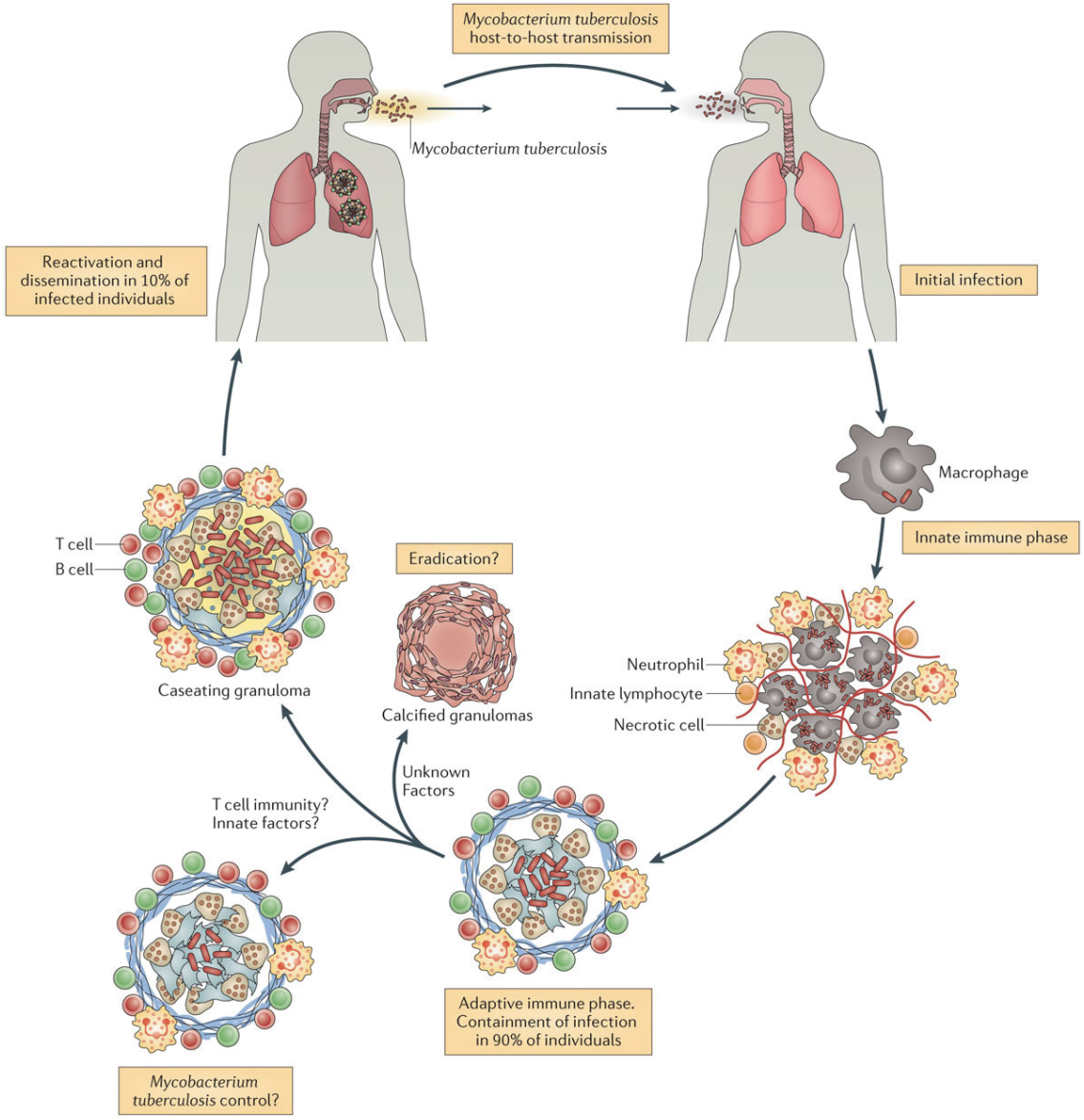

Figure 3: Proposed model of *M. tuberculosis* infection. Figure adapted from Nunes-Alves et al. (2014) and reprinted by permission from *Springer Nature: Nature Reviews Microbiology*. © 2014.

The early response in *M. tuberculosis* infection involves innate immune cells. Alveolar macrophages phagocytise *M. tuberculosis* but may fail to kill virulent bacteria, and cells of the adaptive immune

system is later primed by antigen-presenting cells. Due to the persistence of *M. tuberculosis*, granulomas are formed in which innate and adaptive immune cells cap off and contain the infection, also described as latent infection (Figure 3). This may lead to control of infection with or without eradication of the intracellular bacteria. Alternatively, an inadequate immune response can lead to active tuberculosis and transfer of the bacteria to another host (Nunes-Alves et al., 2014, O'Garra et al., 2013). Together with other cells of the adaptive immune system, IFN-γ-producing Th1 cells have been ascribed a central role in the immunity against *M. tuberculosis*, which is also seen in by the increased risk of active tuberculosis in patients with HIV infection and low number of $CD4^+$ cells (O'Garra et al., 2013).

IL-7 has proven beneficial *in vitro*, where co-incubation with IL-7 in IFN-γ release assays increase the response to *M. tuberculosis*-specific antigens, thereby increasing the sensitivity of the assay that otherwise may show limited response, especially from children and/or immunocompromised patients (Feske et al., 2008).

In an animal model of tuberculosis, Maeurer et al. (2000) showed that administration of IL-7 prolonged the survival of mice infected with *M. tuberculosis*. Further, adoptive transfer of *M. tuberculosis*-experienced splenocytes into *M. tuberculosis*-infected mice showed improved survival when priming with *M. tuberculosis* took place in the presence of IL-7 (Maeurer et al., 2000). Similarly, the importance of IL-7 during initial T cell receptor-stimulation for generation of optimal effector function was also shown in a murine model of experimental autoimmune encephalomyelitis by Lawson et al. (2015). In addition, murine vaccination models also indicated improved Th1 response when IL-7 was included in the vaccine (Hatano et al., 2016, Rao et al., 2013), and co-administration of IL-7 and IL-15 with bacille Calmette-Guérin vaccine prior to infection with *M. tuberculosis* resulted in reduced bacterial load (Singh et al., 2010).

Persistent antigen has been shown to downregulate IL-7Rα in murine LCMV models, leading to T cell exhaustion (Lang et al., 2005). This pattern has also been seen in human tuberculosis, where lower proportions of antigen-specific T cells were IL-$7R\alpha^+$ in patients with active tuberculosis when compared to individual with latent infection (Pollock et al., 2013). However, treatment with IL-7 during chronic viral infection reduced virus titres (Pellegrini et al., 2011), indicating an beneficial effect of IL-7 in the response against chronic infections. Still, the role of IL-7 in tuberculosis remains largely undescribed, but initial studies indicated a high variability of IL-7-promoted T cell-responses of individual donors, and potentially a decreased IL-7-response in children with tuberculosis (own unpublished data).

1.5 IL-7 in type 1 diabetes

Type 1 diabetes is an autoimmune disease, in which the insulin-producing pancreatic β-cells are destroyed. The disease often starts at an early age, and patients become dependent on administration of exogenous insulin in order to regulate blood glucose (Daneman, 2006). A strong genetic component of the disease has been reported, with the human leukocyte antigen (HLA) being the strongest genetic risk

factor, while alleles in genes such as *INS*, *PTPN22*, *CTLA4* and *IL2RA* also have been associated with risk of developing type 1 diabetes (Atkinson et al., 2014, Pociot and Lernmark, 2016).

Several cell subsets are involved in the pathogenesis of type 1 diabetes (Figure 4), including B cells that produce autoantibodies against proteins such as proinsulin and glutamic acid decarboxylase, autoreactive cytotoxic $CD8^+$ T cells that destroy β cells, and $CD4^+$ T helper cells regulating the adaptive immune system (Atkinson et al., 2014, Walker and Herrath, 2016). Since HLA class II contributes the most to the genetic component of HLA genes for type 1 diabetes risk, special attention has been paid to $CD4^+$ T cells since this cell subset recognises antigen presented on HLA class II by antigen-presenting cells (Todd, 2010).

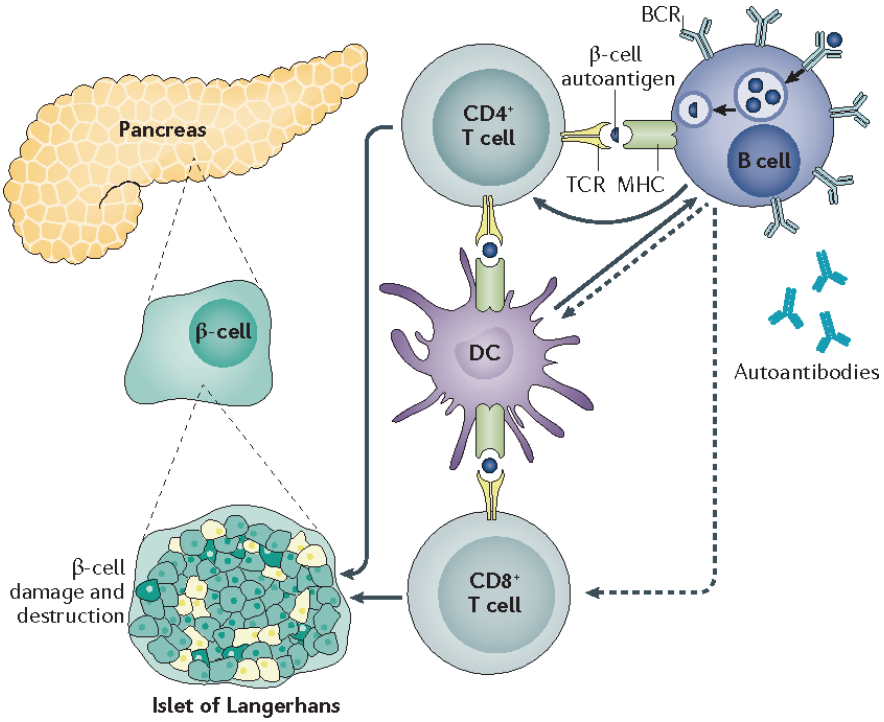

Figure 4: Pathogenesis of type 1 diabetes. Abbreviations: BCR: B cell receptor, TCR: T cell receptor, MHC: Major histocompatibility complex, also known as human leukocyte antigen (HLA). Figure adapted from Katsarou et al. (2017) and reprinted by permission from *Springer Nature: Nature Reviews Disease Primers*. © 2017.

Especially the balance between Treg cells with suppressive functions, and Th1 and Th17 cell subsets with pro-inflammatory functions has been a matter of research, and deficits in suppressive function of Treg cells from type 1 diabetes patients has been reported (Visperas and Vignali, 2016). Further, a role of Th1 and Th17 cells has been described, and especially Th17 cells that show plasticity towards IFN-γ-producing Th1 cells has been shown to induce T1D (Bending et al., 2009, Martin-Orozco et al., 2009, Reinert-Hartwall et al., 2015, Solt and Burris, 2015).

Heninger et al. (2013) reported that naïve $CD4^+$ T cells specific for proinsulin and glutamic acid decarboxylase could be detected already in cord blood from healthy donors, and early generation of

tolerance is therefore needed to avoid an autoimmune response against β-cells. Hence, mediators such as IL-7 that can affect the balance between Treg cells and proinflammatory Th1/Th17 cells are of central relevance. Blocking IL-7-signalling by antibody-treatment against IL-7Rα inhibited diabetes in non-obese diabetic (NOD) mice and could even revert diabetes when administering the antibody after onset of diabetes (Penaranda et al., 2012, Lee et al., 2012). Mainly IFN-γ-producing T cells were affected by the treatment, while minor effect was seen on IL-17-producing cells and the suppressive capacity of Treg cells (Penaranda et al., 2012, Lee et al., 2012). On the other hand, addition of IL-7 impaired the ability of human Treg cells to suppress the response of autoantigen-specific T cells (Heninger et al., 2012). Further, increased levels of IL-7 – e.g. during lymphopenia, where consumption of IL-7 by T cells is reduced – was shown to promote induction of diabetes in mice (Calzascia et al., 2008, King et al., 2004). In humans, IL-7 induced stronger IFN-γ- and TNF-α-responses in T cells from patients with long-term type 1 diabetes when compared to healthy controls (Seyfarth et al., 2017). Overall, IL-7 appears to be able to shift the balance from tolerance towards an autoimmune Th1 response, thereby being able to support progress towards diabetes.

The relevance of IL-7-signalling for development of type 1 diabetes was further supported by the association of *IL7RA* SNPs with disease risk. The minor allele of rs6897932 located in exon 6 of IL-7Rα was associated with protection against type 1 diabetes (Todd et al., 2007), and a similar effect has also been shown in multiple sclerosis (Gregory et al., 2007). The SNP affects splicing of IL-7Rα, and carriers of the minor allele have lower plasma levels of the sIL-7Rα (Lundström et al., 2013). In type 1 diabetes, higher sIL-7Rα concentration was found in serum from new-onset patients with type 1 diabetes when compared to autoantibody-negative relatives (Monti et al., 2013). Further, higher levels of sIL-7Rα were found in autoantibody-positive patients as compared to patients with no autoantibodies. Finally, high glucose concentrations led to non-enzymatic glycation of sIL-7Rα with impaired binding of IL-7, and higher levels of glycation was generally seen on sIL-7Ra from new-onset patients with type 1 diabetes (Monti et al., 2013). Thereby an additional method of regulation of IL-7 signalling was shown in a diabetes-specific context.

Overall, IL-7 has a central function in balancing the immune response, and while enhanced IL-7-signalling may lead to autoimmunity, diminished signalling of IL-7 may impair the adaptive response in infectious diseases. Still, several questions remain about the regulation of IL-7 and IL-7Rα during homeostasis and in disease, and the exact role of sIL-7Rα is also a matter of discussion. Therefore, the role of IL-7 and IL-7-signalling in *M. tuberculosis* infection and in type 1 diabetes will be evaluated in the next chapters.

2. Research objectives

IL-7 is a cytokine important for balancing the immune response, and whereas administration of IL-7 promotes the response against infectious agents, excessive signalling of IL-7 may lead to autoimmunity. Both the availability of IL-7 and the cellular level of its receptor, IL-7Rα, are central for signalling, while sIL-7Rα and genetic variants of the receptor also may play a role. In the four papers included in this thesis, we sought to explore the role of factors relevant for IL-7-signalling in infectious and autoimmune disease. Using two human disease models, i.e. tuberculosis and type 1 diabetes, respectively, the objectives included:

- To describe the levels of circulating IL-7 and sIL-7Rα in patients and healthy individuals, and to characterise the association between these two markers and their potential use as biomarkers for disease status.
- To evaluate the expression of mIL-7Rα on T cells from patients and healthy individuals, and to determine the effect of IL-7-stimulation on downstream signalling process in T cells

In addition to general levels of molecules involved in IL-7-signalling, SNPs in the *IL7RA* gene have ben associated with autoimmune and infectious diseases. To explore the impact of genetic variants in *IL7RA* more in detail, the objectives included:

- To determine the association between *IL7RA* SNPs and risk of disease.
- To evaluate the association between *IL7RA* SNPs and levels of circulating IL-7 and sIL-7Rα, and to other molecules involved in IL-7-signalling.
- To establish a model system for evaluating the functional effects of *IL7RA* SNPs on receptor expression and downstream IL-7-signalling.

3.1 Paper I: Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T-cell response to IL-7 in human tuberculosis

Lundtoft, C.*, Afum-Adjei Awuah, A.*, Rimpler, J.*, Harling, K., Nausch, N., Kohns, M., Adankwah, E., Lang, F., Olbrich, L., Mayatepek, E., Owusu-Dabo, E. & Jacobsen, M. 2017. Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T-cell response to IL-7 in human tuberculosis. *PLOS Pathogens*, 13, e1006425.

 * Shared 1st authorship

Contribution to this publication: 60%

- ELISA
- Analysis of gene expression
- Genotyping
- Data analysis
- Writing of the manuscript

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RESEARCH ARTICLE

Aberrant plasma IL-7 and soluble IL-7 receptor levels indicate impaired T-cell response to IL-7 in human tuberculosis

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Abstract

T-cell proliferation and generation of protective memory during chronic infections depend on Interleukin-7 (IL-7) availability and receptivity. Regulation of IL-7 receptor (IL-7R) expression and signalling are key for IL-7-modulated T-cell functions. Aberrant expression of soluble (s) and membrane-associated (m) IL-7R molecules is associated with development of autoimmunity and immune failure in acquired immune deficiency syndrome (AIDS) patients. Here we investigated the role of IL-7/IL-7R on T-cell immunity in human tuberculosis. We performed two independent case-control studies comparing tuberculosis patients and healthy contacts. This was combined with follow-up examinations for a subgroup of tuberculosis patients under therapy and recovery. Blood plasma and T cells were characterised for IL-7/sIL-7R and mIL-7R expression, respectively. IL-7-dependent T-cell functions were determined by analysing STAT5 phosphorylation, antigen-specific cytokine release and by analysing markers of T-cell exhaustion and inflammation. Tuberculosis patients had lower soluble IL-7R ($p < 0.001$) and higher IL-7 ($p < 0.001$) plasma concentrations as compared to healthy contacts. Both markers were largely independent and aberrant expression normalised during therapy and recovery. Furthermore, tuberculosis patients had lower levels of mIL-7R in T cells caused by post-transcriptional mechanisms. Functional *in vitro* tests indicated diminished IL-7-induced STAT5 phosphorylation and impaired IL-7-promoted cytokine release of *Mycobacterium tuberculosis*-specific $CD4^+$ T cells from tuberculosis patients. Finally, we determined T-cell exhaustion markers PD-1 and SOCS3 and detected increased SOCS3 expression during therapy. Only moderate correlation of PD-1 and SOCS3 with IL-7 expression was observed. We conclude that diminished soluble IL-7R and increased IL-7 plasma concentrations, as well as decreased membrane-associated IL-7R expression in T cells, reflect impaired T-cell sensitivity to IL-7 in tuberculosis patients. These findings show similarities to pathognomonic features of impaired T-cell functions and immune failure described in AIDS patients.

decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Author summary

IL-7 is important for the development and homeostasis of T cells and promotes antigen-specific T-cell responses. Aberrant expression of plasma IL-7 and soluble IL-7R are found in autoimmune diseases and chronic viral infections. In AIDS patients—especially those who fail to reconstitute T-cell numbers during therapy—impaired IL-7-promoted T-cell functions indicated T-cell exhaustion/senescence. In order to evaluate the potential impact of IL-7 on tuberculosis, we characterised various parameters involved in the IL-7-response of tuberculosis patients and healthy contacts. Despite IL-7 being available at higher plasma levels among tuberculosis patients, the T-cell response to IL-7 was impaired when compared to healthy contacts. Soluble IL-7R levels were aberrantly low in plasma during acute tuberculosis but did not account for impaired IL-7 usage. Chronic inflammation in tuberculosis patients—reflected by increased IL-6 plasma levels—did not account for dysfunctional T-cell responses and analysed T-cell exhaustion markers were only moderately correlated. Our findings demonstrate that availability of IL-7 alone is not sufficient to promote protective T-cell immunity against tuberculosis. We describe aberrant IL-7/soluble IL-7R expression and impaired IL-7-mediated T-cell functions in tuberculosis patients with similarities and differences to described IL-7 dysregulation seen in patients with AIDS.

Introduction

T cells are crucial for protection against *Mycobacterium (M.) tuberculosis* infection but biomarkers that characterise T-cell failure and progression towards tuberculosis disease are not available [1]. $CD4^+$ T cells are key to anti-mycobacterial immune protection [2] and $CD4^+$ T-cell deficiency, e.g. of AIDS patients, results in increased susceptibility against tuberculosis [3–5]. There is growing evidence that impaired $CD4^+$ T-cell functions play a role in tuberculosis [6]. Recent studies identified T-cell exhaustion as a feature of tuberculosis [7, 8]. T-cell exhaustion impairs immunity against chronic viral infections and harms memory T-cell potential [9]. IL-7 is central for generation of memory T cells and was shown to revert T-cell exhaustion in chronic viral infections [10]. Notably, IL-7 induced T-cell memory was hampered in the presence of persistent antigen and inflammation as seen for chronic viral infections [11]. In AIDS patients, failure of immune reconstitution is accompanied by a dysfunctional T-cell response that showed features of senescence and exhaustion [12–14]. Recently, persistent inflammation characterised e.g. by increased IL-6 serum concentrations from AIDS patients were found to correlate with T-cell exhaustion/senescence and impaired T-cell response to IL-7 [14, 15]. High IL-7 plasma levels as well as decreased membrane-associated (m)IL-7R expression of T cells were found in AIDS patients with immune failure [16, 17]. Concomitantly impaired T-cell response to IL-7 was detected in immune failure patients [13–15, 18–20].

The regulation of IL-7R expression is central for control of IL-7-mediated effects on T cells [21]. On IL-7 binding, the mIL-7R assembles as a heterodimer (comprising the IL-7Rα (CD127) and the common γ-chain (CD132)) and induces signalling cascades mainly via the Jak/STAT pathway. Jak1 and Jak3 are involved in IL-7R signalling, and STAT5 gets phosphorylated and initiates multiple transcription events [22]. As part of IL-7 signalling, the mIL-7R is rapidly internalised, becomes partly degraded or recycles to the cell surface [23]. Regulation of IL-7R expression is also controlled on the transcriptional level and IL-7 and other cytokines were shown to suppress IL-7R mRNA expression [24]. Alternative splicing of the *IL7RA* gene generates a soluble IL-7R (sIL-7R) variant [25]. The sIL-7R variant binds IL-7 although with

lower affinity as compared to the mIL-7R heterodimer and is present in blood plasma at high molar excess relative to IL-7 [26]. The exact role of the sIL-7R for IL-7 metabolism remains elusive. Competitive inhibition of IL-7 uptake as well as IL-7 reservoir functions have been described [26–28]. Differential sIL-7R plasma concentrations are found in immune pathologies, e.g. autoimmune diseases [26, 29, 30] and AIDS [28, 31]. In addition, a functional *IL7RA* polymorphism (rs6897932) that interferes with IL-7R alternative splicing and thereby leads to reduced sIL-7R levels in plasma was found to be associated with autoimmune diseases [32, 33] and to affect immune reconstitution in AIDS patients [34–36].

Initial results indicating a role of IL-7 during T-cell immunity against tuberculosis were derived from animal models. Increased IL-7 and soluble IL-7R expression in pulmonary tissue of primates with tuberculosis was found, indicating a possible role of IL-7 metabolism in tuberculosis pathogenesis [37, 38]. Furthermore IL-7 was shown to promote survival and to improve BCG vaccination efficacy in *M. tuberculosis*-infected mice [39, 40]. However, a comprehensive understanding of the possible role of IL-7 or IL-7R functions in human tuberculosis has not yet been developed.

This present study aimed to elucidate a possible role of IL-7 modulated T-cell responses in human tuberculosis. We determined sIL-7R and IL-7 plasma concentrations and mIL-7R expression of T cells from tuberculosis patients—before, during, and after chemotherapy—and compared these to healthy contacts. Since results resembled pattern seen in AIDS patients with impaired T-cell response to IL-7, we then performed functional T-cell assays in a second set of tuberculosis patients and healthy contacts to determine IL-7-mediated signalling and promoted cytokine release on *M. tuberculosis*-specific T-cell activation. Finally, mRNA expression of exhaustion markers was compared in $CD4^+$ T cells between the cohorts to evaluate a possible causative role of T-cell exhaustion for impaired IL-7 response in tuberculosis.

Results

Decreased sIL-7R plasma concentrations in acute tuberculosis patients

Aberrant sIL-7R plasma levels indicate pathologic T-cell immunity in autoimmune, inflammatory, and chronic viral diseases. Hence, we determined sIL-7R plasma concentrations in individuals infected with *M. tuberculosis*. Patients with active tuberculosis (n = 57) and healthy contacts (n = 151) were included. Tuberculosis patients had significantly lower sIL-7R concentrations as compared to healthy contacts ($p < 0.001$) (Fig 1a). Since study groups differed in gender distributions (tuberculosis: 30% females; contacts: 56% females; Table 1), we compared sIL-7R between male and female subgroups. Female patients with tuberculosis showed moderately lower sIL-7R concentrations as compared to male patients, whereas no differences were detected for healthy contacts (S1 Fig). Therefore, differences in plasma sIL-7R were not due to gender differences. Next we determined the influence of anti-tuberculosis therapy and recovery on plasma sIL-7R in tuberculosis patients (i.e. 2 months and 6 months after therapy onset). Analyses revealed significantly increased sIL-7R plasma levels after 2 months ($p = 0.03$) and after recovery ($p = 0.009$) (Fig 1b). sIL-7R plasma concentrations of recovered tuberculosis patients were comparable to healthy contacts (Fig 1b). To determine if changes in sIL-7R under therapy were dependent on sIL-7R concentrations prior to treatment, we compared initial sIL-7R concentrations with changes of sIL-7R expression between 0 and 6 months. Absolute differences and ratios were calculated. Absolute differences (month 6 –month 0) showed only moderate negative correlation with initial sIL-7R levels (rho = -0.26; $p = 0.13$) (S2a Fig), but changes of ratios (month 6 / month 0) were strongly associated with sIL-7R levels prior to treatment (rho = -0.61, $p < 0.001$) (S2a Fig). Therefore, especially tuberculosis patients with

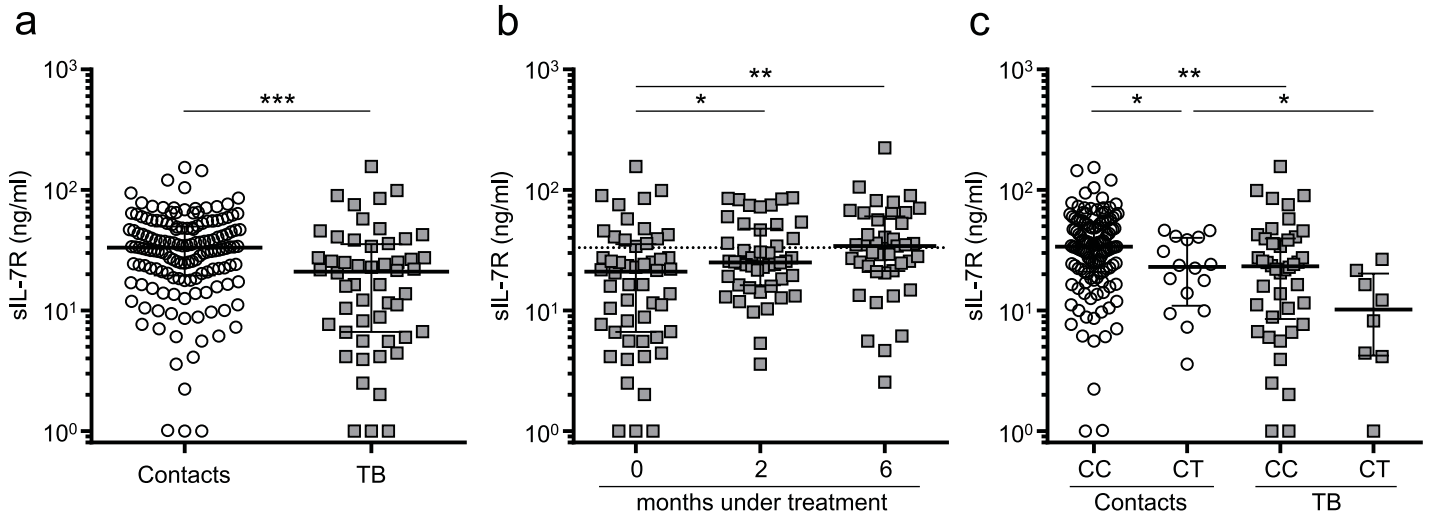

Fig 1. Plasma sIL-7R level in healthy contacts and tuberculosis during chemotherapy. (a) Concentrations of sIL-7R in plasma from tuberculosis patients (n = 52) and healthy contacts (n = 149) determined by cytometric bead array. **(b)** sIL-7R plasma concentration prior to (0 months, n = 52), during (2 months, n = 46) and after (6 months, n = 41) treatment of tuberculosis patients. Median sIL-7R plasma concentration of healthy contacts is indicated with a dotted line. **(c)** Plasma sIL-7R levels stratified for the *IL7RA* exon 6 single nucleotide polymorphism rs6897932C>T for healthy contacts (n = 142) and tuberculosis patients (n = 50). Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test was used for comparison of groups, while paired data was evaluated by Wilcoxon Signed-Rank test. Nominal p-values are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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low sIL-7R concentrations prior to treatment showed increased sIL-7R levels after recovery and a relative gain of sIL-7R plasma concentration was detected.

The IL7RA functional polymorphism rs6897932 contributed to differential sIL-7R plasma levels

A functional single nucleotide polymorphism (SNP, rs6897932C>T) in exon 6 of the *IL7RA* gene interferes with splicing and impairs sIL-7R expression [32]. Therefore, we determined

Table 1. Patient characteristics.

Cohort 1	**Healthy Contacts**	**TB**
Number of participants	151	57
Age (y)	31 [18–68]	33 [18–71]
Gender		
Female	84 (56%)	17 (30%)
Male	67 (44%)	40 (70%)
BCG vaccination		
Yes	90 (60%)	28 (49%)
No	50 (33%)	28 (49%)
No information	11 (7%)	1 (2%)
Cohort 2		
Number of participants	24	22
Age (y)	41 [21–65]	39 [15–72]
Gender		
Female	14 (58%)	15 (68%)
Male	10 (42%)	7 (32%)

Median [range] or number (proportion) is shown.

https://doi.org/10.1371/journal.ppat.1006425.t001

the rs6897932 minor T allele (rs6897932T) frequency in tuberculosis patients and healthy contacts. Tuberculosis patients had a marginally higher MAF proportion (7.3%) as compared to healthy contacts (5.6%). No homozygous rs6897932T/T carriers were identified in the study groups. As expected, lower levels of plasma sIL-7R were detected for rs6897932C/T healthy contacts as compared to rs6897932C/C wild type healthy contacts ($p = 0.02$), and the same tendency was seen for the tuberculosis patients ($p = 0.06$) (Fig 1c). However, stratification for SNP genotypes confirmed lower plasma sIL-7R among tuberculosis patients when compared to healthy contacts ($p < 0.001$). We concluded that increased frequencies of IL-7R rs6897932T alleles in tuberculosis patients contributed to differential sIL-7R levels but did not account for lower sIL-7R plasma concentrations of tuberculosis patients.

Increased IL-7 plasma concentrations in tuberculosis patients but no correlation with sIL-7R

We hypothesised that differential sIL-7R plasma levels would affect IL-7 consumption. Consequently we next determined IL-7 plasma concentrations in tuberculosis patients and healthy contacts. Tuberculosis patients showed significantly increased IL-7 concentrations prior to therapy as compared to healthy contacts ($p < 0.001$) (Fig 2a). IL-7 concentrations decreased under therapy and recovery (0 vs. 6 months, $p < 0.001$) and reached levels comparable to healthy contacts (Fig 2b). Higher initial IL-7 levels were associated with stronger decrease rates until month 6 ($rho = -0.58$, $p < 0.001$; S2b Fig). Notably, and in contrast to sIL-7R results, also absolute differences between month 0 and 6 correlated strongly with IL-7 levels prior to therapy ($rho = -0.79$, $p < 0.001$; S2b Fig). This indicated different mechanisms involved in IL-7 and sIL-7R regulation during tuberculosis. In accordance, no dependency was detected between IL-7 and sIL-7R plasma concentrations for tuberculosis patients or healthy contacts (Fig 2c).

These results suggested that IL-7 and sIL-7R could be useful as biomarkers for diagnosis of tuberculosis patients. Comparison of tuberculosis patients and healthy contacts revealed moderate discrimination capacity for both sIL-7R (AUC = 0.67) and IL-7 (AUC = 0.73) using Receiver Operating Characteristic (ROC) analysis (Fig 2d). Independency of IL-7 and sIL-7R plasma levels (Fig 2c) prompted us to calculate the combined efficacy of both markers using Random Forest analysis (for details see Methods section). Correct prediction of tuberculosis patients and healthy contacts was achieved for 73% of all donors, and IL-7 was about two times more influential on prediction than sIL-7R. These results indicated that IL-7 and sIL-7R plasma concentrations were largely independent and may contribute to tuberculosis diagnosis.

Increased proportions of mIL-7R$_{low}$ CD4$^+$ and CD8$^+$ T cells in tuberculosis patients

Increased IL-7 plasma concentrations are likely caused by decreased T-cell consumption of IL-7. Low T-cell numbers or impaired T-cell receptivity of IL-7 may account for this. Hence we compared mIL-7R protein expression for subgroups of tuberculosis patients and healthy contacts by flow cytometry. We detected lower mean mIL-7R expression for CD8$^+$ T cells ($p = 0.02$) and a tendency for CD4$^+$ T cells ($p = 0.05$) (Fig 3a). Analysis of mIL-7R on T-cell subpopulations revealed increased proportions of mIL-7R$_{low}$ CD4$^+$ ($p = 0.006$) and CD8$^+$ T cells ($p = 0.02$) from tuberculosis patients as compared to healthy contacts (Fig 3b). To confirm these observations, we performed mIL-7R analysis in a second independent cohort study including additionally recruited tuberculosis patients ($n = 22$) and healthy contacts ($n = 24$). Due to restriction in the number of flow cytometry parameters, CD4$^+$ and CD4$^-$ T cells were analysed for mIL-7R protein expression. Tuberculosis patients showed significantly decreased

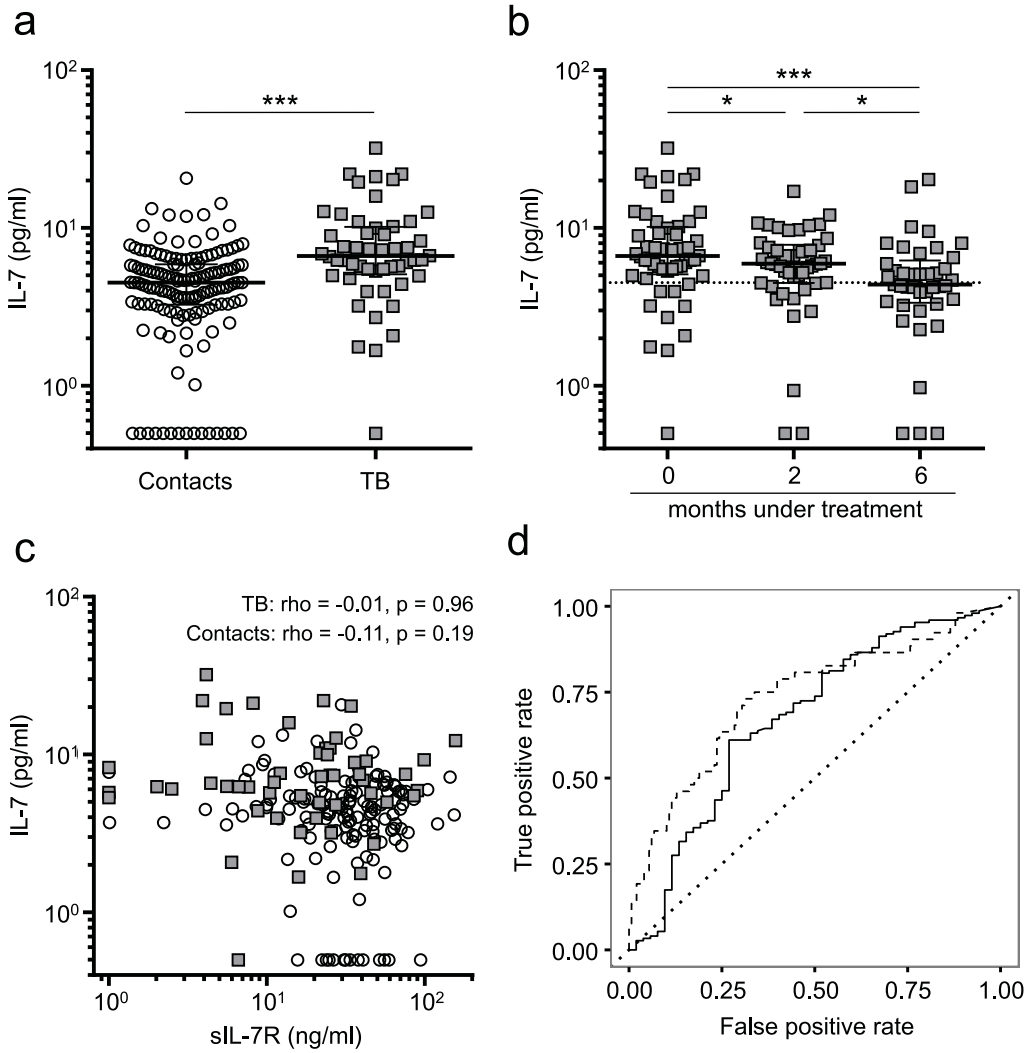

Fig 2. Plasma IL-7 concentration of tuberculosis patients and healthy contacts. (a) Comparison of IL-7 plasma concentrations between tuberculosis patients (n = 52) and healthy contacts (n = 148). **(b)** Plasma IL-7 concentrations prior to (0 months, n = 52), during (2 months, n = 46), and after (6 months, n = 41) treatment of tuberculosis patients. Median IL-7 plasma level of healthy contacts is represented with a dotted line. **(c)** Spearman correlation between plasma IL-7 and sIL-7R for tuberculosis patients prior to treatment (squares), and healthy contacts (circles). **(d)** Receiver operating characteristic (ROC) curve indicates sensitivity and specificity of plasma sIL-7R (solid line) and plasma IL-7 (dashed line) to discriminate between patients with tuberculosis and healthy contacts. The line of no discrimination is indicated as a dotted line. Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test was used for comparison of groups, whereas paired data was evaluated by Wilcoxon Signed-Rank test. Nominal p-values are indicated as: * $p < 0.05$, *** $p < 0.001$.

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mIL-7R expression for both $CD4^+$ ($p = 0.01$) and $CD4^-$ ($p = 0.006$) T cells (S4 Fig). This confirmed initial results and led us to the conclusion that impaired mIL-7R expression of T cells resulted in increased proportions of $mIL\text{-}7R_{low}$ $CD4^+$ and $CD8^+$ T cells in tuberculosis patients.

Differential mIL-7R expression may be affected by plasma IL-7 and sIL-7R levels. We determined correlation between these parameters to identify possible interactions. A tendency of positive correlation between mIL-7R expression and sIL-7R plasma (rho = 0.42, p = 0.06) was

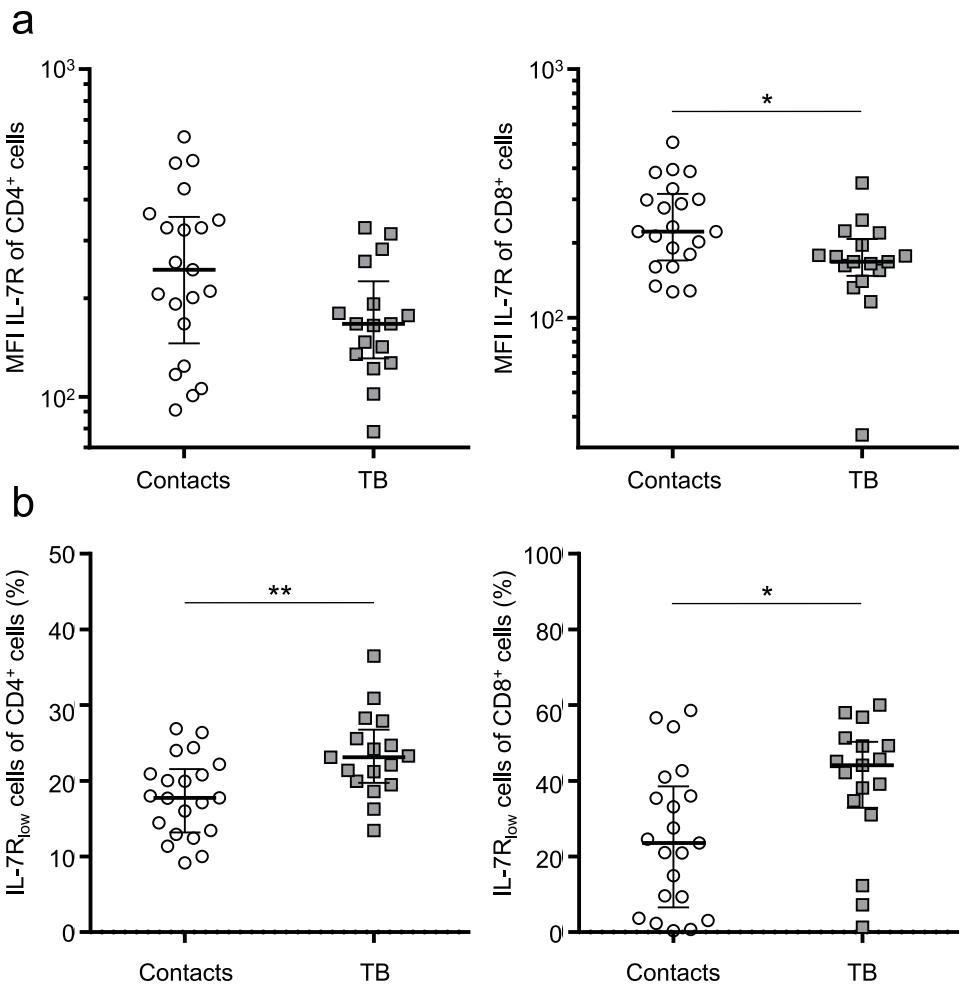

Fig 3. Membrane-associated IL-7R expression on CD4⁺ and CD8⁺ T cells. Membrane-associated IL-7R expression of $CD4^+$ (left graphs) and $CD8^+$ (right graphs) T cells from tuberculosis patients (n = 17) and healthy contacts (n = 21) analysed by flow cytometry. **(a)** Mean fluorescence intensity (MFI) analyses of IL-7R. **(b)** Proportions of $IL\text{-}7R_{low}$-expressing $CD4^+$ and $CD8^+$ T cells from tuberculosis patients and healthy contacts. Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test used for comparison of groups. Nominal p-values are indicated as * $p < 0.05$, ** $p < 0.01$.

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found only in the group of contacts, whereas mIL-7R and IL-7 showed a marginal negative correlation (rho = -0.38, p = 0.10) (S1 Table) in this study group. No correlation between any parameters was found for tuberculosis patients (S1 Table).

Similar IL-7R isoform mRNA expression of $CD4^+$ T cells from tuberculosis patients and healthy contacts

High IL-7 plasma levels and low mIL-7R expression of T cells have previously been described for HIV/AIDS patients [16, 17, 41, 42]. In AIDS patients these differences are accompanied with mIL-7R regulatory dysfunctions [43]. Therefore we questioned whether aberrant expression of IL-7R variants in tuberculosis patients is caused by differential regulation on the transcriptional or post-transcriptional level. Hence, we analysed IL-7R mRNA transcripts of purified $CD4^+$ T cells from tuberculosis patients and healthy contacts. Three IL-7R transcripts

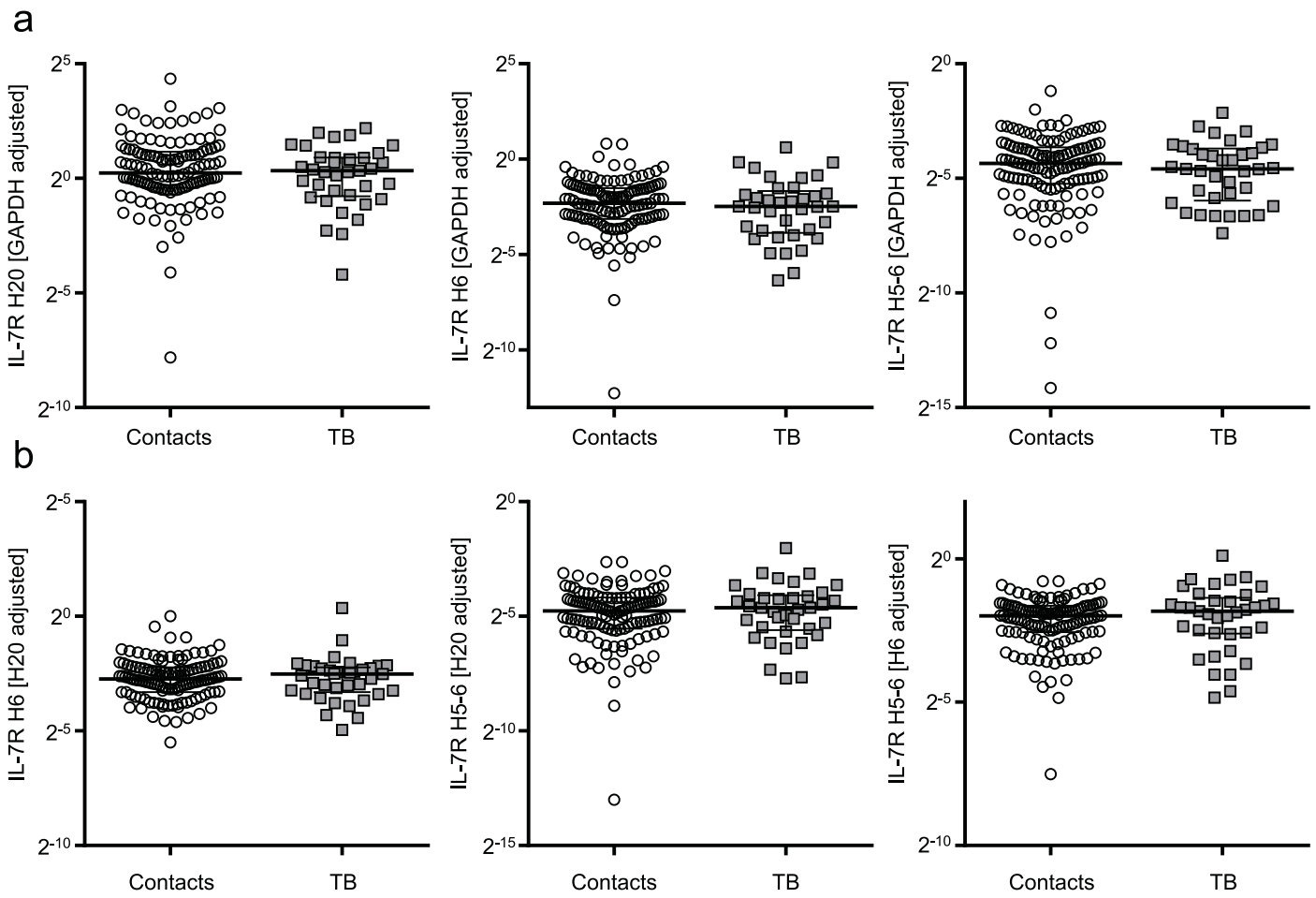

Fig 4. mRNA expression of IL-7R variants of CD4⁺ T cells from tuberculosis patients and healthy contacts. The expression of full length IL-7R (H20), and IL-7R lacking exon 6 (H6) or exon 5–6 (H5-6) was evaluated using mRNA isolated from CD4⁺ T cells. **(a)** Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or **(b)** IL-7R variants were applied as references. Cycle threshold differences ($2^{-\Delta Ct}$) are shown for tuberculosis patients (n = 37) and healthy contacts (n = 120). Median and interquartile range is depicted, and each symbol indicates mean values of duplicates from each individual donor. Exact Mann-Whitney U test used for comparison of groups.

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coding for the mIL-7R (all 8 exons included; H20) and a sIL-7R (H6 and H5-6; for details see Methods section [25]) were measured. None of the IL-7R variants were differentially expressed on the mRNA level of CD4⁺ T cells between tuberculosis patients and healthy contacts (Fig 4a). Also relative expression of sIL-7R vs. mIL-7R transcripts was similar between study groups (Fig 4b). These results indicated that differential IL-7R mRNA expression is not the cause for aberrant sIL-7R and mIL-7R expression in tuberculosis patients and render causative post-transcriptional mechanisms likely.

Impaired IL-7-induced STAT5 phosphorylation and IL-7-promoted T-cell cytokine release in tuberculosis patients

Impaired IL-7 signalling has been associated with diminished IL-7R$_{low}$ expression of T cells from AIDS patients, but different mechanisms about the role of STAT5 were described [18, 44, 45]. To evaluate the effect of IL-7 signalling, we recruited a second cohort of tuberculosis patients (n = 22) or healthy contacts (n = 24) (Table 1). A lower surface level of mIL-7R on T

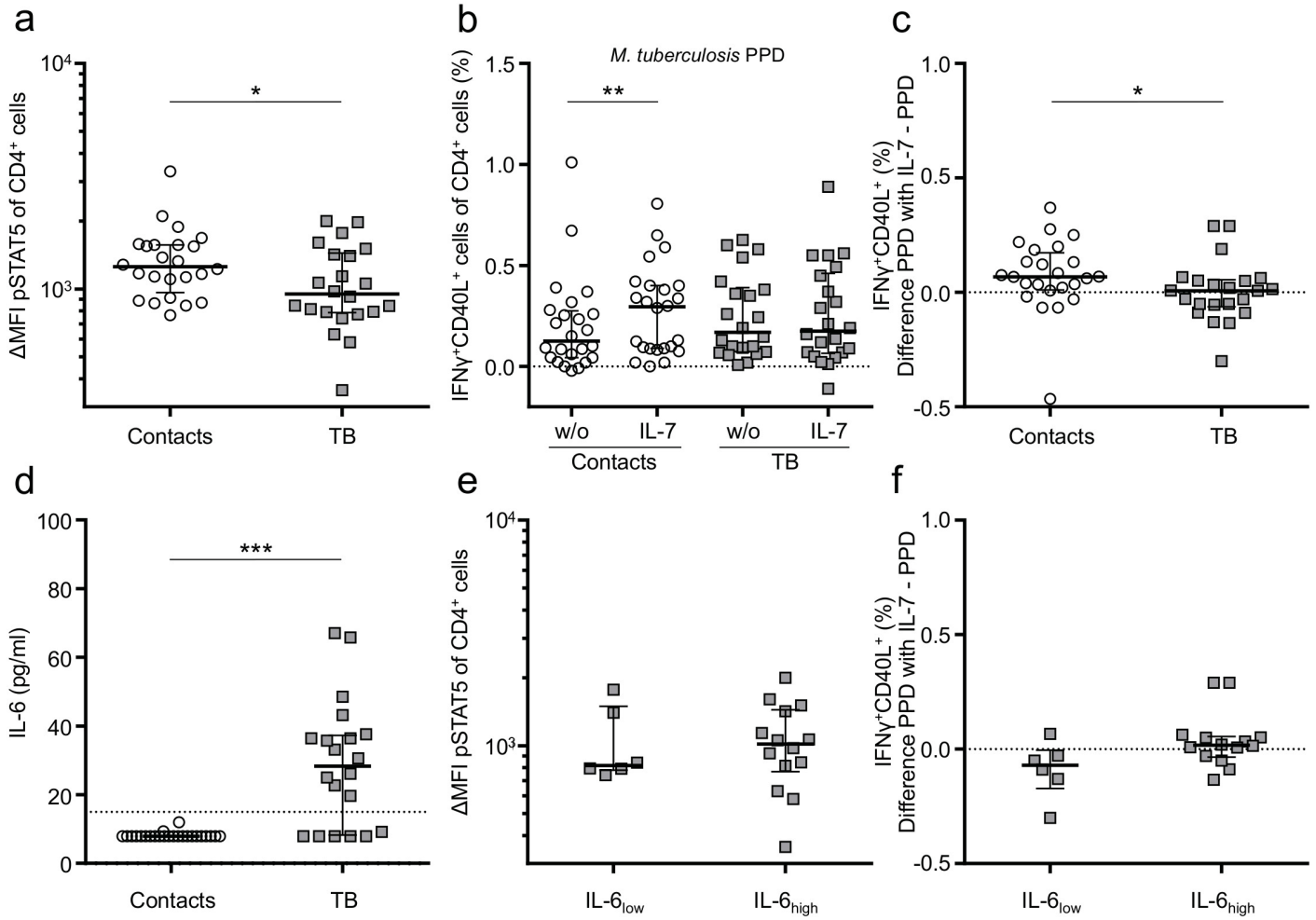

Fig 5. IL-7 response of CD4⁺ T cells from tuberculosis patients and healthy contacts. (a) IL-7-induced (10 ng/ml) STAT5 phosphorylation of CD4⁺ T cells from tuberculosis patients (n = 22) and healthy contacts (n = 24) measured by flow cytometry. The STAT5 phosphorylation level of non-stimulated cells has been subtracted of all values. **(b)** IFNγ/CD40L-expressing CD4⁺ T cells after PPD re-stimulation in the presence or absence of IL-7 detected by flow cytometry. Non-stimulated values with or without IL-7 have been subtracted. **(c)** Induction of IFNγ/CD40L-expressing CD4⁺ T cells by IL-7 and PPD stimulation. Absolute differences as compared to PPD alone are shown. **(d)** Plasma IL-6 levels of tuberculosis patients (n = 20) and healthy contacts (n = 24). An arbitrary threshold indicated by a dotted line was set to define tuberculosis patients with high ($IL\text{-}6_{high}$) and low ($IL\text{-}6_{low}$) concentrations of plasma IL-6. **(e)** STAT5 phosphorylation, and IL-7-induced PPD response **(f)** for tuberculosis patients with high or low plasma IL-6 level, as defined in (d). Median and interquartile range is depicted, and exact Mann-Whitney U test was applied for comparison of groups, whereas paired data was evaluated by Wilcoxon Signed-Rank test. Nominal p-values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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cells from tuberculosis patients was confirmed in this cohort (S4 Fig). Next, we measured IL-7-induced STAT5 phosphorylation and detected decreased phosphorylated STAT5 in CD4⁺ T cells from tuberculosis patients as compared to healthy contacts ($p = 0.04$) (Fig 5a). Since IL-7 was shown to enhance specific T-cell cytokine release [46], we determined intracellular cytokines after *M. tuberculosis* antigen (PPD) *in vitro* stimulation in the presence or absence of IL-7. No differences were detected for PPD-specific T cells co-expressing IFNγ and CD40L when comparing tuberculosis patients and healthy contacts (Fig 5b). However, co-stimulation with IL-7 induced increased proportions IFNγ-producing T cells solely in the study group of healthy contacts ($p = 0.003$), but not in tuberculosis patients ($p = 0.94$) (Fig 5b). Next, IL-7-specific effects were quantified by calculating the difference of PPD induced T cells with or without IL-7 for each individual (Fig 5c). We found a significantly stronger effect of IL-7 on

cytokine release in healthy contacts as compared to tuberculosis patients ($p = 0.02$). These results suggested impaired T-cell responses to IL-7 in patients with tuberculosis.

Increased IL-6 plasma concentrations in tuberculosis patients were not associated with impaired T-cell responses to IL-7

Chronic inflammation and increased IL-6 serum concentrations were found in AIDS patients with impaired T-cell immunity to IL-7 [14, 15]. One study found a direct inhibitory effect of IL-6 on IL-7-mediated T-cell functions [15]. Since increased IL-6 plasma levels were described in tuberculosis previously [47], we measured plasma IL-6 levels and detected increased IL-6 concentrations in tuberculosis patients as compared to healthy contacts ($p < 0.001$) (Fig 5d). The distribution of IL-6 plasma concentrations indicated two subgroups of tuberculosis patients. Hence we set an arbitrary threshold (15 pg/ml) and compared IL-6_{high} and IL-6_{low} tuberculosis patients for IL-7-promoted T-cell responses. No significant differences in IL-7-induced STAT5 phosphorylation or IL-7 co-stimulated IFNγ/CD40L expression was found between the two IL-6_{high} and IL-6_{low} subgroups of tuberculosis patients (Fig 5e and 5f). Therefore differential IL-6 serum levels were not associated with impaired IL-7-promoted T-cell responses in tuberculosis patients.

Exhaustion markers PD-1 and SOCS3 were not associated with IL-7-impaired T-cell response in tuberculosis patients

Programmed cell death (PD)-1, a marker of T-cell exhaustion and senescence was recently found to be expressed on T cells with impaired response to IL-7 [14]. We determined PD-1 mRNA expression of purified $CD4^+$ T cells and found similar PD-1 expression among healthy contacts and tuberculosis patients prior to therapy (Fig 6a). Under therapy, a decrease of PD-1 expression was found for tuberculosis patients ($p = 0.007$) followed by an increase until recovery ($p < 0.001$). PD-1 levels in recovered tuberculosis patients were even higher as compared to healthy contacts ($p = 0.04$). We found a moderate but significant positive correlation of PD-1 ($rho = 0.22$, $p = 0.005$) with IL-7 (Fig 6b). Previously, we identified SOCS3 as a marker of $CD4^+$ T cells in tuberculosis [48], and others described SOCS3 as a central regulator of T-cell exhaustion and target of IL-7 in chronic viral infections [10]. Therefore we determined SOCS3 mRNA expression of $CD4^+$ T cells. Marginal increased SOCS3 expression was detected in tuberculosis patients prior to therapy ($p < 0.16$), and significantly increased SOCS3 levels were detected at two months under therapy ($p < 0.001$) and after six months ($p = 0.04$) as compared to healthy contacts (Fig 6c). As for PD-1, a moderate positive correlation between SOCS3 expression and IL-7 concentrations was found ($rho = 0.22$, $p = 0.005$) (Fig 6d). We concluded that expression of T-cell exhaustion marker SOCS3 was increased in tuberculosis patients during therapy but was only moderately associated with aberrant IL-7 plasma concentrations. These observations indicated similarities and differences of aberrant IL-7 pathway features in tuberculosis patients as compared to AIDS patients.

Discussion

In the presented study, we identified alterations in the IL-7 pathway and impaired T-cell response to IL-7 co-stimulation in tuberculosis patients.

First, we detected higher IL-7 plasma concentrations in tuberculosis patients that decreased during therapy and recovery. Lymphopenia may cause high IL-7 plasma levels [49, 50] and few reports indicated a role of lymphopenia in tuberculosis [51–53], but this has not been verified by others [54]. We did not determine lymphocyte counts in the present study and cannot

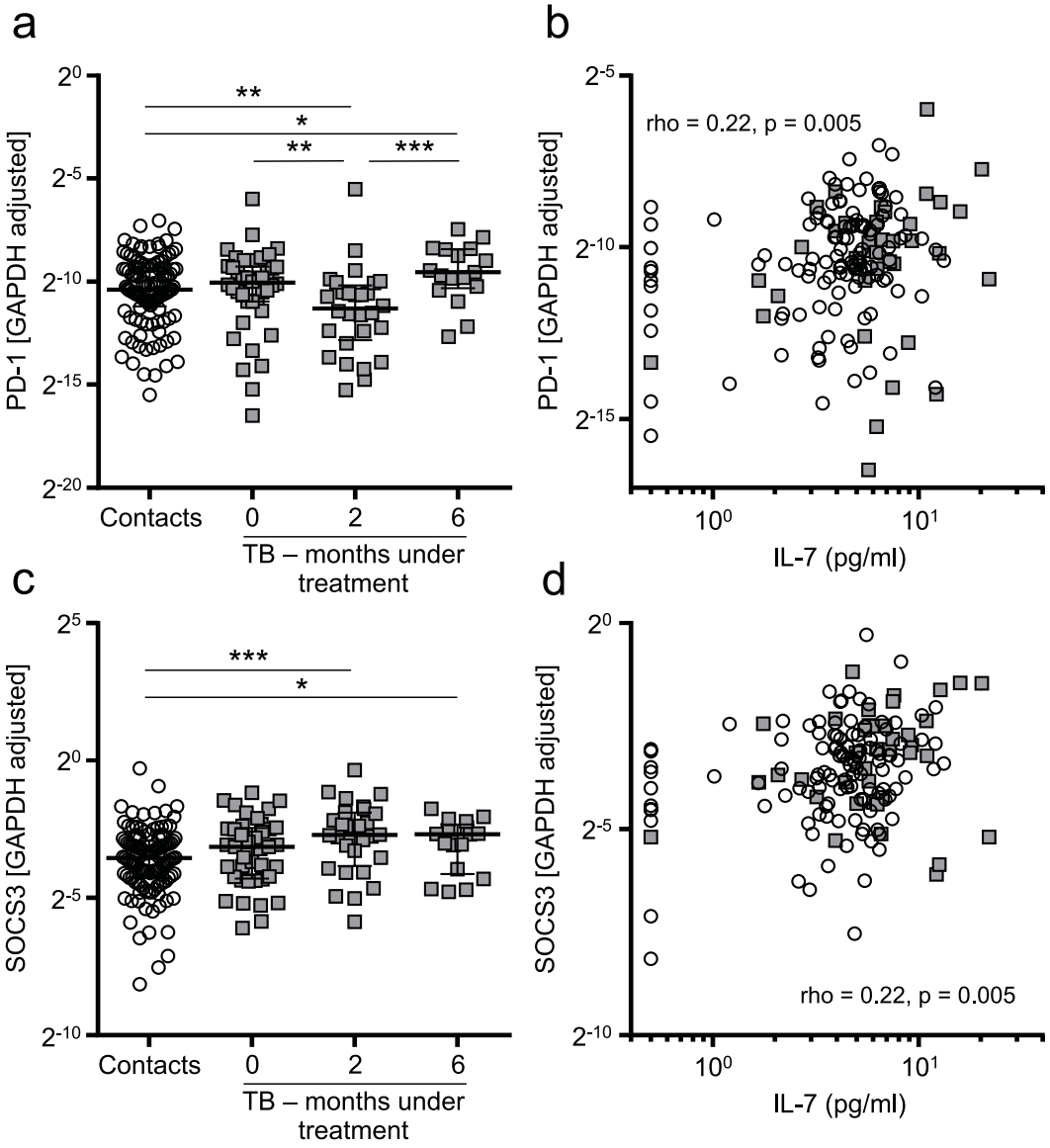

Fig 6. PD-1 and SOCS3 mRNA expression of CD4+ T cells from tuberculosis patients and healthy contacts. The expression of PD-1 **(a)** and SOCS3 **(c)** was determined for mRNA isolated from $CD4^+$ T cells, using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference. Cycle threshold differences ($2^{-\Delta Ct}$) are shown for healthy contacts [n = 117 (PD-1), n = 119 (SOCS3)], and for tuberculosis patients prior to (0 months, n = 40), during (2 months, n = 28), and after (6 months, n = 17) treatment. Median and interquartile range is depicted. Spearman correlation between plasma IL-7 and **(b)** PD-1 or **(d)** SOCS3 for healthy contacts (circles) or tuberculosis patients (squares) prior to treatment. Each symbol indicates mean values of duplicates from each individual donor. Due to a low overlap between tuberculosis patients, exact Mann-Whitney U test used for comparison of all groups. Nominal p-values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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prove or refute lymphopenia as a possible cause for high IL-7 levels. However, there is evidence that IL-7 serum concentrations are affected only at very low $CD4^+$ T-cell numbers in AIDS patients [41, 55], and these levels are far below lymphopenia described in tuberculosis [49, 50]. Another possible explanation for higher IL-7 plasma concentrations is impaired receptivity/consumption of IL-7 by T cells [21]. Our investigations provide evidence for reduced mIL-7R expression and impaired IL-7 co-stimulatory effects on T cells from tuberculosis patients.

Strong evidence for impaired IL-7 regulation and T-cell function was found for chronic viral infections, especially AIDS [56]. In AIDS patients increased IL-7 plasma levels and decreased mIL-7R expression of T cells were described [17, 42, 43, 57–59]. Furthermore, impaired T-cell response to IL-7 in AIDS patients was shown to affect immune reconstitution during anti-retroviral therapy [13, 60, 61]. In order to determine possible dependencies between mIL-7R expression on T cells and IL-7/sIL-7R plasma concentrations, we performed correlation analyses. For contacts there was a tendency of positive correlation between mIL-7R and sIL-7 levels, whereas IL-7 plasma levels showed a marginal negative correlation with mIL-7R expression. Given the described regulatory influence of IL-7/sIL-7R on mIL-7R expression [21], we speculate that IL-7 and sIL-7R plasma level alterations caused by tuberculosis disrupted this dependency that indicates the homeostatic balance in healthy individuals. The low number of samples included for mIL-7R analyses restricted the validity of these results. In addition, analyses of mIL-7R during disease course and after recovery are needed to confirm this thesis.

Several mechanisms and T-cell phenotypes were described to play a role in impaired IL-7 functions of AIDS patients. Chronic inflammation and increased serum concentrations of IL-6 were found in HIV/AIDS [14, 15], and functional *in vitro* assays indicated inhibitory effects of the pro-inflammatory cytokines IL-6 and IL-1β on IL-7-mediated signal transduction [15]. Higher IL-6 plasma concentrations were described for tuberculosis [47, 62], and we confirmed higher IL-6 plasma concentrations in a subgroup of tuberculosis patients in the present study. However, we did not detect IL-7 response differences between IL-6_{high} and IL-6_{low} subgroups among tuberculosis patients (Fig 5e and 5f). Hence there was no indication for an association between IL-6 plasma concentrations and impaired IL-7 T-cell response of tuberculosis patients.

T-cell exhaustion was found in AIDS patients [12, 13, 60, 63–65] and was associated with decreased IL-7R expression [60, 64] and impaired IL-7 response [13]. Initial studies indicated a role of T-cell exhaustion in tuberculosis animal models [7, 8]. Our results on SOCS3 and PD-1 expression did not support a major role of T-cell exhaustion in human tuberculosis and this is in accordance with a previous study [66]. These differences might at least partly be due to the fact that exhaustion is poorly defined for $CD4^+$ T cells in contrast to $CD8^+$ T cells [67]. Therefore, other marker molecules may be indicative for exhaustion in $CD4^+$ T cells. We were not able to study the phenotype of $CD8^+$ T cells in detail in the present study but decreased mIL-7R expression (Fig 3a) may indicate exhaustion of $CD8^+$ T cells in tuberculosis patients.

Impaired mIL-7R signalling was described for T cells from AIDS patients [13, 14, 18, 68]. We detected lower STAT5 phosphorylation and showed also impaired IL-7 promoted cytokine release in T cells from tuberculosis patients. The capacity of IL-7 to promote IFNγ-expressing T cells for detection of *M. tuberculosis* infection has been shown before [46]. Here we provide first evidence that IL-7 mediated increased sensitivity of T cells to stimulation (e.g. by decreasing the T-cell receptor activation threshold [69]) was impaired in tuberculosis patients. One may therefore speculate that impaired IL-7 response not only hampered generation of effective memory but also effector T-cell response against acute tuberculosis. This raised the question if impaired T-cell response to IL-7 can be interpreted as a feature of T-cell anergy. Anergy is defined as unresponsiveness of T cells to their cognate antigen and anergy against PPD—measured by tuberculin skin test—has been described for tuberculosis patients before [70]. We did not detect differences in the PPD response of $CD4^+$ T cells between tuberculosis patients and healthy contacts in the present study (Fig 5b). However, we would speculate that impaired T-cell responses to IL-7 contributed to the phenomenon of diminished tuberculin reactivity in tuberculosis patients as this *in vivo* test would be better reflected by IL-7-supplemented PPD stimulation in our *in vitro* assay. Since IL-7 effects on T-cell function include a decreased T-cell receptor activation threshold [69], impaired mIL-

7R signaling may contribute to diminished T-cell receptor signaling characteristic for T-cell anergy [71]. Therefore impaired mIL-7R signaling may contribute to tuberculin skin test anergy described for tuberculosis patients but additional studies are needed to further clarify the exact role of IL-7.

We also detected lower sIL-7R plasma concentrations in tuberculosis patients and normalisation during therapy and recovery. sIL-7R levels were previously shown to affect IL-7-availability for T cells, but the role of aberrant sIL-7R levels in immune pathologies is a matter of controversy [26–28]. Crawley et al. detected increased sIL-7R concentrations in plasma samples from AIDS patients and described sIL-7R-Fc chimera-mediated inhibition of IL-7 bioactivity [28]. They hypothesised that increased sIL-7R concentrations limited availability of IL-7 for T cells [28]. In contrast, Rose et al. found decreased sIL-7R plasma concentrations in HIV/AIDS patients as compared to controls [31]. sIL-7R plasma concentrations of this study were similar to the present study and 5 to 10 times lower for both study groups as compared to the study published by Crawley et al. [28]. Recently, Lundstrom et al. proposed an alternative model of IL-7 storage provided by the sIL-7R [26]. They demonstrated that sIL-7R even potentiates the bioactivity of IL-7 by forming a reservoir of accessible IL-7 [26]. In accordance, high sIL-7R as well as IL-7 plasma concentrations were associated with multiple sclerosis, and sIL-7R had potentiating effects on exacerbation of experimental autoimmune encephalomyelitis [26]. From this, they concluded that increased plasma concentrations of sIL-7R supported generation of autoimmunity by promoting IL-7-dependent T cells [26]. Since IL-7 serum levels are predominantly regulated by T-cell consumption [21], both restriction and reservoir hypotheses suggest dependency of IL-7 on sIL-7R levels. In the present study, we did not detect a correlation between IL-7 and sIL-7R plasma levels in tuberculosis patients or healthy contacts, although both factors were affected during tuberculosis pathogenesis. It is therefore tempting to speculate that sIL-7R has either no regulatory activities on IL-7, or that additional factors influence sIL-7R and/or IL-7 serum levels. In accordance, the proposed regulatory function of sIL-7R on IL-7 has been questioned by others [72].

We evaluated the utility of IL-7 and sIL-7R plasma concentrations as biomarkers for diagnosis of active tuberculosis using ROC curve and Random Forest-based statistics. Both markers showed moderate classification capacity and the combined efficacy of both markers revealed correct prediction for 73% of all donors. Since normalization of low sIL-7R and high IL-7 plasma concentrations during recovery from tuberculosis was found, these parameters may qualify as biomarker candidates for successful tuberculosis chemotherapy. This study was not designed to evaluate markers for the efficacy of tuberculosis therapy but future studies may address this important question.

Immunomodulatory therapies of tuberculosis gained increasing interest during recent years to complement antibiotic therapy that is periled e.g. by multi-drug resistant mycobacteria [73]. IL-7 is a promising candidate for immunotherapies and is already applied in clinical trials against chronic viral infections [74, 75]. However, the mechanisms underlying impaired IL-7 signalling pathways during chronic infections may antagonise IL-7-based novel therapy strategies. Our study contributed to the characterisation of impaired IL-7 T-cell response that may indeed counteract IL-7 treatment in tuberculosis.

We provide initial evidence that IL-7-availability is not critical during tuberculosis. Instead, T-cell functions in response to IL-7 are impaired, and therefore approaches targeting T-cell abnormalities—causative for reduced IL-7 response—may be helpful. Since IL-7 availability is a crucial factor for the development of memory T-cell induction [76], such an approach might also aim at improving protection against recurrent *M. tuberculosis* infection and disease.

Methods

Study design and samples

In this hospital-based observational study, we recruited adult tuberculosis patients (n = 57; Table 1) and exposed but healthy household contacts (healthy contacts) (n = 151). Tuberculosis patients were recruited at the Komfo Anokye Teaching Hospital (KATH), the Kumasi South Hospital (KSH), and the Kwame Nkrumah University of Science and Technology (KNUST) Hospital, Ghana, in 2011–2012. Diagnosis of tuberculosis was based on patient history, chest X-ray, and sputum smear test. For sputum smear negative cases, laboratory confirmation by *M. tuberculosis* sputum culture was performed. Tuberculosis patients with a known history of HIV infection were excluded from this study. Chemotherapy according to the Ghanaian guidelines was initiated immediately after the first blood sample was taken. For the patient study group, peripheral heparinised blood was taken consecutively (i.e. prior to treatment, under treatment (at 2 months), and after recovery (at 6 months)). Only a subgroup of tuberculosis patients (n = 36) completed the study procedure. Twenty-one tuberculosis patients were not included at all time points, including nine patients included only prior to treatment; six patients prior to treatment and under treatment; two patients prior to treatment and after recovery, and four patients during treatment and after recovery. Healthy tuberculosis patient contacts (short: healthy contacts) were recruited at the homes of tuberculosis index cases and showed no clinical symptoms of tuberculosis. A subgroup of healthy contacts (n = 19) and tuberculosis patients (n = 32) was tested for *M. tuberculosis* PPD-specific immune response before and showed significant IFNγ expression [77]. We took heparinised blood (up to 30 ml) from each donor. Not all samples were included for all experiments, and the respective numbers of samples included are given in the figure legends. A second cohort of tuberculosis patients (n = 22) and healthy contacts (n = 24) were recruited in the period of October 2015 to March 2016. HIV-positive individuals were excluded from the analysis (First Response HIV 1–2.0 Card Test, Premier Medical Corporation).

Ethics statement

All study participants were adults who gave written informed consent. All participants were free to drop out at any time of the study. The studies were approved by the Committee on Human Research, Publication and Ethics (CHRPE) at the School of Medical Sciences (SMS) at the Kwame Nkrumah University of Science and technology (KNUST) in Kumasi, Ghana.

Measurement of sIL-7R concentrations using cytometric bead assay

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised whole blood (diluted 1:1 in PBS) by density centrifugation (Ficoll, Biochrom) according to manufacturer's instructions. PBMCs were cryopreserved in DMSO/FCS (each 10%) containing medium. The plasma layer (diluted 1:1 in PBS) were collected and frozen at -80˚C until processing. Diluted plasma samples were thawed in parallel and analysed for sIL-7R expression. Quantification of sIL-7R was performed according to the protocol of Faucher et al. [78] with minor modifications. In brief, we applied cytometric bead array (CBA) (Bead A4, BD Biosciences). Conjugation of beads with polyclonal goat anti-human CD127 (IL-7Rα) antibody (R&D Systems, AF306) was done according to manufacturer's instructions. Biotinylated mouse anti-human CD127 (clone HIL-7R-M21, BD Biosciences) was used as detection antibody. Samples were incubated with labelled beads in PBS for 1 hour at room temperature and then the detection antibody (5 μl) was added for overnight incubation in the fridge. Afterwards, Streptavidin-PE (1 μl) (Southern Biotech) was added and incubated for 30 min at room

temperature. Finally the beads were washed twice in PBS. For analyses, the bead pellets were resuspended in 80 μl PBS and analysed using a BD LSRFortessa flow cytometer (BD Biosciences) and the FCS Express 4 (De Novo Software) software. For absolute quantification, the assay was calibrated with dilutions of rhIL-7R alpha-Fc chimera (R&D Systems). sIL-7R concentrations were calculated using the non-linear regression tool of GraphPad Prism 6 (Graphpad Software Inc.). Possible effects of IL-7 on sIL-7R measure were excluded by Faucher et al. [78].

Measurement of plasma IL-6 and IL-7

IL-6 and IL-7 was determined in duplicate for diluted plasma samples using Human IL-6 ELISA Ready-SET-Go! (eBioscience) and Human IL-7 Quantikine HS ELISA kit (R&D Systems), respectively, according to manufacturer's instructions. Samples were measured using the Infinite M200 ELISA reader (Tecan). Concentrations were calculated from the respective standard curves by applying 4-parametric logistic regression. Samples outside the detection range were set to the corresponding lower or upper range value.

Real-Time PCR of IL-7R variants and T-cell exhaustion markers

$CD4^+$ cells were isolated from freshly isolated PBMCs (1.5 x 10^7 cells) using anti-human CD4 magnetic particles (BD Biosciences) according to manufacturer's recommendations. Cell purity was evaluated by flow cytometry and was generally higher than 95%. miRNA was isolated from at least 5 x 10^6 enriched $CD4^+$ cells using mirVanaTM miRNA Isolation Kit (Life Technologies) following manufacturer's instructions. cDNA was generated by Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific), while RT-PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) for full-length IL-7R (H20: forward 5'-AATAATAGCTCAGGGGAGATGG-3', reverse 5'-ATGACCAACAGAGCGACAGAG-3'), IL-7R lacking exon 6 (H6: forward 5'-GATCAATAATAGCTCAGGATTAAGC-3', reverse 5'-AAGATGTTCCAGAGTCTTCTTATG-3'), and IL-7R lacking exon 5–6 (H5-6: forward 5'-ATGAAAACAAATGGACGGATTAAGC-3', reverse 5'-AAGATGTTCCAGAGTCTTCTTATG-3'), PD-1 (forward 5'-CTCAGGGTGACAGAGAGAAG-3', reverse 5'-GACACCAACCACCAGGGTTT-3'), SOCS3 (forward 5'-GACCAGCGCCACTTCTTCAC-3', reverse 5'-CTGGATGCGCAGGTTCTTG-3') using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping control gene (forward 5'-CACCATCTTCCAGGAGCGAG-3', reverse 5'-GACTCCACGACGTACTCAGC-3'). The reaction with a final volume of 25 μl was run 2 min. at 50˚C, 10 min. at 95˚C, 45 cycles of 15 s at 95˚C, 30 s at 53˚C and 30 s at 72˚C, followed by a melt curve sequence of 15 s at 95˚C, 60 s at 60˚C with a slow gradient to 95˚C and finally 15 s at 60˚C. Data from duplicate reactions was evaluated using the $2^{-\Delta Ct}$ method. A 7500 Real-Time PCR machine (Applied Biosystems) was used for quantitative PCR analyses.

Genotyping of IL7RA single nucleotide polymorphism

DNA was isolated from PBMCs using QIAamp DNA Mini Mini kit (Qiagen) followed by rs6897932C>T genotyping using a predesigned TaqMan SNP Genotyping Assay (Applied Biosystems) following manufacturer's instructions.

Staining of PBMCs

Frozen PBMCs were thawed and washed with RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-Glutamine, 10 mM HEPES, and 50 U/ml Penicillin-Streptomycin (all from Thermo Fisher). Cells were stained with Viability Dye eFluor 780 (eBioscience) and

antibodies against CD3 (PE-labelled, clone HIT3a, BD Biosciences), CD4 (BrilliantViolet510-labelled, clone OKT4, BioLegend), CD8 (PerCP-Cy5.5-labelled, clone HIT8a, BioLegend), CD25 (PE-Cy7-labelled, clone 2A3, BD Biosciences) and CD127 (AlexaFluor647-labelled, clone HIL-7R-M21, BD Biosciences). After cell wash, PBMCs were fixed with Fixation Buffer (BioLegend) and subsequently analysed using a BD LSRFortessa flow cytometer (BD Biosciences). Gating procedures are depicted in S3 Fig. For detection of mIL-7R in the second independent cohort of tuberculosis patients and healthy contacts we used the CD127 antibody clone A019D5 (BioLegend). Comparison of both antibody clones revealed similar T-cell binding pattern as well as percentages of mIL-7R_{high} and mIL-7R_{low} T cells.

STAT5 phosphorylation by IL-7

Freshly isolated PBMCs were stained for CD4 (AlexaFluor488, clone RPTA-4, BioLegend) followed by addition of 100 μl pre-warmed X-VIVO 15 medium (Lonza) added 50 U/ml Penicillin-Streptomycin with or without human recombinant IL-7. The concentration of IL-7 was titrated prior to the study and a concentration of 10 ng/ml was sufficient to induce pSTAT5 in 94% of the T cells (S5 Fig). Higher IL-7 concentrations (25 or 50 ng/ml) did not further increase STAT5 phosphorylation (S5 Fig). Therefore we cultured the samples with and w/o 10 ng/ml of recombinant human IL-7 in this study. After 15 min incubation at 37˚C, 5% CO_2, cells were fixed for 15 min. with 100 μl 1x True-Nuclear Transcription Factor buffer (BioLegend). Subsequently, cells were permeabilised with 100% methanol, washed in PBS/10% FCS and stained for p-STAT5 Y694 (PE, clone SRBCZX, eBioscience). Analysis was performed on a BD Accuri C6 flow cytometer. Gating procedure is shown in S5 Fig.

Ex vivo stimulation of whole blood

Heparinised blood was diluted 1:2 in RPMI 1640 supplemented with 2 mM L-Glutamine and 50 U/ml Penicillin-Streptomycin in a 96-well U bottom plate. Cells were stimulated with 10 μg/ml PPD (Statens Serum Institute) and/or 10 ng/ml recombinant human IL-7 (BioLegend), or left unstimulated. After 2.5 hours of stimulation at 37˚C, 5% CO_2, Brefeldin A (Sigma Aldrich) was added at a concentration of 3.75 μg/ml followed by 16 hours of incubation. Erythrocytes were subsequently lysed in two rounds by resuspending pelleted cells in 100 μl RBC Lysis Buffer (Roche) followed by 10 min incubation at room temperature. Next, cells were fixed and permabilised (BioLegend) and stained with antibody against CD4 (AlexaFluor488, clone RPTA-4, BioLegend), IFNγ (PE, clone 25723.11, BD Biosciences) and CD154 (APC, clone 24.31, BioLegend). Cells were analysed using a BD Accuri C6 flow cytometer (BD Biosciences). Gating procedure is shown S6 Fig.

Statistical analysis

Statistical analyses were performed using R version 3.3.0, applying Exact Mann-Whitney U test from the package *coin* for comparison between groups and Wilcoxon signed-rank test for evaluation of repeated measurements. Spearman correlation was used to evaluate association between continuous variables, while Receiver Operating Characteristic (ROC) was performed using the package *ROCR*. Random forest analysis was performed with the package *ranger*, applying 10^5 random trees and adjusting the importance measure by permutation. Plots were generated in R and GraphPad Prism version 6.07.

Supporting information

S1 Table. Correlation between mIL-7R expression on T cells and plasma IL-7 or sIL-7R concentrations.
(EPS)

S1 Fig. Gender differences in plasma sIL-7R levels. Plasma concentrations of sIL-7R from TB contacts (n = 149) and tuberculosis patients prior to (0 months, n = 52), during (2 months, n = 46) and after (6 months, n = 41) treatment was determined by cytometric bead array. Exact Mann-Whitney U test used for comparison of gender differences.
(PDF)

S2 Fig. Changes in plasma sIL-7R and plasma IL-7 during chemotherapy. Absolute (left panel) and relative (right panel) differences of a) plasma sIL-7R and b) plasma IL-7 level after (6 months) and prior treatment for tuberculosis. Concentration of sIL-7R in plasma from TB patients was determined by cytometric bead array, while plasma IL-7 level was determined by ELISA (n = 36). p-values for Spearman correlation are shown, while linear regression lines are shown for guidance.
(PDF)

S3 Fig. Gating strategy for IL-7R$_{low}$, and IL-7R MFI of CD4$^+$ and CD8$^+$ cells. Proportions (%) of cells within the individual gates are indicated.
(PDF)

S4 Fig. Surface level of IL-7R on CD3$^+$CD4$^+$ and CD3$^+$CD4$^-$ cells. Heparinised blood from TB patients (n = 22) and contacts to TB patients (n = 24) was lysed (RBC Lysis Buffer, Roche) and leukocytes were stained for CD3 APC (clone UCHT1, BD Biosciences), CD4 AlexaFluor 488 (clone RPTA-4, Biolegend) and IL-7R (CD127) PE-Cy7 (clone A019D5, Biolegend). Cells were analysed on a BD Accuri C6 Flow Cytometer (BD Biosciences). Mean Fluoresence Intensity (MFI) of IL-7R is shown for (a) CD3$^+$CD4$^+$ and (b) CD3$^+$CD4$^-$ cells. Exact Mann-Whitney U test is used for comparison of groups.
(PDF)

S5 Fig. STAT5 phosphorylation of CD4$^+$ cells after IL-7 stimulation. (a) Gating strategy for STAT5 phosphorylation (pSTAT5) on CD4$^+$ cells stimulated with (solid line) or without (shaded) 10 ng/ml IL-7 for 15 min. Proportions (%) of cells in the individual gates are indicated, and mean fluorescence intensity (MFI) is shown for the two stimulations. (b) Titration of IL-7. PBMCs stimulated as in (a) with various concentrations of IL-7 shown for CD4$^+$ cells.
(PDF)

S6 Fig. Gating strategy for IFNγ$^+$CD40L$^+$ cells. Gating strategy for IFNγ$^+$CD40L$^+$ cells of CD4$^+$ cells after overnight stimulation of whole blood with PPD. Proportions (%) of cells in the individual gates are indicated.
(PDF)

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Author Contributions

Conceptualization: MJ CL.

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3.2 Paper II: Interleukin-7 receptor α-chain haplotypes differentially affect soluble IL-7 receptor and IL-7 serum concentrations in children with type 1 diabetes

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ORIGINAL ARTICLE

Interleukin-7 receptor α-chain haplotypes differentially affect soluble IL-7 receptor and IL-7 serum concentrations in children with type 1 diabetes

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Background: Interleukin-7 receptor α-chain (*IL7RA*) haplotypes are associated with susceptibility for development of autoimmune diseases, including type 1 diabetes (T1D). A protective *IL7RA* haplotype which causes lower soluble IL-7R (sIL-7R) serum levels is hypothesized to restrict IL-7-availability for self-reactive T cells. Functional mechanisms affected by a risk-associated *IL7RA* haplotype are unknown.

Methods: We investigated the influence of *IL7RA* haplotypes (tagged by rs6897932T for the protective or by rs1494555G for the risk haplotype) on sIL-7R and IL-7 serum concentrations as well as disease manifestation of children with T1D (n = 259). Possible effects of differential IL-7 serum concentrations on IL-7-mediated *in vitro* T cell functions (i.e. IL-7R regulation and cytokine expression) were measured in a second study group of children with T1D (n = 42).

Results: We detected lower sIL-7R serum concentrations in children with T1D carrying protective or risk haplotypes as compared to reference haplotypes. sIL-7R levels were lowest in T1D children with the protective haplotype and lower IL-7 serum levels were exclusively detected in this study group. We found no evidence for dependency between IL-7 and sIL-7R serum concentrations and no association with T1D manifestation. Neither IL-7 nor sIL-7R serum levels were associated with mIL-7R regulation or IL-7-promoted T cell cytokine expression.

Conclusions: Children with T1D carrying autoimmunity risk- or protection-associated *IL7RA* haplotypes had both lower sIL-7R serum concentrations as compared to the reference haplotype, but only T1D children with the protective haplotype had lower IL-7 serum levels. Our results suggest additional functional mechanisms of autoimmunity-associated *IL7RA* variants independent from sIL-7R mediated regulation of IL-7 availability for T cells.

KEYWORDS

haplotype, IL-7, sIL-7R, *IL7RA*, T cells

1 | INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by T cell mediated destruction of pancreatic β-cells. There is longstanding evidence that lymphopenia promotes the development of autoimmunity, and IL-7 is a central factor for self-reactive T cells in lymphopenic patients.[1–3] The important role of IL-7 in the pathogenesis of T1D is well established.[4,5] Inhibition of IL-7 signaling protected NOD mice from developing T1D and even reversed established disease.[6] Calzascia et al[7] showed that IL-7 promoted the

generation of self-reactive CD4+ T cells in T1D. Furthermore, IL-7-dependent expansion of self-reactive memory T cells in patients with T1D after islet transplantation has been described.[8] Mechanistically, Deshpande et al[9] showed that IL-7 lowered the activation threshold of antigen-specific CD4+ T cells and concluded that especially low-avidity autoantigen-specific T cells profit from increased IL-7 availability.

IL-7 is mainly produced by stromal cells of the bone marrow and peripheral lymphoid organs.[10] Importantly, IL-7 production was shown to be largely constant, and IL-7 uptake and consumption-mainly by T cells-is hypothesized to affect availability of IL-7.[10,11] The T cell response to IL-7 is tightly controlled, and this is mainly exerted on the level of the IL-7R. The IL-7R pathway is regulated by several mechanisms, including expression of the membrane-associated (m)IL-7R and release of the sIL-7R variant (ie, IL-7R α-chain monomers lacking transmembrane and intracellular domains) (reviewed in[12]). The mIL-7R is expressed on naïve and memory T cell subsets.[12] On IL-7 binding, the mIL-7R complex is rapidly internalized and partly degraded.[13,14] Because naïve and memory T cells require IL-7 for homeostatic proliferation, the quantity of these T cell subsets is hypothesized to regulate IL-7 serum levels.[12]

The importance of the sIL-7R for T1D-as for other autoimmune diseases[15,16]-was strengthened by studies demonstrating associations of *IL7RA* single nucleotide polymorphisms (SNPs) with T1D susceptibility.[17,18] A functional SNP in the exon 6 of *IL7RA* (ie, rs6897932C > T; Thr244Ile) was shown to interfere with alternative splicing of the IL-7R.[15] *IL7RA* rs6897932T variant carriers show less sIL-7R expression and were partially protected from the development of T1D.[17] In accordance with these findings, higher sIL-7R serum concentrations were found in autoimmune and inflammatory diseases.[19–21] The main hypothesis for the role of sIL-7R in autoimmune diseases is based on its capacity to serve as an IL-7 reservoir.[20] The sIL-7R limits uptake of IL-7 and therefore, promotes IL-7 availability.[20,22] In accordance, lower IL-7 serum levels were found in multiple sclerosis patients with the protection-associated *IL7RA* haplotype.[20] Differences in IL-7-availability may be of special importance for self-reactive T cells in autoimmunity because haplotype specific differences in serum IL-7 levels were not found in healthy controls.[20]

Additional *IL7RA* SNPs were found to be associated with autoimmune diseases, and this led to the assignment of different *IL7RA* haplotypes.[15,23–25] Besides the protective haplotype, a second haplotype tagged by rs1494555A > G (exon 4, Ile138Val) was associated with increased risk to develop multiple sclerosis.[15,26] Only few studies addressed the question how risk and protective *IL7RA* haplotypes affect T cell functions in autoimmunity. One study found higher mIL-7R expression of NK cells from multiple sclerosis patients with the protective haplotype.[24] Another study detected differential mIL-7R expression on monocyte-derived dendritic cells.[23]

So far no functional differences have been associated with the multiple sclerosis risk-associated haplotype. To address this question we combined T cell phenotype and functional assays with concomitant IL-7/sIL-7R serum analyses from children with T1D in the present study. Furthermore, we determined *IL7RA* haplotype effects on sIL-7R and IL-7 serum concentrations for children with T1D.

2 | METHODS

2.1 | Study design and samples

SNP and serum analyses were performed in a cohort of children and adolescents with T1D (*n* = 259; >10 years after clinical onset, onset age <5 years) recruited for the Pediatric Diabetes Biobank within the German Center for Diabetes Research (DZD Biobank).[27]

For functional T cell assays, a cohort of children and adolescents with T1D (*n* = 42) recruited at the University Childrens Hospital in Duesseldorf (UKD) was included. All patients received subcutaneous insulin therapy at recruitment. Children with T1D were recruited at onset of T1D (*n* = 25) or at least 6 months after clinical onset of the disease (*n* = 17). Detailed study cohort characteristics were published previously.[28] The ethics committee of the medical faculty, Heinrich-Heine-University Duesseldorf approved this study (ID 4844). Written informed consent was received from all donors (older than 14 years) and the legal guardians. All methods were performed in accordance with the relevant guidelines and regulations.

2.2 | SNP genotyping by real-time PCR

DNA was isolated from blood using QIAamp DNA Mini Kit (Qiagen). Genotyping of rs1494555A > G and rs6897932C > T was performed using predesigned TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions and analyzed on a 7500 real-time PCR machine (Applied Biosystems). Analyzed SNPs did not deviate from the Hardy-Weinberg equilibrium.

2.3 | Measurement of sIL-7R serum concentrations using cytometric bead assay

Serum samples from both cohorts were concomitantly thawed and quantification of sIL-7R was performed as previously described.[29] In brief, anti-human CD127 (IL-7Rα)-labeled beads (1 μL) were incubated with serum samples (50 μL) for 1 hour at room temperature. Afterward, detection antibody (anti-human CD127-Biotin, 5 μL) was added for overnight incubation at 4°C. Streptavidin-PE (Southern Biotech, Birmingham, AL, USA, 1 μL) was added and incubated for 30 minutes at room temperature. The samples were washed twice in PBS and measured by flow cytometry (LSRFortessa, BD Biosciences, San Jose, CA, USA). All samples were measured in duplicate. Serial dilutions of rhIL-7Rα-Fc chimera (R&D Systems, Minneapolis, MN, USA) were applied as standards included on each 96-well plate. Analyses were performed using FlowJo software (Miltenyi Biotec, Bergisch Gladbach, Germany). sIL-7R concentrations were calculated using a non-linear regression model (Graph Pad Prism 7, Graphpad Software Inc., La Jolla, CA, USA). Restrictions in available serum volumes limited the DZD Biobank sample group size for sIL-7R analyses (*n* = 244).

2.4 | Measurement of serum IL-7 concentration using high-sensitive ELISA method

Serum IL-7 concentrations for both study groups were determined by the ELISA (Human IL-7 Quantikine HS ELISA kit, R&D Systems) according to manufacturer's instructions. According to the age group

and expected IL-7 levels, Biobank samples were prediluted 1:2 and UKD samples 1:4 in provided calibrator diluent. Plates were measured using an ELISA reader (Infinite M200, Tecan, Männedorf, Switzerland). Because of moderate IL-7 serum concentrations, we included standards on each plate to increase the accuracy of the assays. A representative standard curve is shown as Figure S1 in Appendix S1. IL-7 concentrations were calculated from the standard curve fitted by a 4-parametric logistic regression (Magellan version 7.2, Tecan). Restrictions in available serum volumes limited the DZD Biobank sample group size for IL-7 analyses (n = 228).

2.5 | T cell phenotyping and IL-7 functional assays

For T cell assays, blood samples were processed within 1 hour after bleeding. Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Biocoll, Biochrom AG, Berlin, Germany) according to manufacturer`s instruction. mIL-7R expression was determined after overnight in vitro culture with or without recombinant human (rh)IL-7 internalization (BioLegend, San Diego, CA, USA). Procedures, material and data analyses have been described previously.[28] In brief, $CD4^+$ T cells were analyzed for mIL-$7R_{high}$ expressing proportions. A representative plot is shown as Figure S4A in Appendix S1. Mean fluorescence Intensity (MFI) values of mIL-7R measures were not determined because the experiments on patients samples have been performed serially (over a period of 1 year and 3 months of recruitment) and not in parallel for all samples. This experimental design precludes the usage of MFI because of possible changes in the instrument settings (mainly laser power, photomultiplier sensitivity and accordingly adjusted multiparameter compensation) during this period.

Intracellular T cell cytokines were measured after overnight culture with or without rhIL-7 followed by T cell activation for 6 hours as described previously.[28] Samples were measured by flow cytometry (LSRFortessa, BD Biosciences) and data analysis was performed using FlowJo software (Miltenyi Biotech).

2.6 | Statistics

GraphPad Prism (Version 7.0a, GraphPad Software) as well as R (version 3.3.0) was used for statistical analyses. For comparison of genotype distribution between T1D children and 1000 Genomes control group, Fisher's exact test was used. Effects of haplotype on serum sIL-7R/IL-7 and T1D susceptibility was analyzed by linear regression and binomial logistic regression, respectively, in R using the package *SNPassoc* version 1.9-2.[30]

Because serum sIL-7R and IL-7 values did not pass normality test (D´Agostino & Pearson normality test), non-parametric distributions were assumed and statistical tests were chosen accordingly. The non-parametric Mann-Whitney U test (2-tailed) was used to compare sIL-7R and IL-7 between homozygous haplotype carrier groups. For correlation analyses, Spearman`s rank correlation was used. Two-tailed P-values below 0.05 were considered statistically significant.

2.7 | Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on request.

3 | RESULTS

3.1 | *IL7RA* SNP allele and haplotype frequencies in children with T1D

We recruited T1D children with early onset of disease (disease manifestation <5 years) and disease duration of more than 10 years (n = 259, mean age 16.8 years, range 12.0-22.7 years) and determined *IL7RA* alleles for SNPs rs6897932 and rs1494555. Three haplotypes were deduced including the well-established haplotype associated with protection against T1D (termed “protective haplotype”), a haplotype associated with increased risk of developing multiple sclerosis (termed “risk haplotype”), as well as a third haplotype not associated with autoimmune or inflammatory diseases (termed “reference haplotype”) by previous studies (Figure 1A). Minor allele frequencies (MAFs) are given in Table 1. Although this study was not designed to detect association of *IL7RA* alleles with T1D susceptibility, we detected moderate differences compared to a reference cohort from the 1000 Genomes Project.[31] A higher MAF was detected for the risk allele in children with T1D compared to the reference group (34.0% vs 29.6%) whereas only minor differences were found for the protective allele (25.9% of T1D children; 27.1% of controls) (Table 1). In accordance, comparisons of haplotype frequencies revealed a tendency of an increased risk haplotype frequency in the group of T1D children (odds ratios [OR] = 1.24, 95% confidence interval 0.96-1.59, P = .098) (Table 2). No difference was seen for the protective haplotype (Table 2). These results strengthened the underlying assumption for a role of the *IL7RA* risk haplotype in T1D disease pathogenesis.

3.2 | Lower sIL-7R serum concentrations in T1D children with the protective and the risk haplotype

Functional effects of the protective haplotype on *IL7RA* alternative splicing and differential sIL-7R serum levels were shown.[15] We determined sIL-7R serum concentrations for children with T1D and compared concentrations between different haplotypes. Results for homozygous haplotype carriers are shown in Figure 1B. As expected, lower sIL-7R serum concentrations were detected for children with the protective haplotype as compared to the reference haplotype (P < .001 for homozygous, Figure 1B; overall haplotype effect, P < .001) and those with the risk haplotype (P = .022 for homozygous, Figure 1B; overall haplotype effect, P = .004). Notably, risk haplotype carriers had also lower sIL-7R serum concentrations as compared to the reference haplotype (P = .026 for homozygous, Figure 1B; overall haplotype effect, P = .014). These results indicated effects of the risk haplotype on the sIL-7R serum level in patients with T1D.

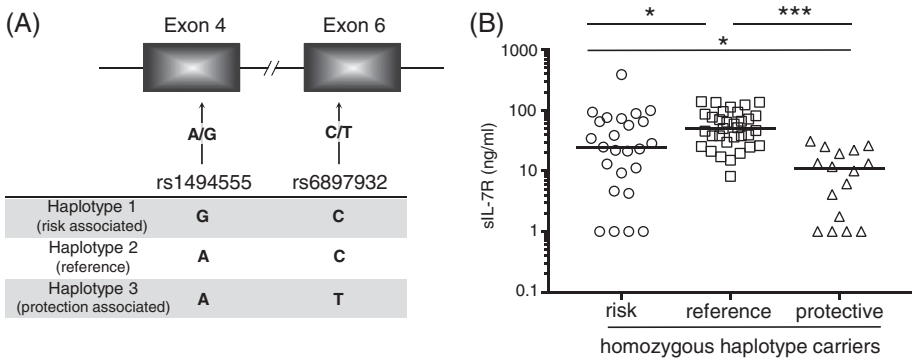

FIGURE 1 *IL7RA* gene region map, assigned haplotypes, and sIL-7R serum concentrations of children with T1D homozygous for risk, reference, or protective haplotypes. (A) Schematic depiction of the *IL7RA* gene and localization of SNPs rs1494555 and rs6897932 within exons 4 and 6, respectively. Haplotypes were assigned according to reported association with risk (risk haplotype; tagged by the rs1494555G allele) or protection (protective haplotype; tagged by the rs6897932T allele) to develop autoimmune diseases. For the reference haplotype, no association with autoimmune diseases has been reported. (B) sIL-7R of homozygous haplotype carriers (risk haplotype, n = 25; reference haplotype, n = 33; protective haplotype, n = 16). Each symbol represents data of an individual donor. Median values for groups are indicated. Nominal P-values for the Mann-Whitney U test (2-tailed) are indicated as *for P<.05 and ***for P<.001

TABLE 1 SNP frequencies of children with T1D and controls

SNP ID	Genotype	Frequencies T1D	MAF T1D	Frequencies control group[a]	MAF control group[a]
rs1494555	G/G	26 (10.0%)	34.0%	40 (8.0%)	29.6%
	A/G	124 (47.9%)		218 (43.3%)	
	A/A	109 (42.1%)		245 (48.7%)	
rs6897932	T/T	17 (6.6%)	25.9%	40 (8.0%)	27.1%
	C/T	100 (38.6%)		193 (38.4%)	
	C/C	142 (54.8%)		270 (53.7%)	

[a] according to 1000 Genomes database (EUR cohort [CEU, TSI, FIN, GBR and IBS combined], n = 503); 259 T1D children were included.

3.3 | IL-7 serum concentrations are lower in T1D children with the *IL7RA* protective haplotype

Differential sIL-7R serum levels are hypothesized to affect IL-7-availability and consumption by T cells.[20] Therefore, we determined IL-7 serum concentrations for T1D children with different haplotypes. A tendency of lower IL-7 serum concentrations was found for children with the protective haplotype as compared to the reference haplotype (P = .063 for homozygous; overall haplotype effect, P = .08) (Figure 2A) and similar IL-7 serum concentrations between children with risk and reference haplotypes were detected (Figure 2A). However, significantly lower IL-7 serum concentrations were detected in T1D children with the protective haplotype as compared to risk haplotype carriers (P = .004 for homozygous; overall haplotype effect, P = .006). Higher glycemia levels in T1D patients may influence IL-7 serum concentrations by causing sIL-7R glycation that affects sIL-7R reservoir capacity.[21] To avoid a possible bias of differential serum glycemia levels in children with T1D, we compared individual hemoglobin A1c proportions with IL-7 and sIL-7R serum concentrations, but found no evidence of correlation between these factors (Figure S2 in Appendix S1). We concluded that both T1D children with risk and protective haplotypes had lower sIL-7R serum concentrations as compared to the reference haplotype, whereas lower IL-7 serum levels were exclusively detected in protective haplotype carriers. These results did not suggest a dependency between sIL-7R and IL-7 concentrations. To test this, we compared sIL-7R and IL-7 serum concentrations for individuals. No correlation was detected, neither for subgroups with different homozygous haplotypes (Figure 2B). These results suggested protective haplotype effects on IL-7 availability but no association between sIL-7R and IL-7 serum concentrations.

TABLE 2 Haplotype analysis of children with T1D and controls

Haplotype (rs1494555/ rs6897932)	T1D (%)	Control group[a] (%)	OR (95% CI)	P-value
Risk (G/C)	34	30	1.24 (0.96-1.59)	0.098
Reference (A/C)	40	43	Reference	na
Protection (A/T)	26	27	1.02 (0.79-1.32)	0.875

Abbreviations: CI, confidence interval; na, not applicable; OR, odds ratios.

[a] 1000 Genomes database (EUR cohort [CEU, TSI, FIN, GBR and IBS combined], n = 503); 259 T1D children were included. OR, 95% CI and P-values were calculated with the package *SNPassoc* version 1.9-2.

3.4 | mIL-7R expression and internalization are not affected by differential IL-7/sIL-7R serum concentrations

Differential IL-7-availability may affect T cell functions including mIL-7R internalization and cytokine expression in autoimmune

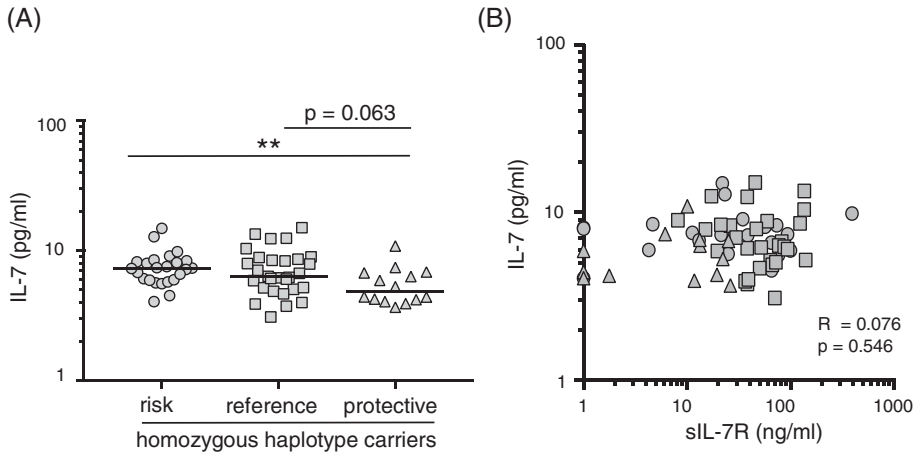

FIGURE 2 IL-7 serum concentrations of haplotypes and correlation with sIL-7R levels. (A) IL-7 serum levels of homozygous haplotype carriers (risk haplotype, circle, *n* = 24; reference haplotype, square, *n* = 29; protective haplotype, triangle, *n* = 14). Median values for groups are indicated. Nominal *P*-values for the Mann-Whitney *U* test (2-tailed) are indicated as ** for *P*<.01. (B) Correlation of serum IL-7 and sIL-7R is shown for all homozygous haplotype carriers (*n* = 67). Every symbol (circle: risk haplotype, square: reference haplotype, triangle: protective haplotype) represents data of an individual donor. Spearman`s rank correlation coefficient R and the respective P-value are indicated

diseases.[9,13,14,21] Consequently, we recruited a second cohort of children with T1D (*n* = 42; mean age 10.6 years; range 3.0-16.5 years) and performed functional T cell analyses in children characterized for IL-7/sIL-7R serum levels. As for the first cohort, we detected no correlation between sIL-7R and IL-7 concentrations (Figure S3A in Appendix S1). Children with T1D comprised individuals at disease onset as well as individuals with established symptomatic disease (> 6 months, median diabetes duration 4.5 years). In addition, IL-7 and sIL-7R serum levels were similar between these subgroups (Figure S3B in Appendix S1). Analyses of mIL-7R expression on $CD4^+$ and $CD8^+$ T cells showed no correlation of mIL-7R expression with IL-7 serum concentrations for $CD4^+$ or $CD8^+$ T cells (Figure 3A, for gating of mIL-$7R_{high}$ cells see Figure S4A in Appendix S1). In addition, no correlation was found between mIL-7R expression and sIL-7R

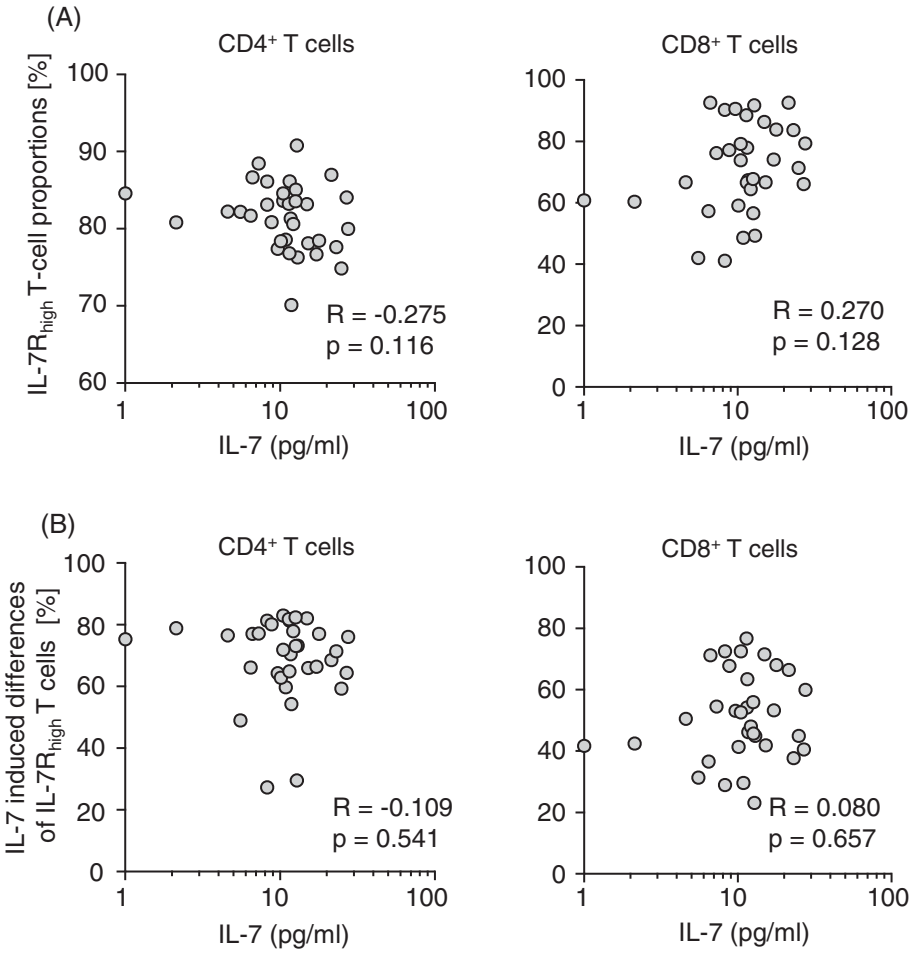

FIGURE 3 Membrane-associated (m)IL-7R expression and IL-7-induced mIL-7R internalization correlated with IL-7 serum concentrations. (A) IL-$7R_{high}$-expressing $CD4^+$ (left graphs) and $CD8^+$ (right graphs) T cell proportions of T1D children (*n* = 34) were correlated with IL-7. Spearman`s rank correlation coefficient R and the respective *P*-values are indicated. (B) IL-7-induced differences of IL-$7R_{high}$ cells were correlated with IL-7 serum concentrations. Spearman´s rank correlation coefficient R and the respective *P*-values are indicated

serum concentrations (Figure S4B in Appendix S1). Next, we determined the mIL-7R internalization rate of T cells on IL-7 exposure in vitro. Significantly decreased mIL-7R expression was detected after in vitro culture with IL-7 (data not shown). However, we detected no differences in the mIL-7R reduction for $CD4^+$ or $CD8^+$ T cells associated with differential serum IL-7 concentrations (Figure 3B). The same holds true for sIL-7R serum levels (Figure S4C in Appendix S1).

3.5 | IL-7-promoted T cell cytokine expression

Recently, we showed that IL-7 differentially affected cytokine expressing T cell proportions of children with T1D in vitro.[28] Hence, we compared IL-7 effects on IFNγ, TNFα, and IL-2 secretion of $CD4^+$ and $CD8^+$ T cells with differential IL-7 serum levels. We detected no correlation between serum IL-7 concentrations and in vitro IL-7-induced differential IFNγ, TNFα or IL-2 secretion for $CD4^+$ or $CD8^+$ T cells (Figure 4A-C). Similar results were obtained for the sIL-7R (Figure S5A-C in Appendix S1). We concluded that differential sIL-7R and IL-7 serum concentrations were affected by protective and risk haplotypes of the IL-7R, but no evidence for IL-7-dependent T cell functions was found.

4 | DISCUSSION

IL-7 is an important factor for the development of autoimmune T cells, and the sIL-7R variant was shown to regulate IL-7 availability. *IL7RA* genetic variants affect susceptibility for autoimmune diseases like T1D, but the mechanisms how different haplotypes influence T cell function are poorly understood. Here we detected lower sIL-7R serum levels in children with T1D carrying the risk haplotype, tagged by the rs1494555G variant, albeit less pronounced compared to the known effect of the protective haplotype, tagged by the rs6897932T variant.

For the first time, we detected lower IL-7 serum levels in T1D patients with the protective haplotype when compared to risk haplotype carriers. Lower IL-7 serum levels in multiple sclerosis patients with the protective haplotype have been described by Lundstrom

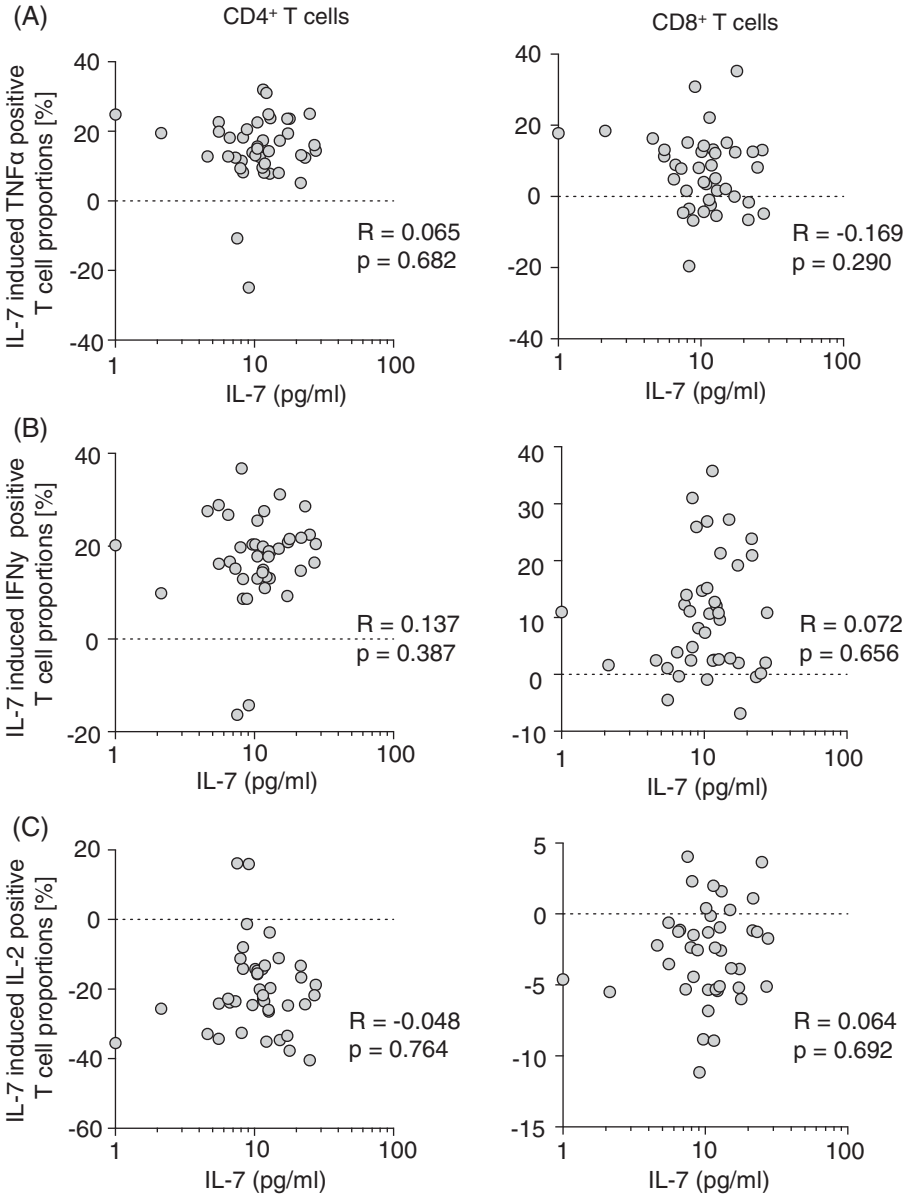

FIGURE 4 IL-7-promoted T cell activation (i.e. anti-CD3/CD28) induced cytokine expression and IL-7 serum concentrations. IL-7-induced proportions of TNFα-producing (A), IFNγ-producing (B) and IL-2-producing (C) memory $CD4^+$ (left graphs) and $CD8^+$ (right graphs) T cells in T1D children (n = 42) were correlated with serum IL-7. Spearman´s rank correlation coefficient R and the respective P-values are indicated

et al.[20] Notably, the study detected no effects of the protective haplotype on IL-7 serum concentrations for healthy controls.[20] Lower IL-7 serum concentrations have also been described for rheumatoid arthritis.[32] This group assumed a possible genetic cause for differential IL-7 levels but *IL7RA* haplotypes were not analyzed.[32] Therefore, our results and previous studies suggested that carriers of the protective *IL7RA* haplotype have lower IL-7 serum only under pathogenic autoimmune conditions.

IL-7 production-predominantly by stromal cells-is largely constant[33] and regulation of IL-7 availability has been shown to be dependent on T cell consumption.[11] Thus, we hypothesize that low IL-7 serum concentrations in T1D children with the protective haplotype are due to increased IL-7 uptake by T cells. Autoimmune T cells are likely more dependent on IL-7 because of low self-antigen affinity,[9] but low numbers of self-reactive T cells present in T1D render differences caused solely by autoimmune T cells unlikely.[34] However, recent studies indicate more profound differences concerning the T cell reservoir from T1D patients.[35] Bian et al[35] described a generally high-activation pattern of memory T cells from T1D patients. Accordingly, we demonstrated that memory T cells from symptomatic T1D patients produced generally higher cytokine levels and were more sensitive to IL-7-mediated T cell costimulation.[28] IL-7 especially promoted the effector cytokines IFNγ and TNFα in these T1D patients, whereas relative IL-2 production was lower as compared to healthy controls.[28] In the present study, we addressed the question if aberrant IL-7-promoted memory T cell response in children with T1D was due to differential IL-7 serum availability. We found no evidence for a dependency between IL-7 serum concentrations and mIL-7R expression, IL-7-induced mIL-7R internalization or IL-7 promoted T cell cytokine expression. However, moderately sized study groups for functional T cell analyses did not allow concurrent evaluation of *IL7RA* haplotype association. Therefore, additional studies are needed to clarify if dependency between IL-7 serum concentrations and IL-7-mediated T cell functions may only be apparent for certain haplotypes.

The current paradigm for the role of the sIL-7R in autoimmunity is that sIL-7R serum concentrations regulate the availability of IL-7. Lundstrom et al[20] showed that sIL-7R can form a reservoir of IL-7 in the serum and this ensures IL-7 availability as well as increased bioactivity of IL-7. Although the sIL-7R is present at about 1000-fold higher levels as compared to IL-7, differences in the avidity may counterbalance concentration differences and strengthen this assumption.[20] In accordance, lower sIL-7R serum concentrations of the protective haplotype would lead to decreased IL-7 reservoir capacity, and this may hamper especially self-reactive T cells. Against this background, we assume that low IL-7 serum concentrations of T1D children with the protective haplotype are a consequence of genetically determined low sIL-7R reservoir and increased consumption of IL-7 by T1D pathognomonic memory T cells found in T1D patients.[28] Previous findings about a possible influence of sIL-7R glycation and decreased IL-7-binding in children with T1D as compared to healthy controls[21] would contribute to this proposed mechanism by further impairing the IL-7 reservoir.

The finding, that T1D children carrying the risk haplotype also showed decreased sIL-7R serum concentrations but not decreased IL-7 serum levels is interesting and seemingly controversial. This may be explained by less pronounced differences of risk haplotype carriers, who have only moderate reduction in sIL-7R serum levels as compared to individuals with the protective haplotype. Another explanation could be that sIL-7R reservoir functions are not the only mechanism that leads to differential IL-7 availability and consumption as the proposed mechanism.[20] This is supported by the absence of correlation between sIL-7R and IL-7 serum concentrations. Similarly, we did not find any correlation between IL-7 and sIL-7R serum concentration in a previous study of tuberculosis patients and controls, although both IL-7 and sIL-7R serum concentration showed pathognomonic changes and normalization during recovery.[29] This suggested disease-specific but largely independent regulation of serum IL-7 and sIL-7R levels.[29] As an alternative explanation, one could hypothesize that the protective haplotype affects mIL-7R functions. In this regard, it has been shown that the protective haplotype changes the amino acid sequence (Thr → Ile) in a region crucial for IL-7R dimerization.[36] There is initial evidence that T cells carrying the protection-associated *IL7RA* haplotype differ in IL-7 binding and signaling.[37] Additional studies are needed to investigate a sIL-7R reservoir-independent role of *IL7RA* haplotypes.

This study found lower sIL-7R serum levels in T1D children with the *IL7RA* risk haplotype. In contrast to the protective haplotype, decreased sIL-7R serum levels in risk haplotype carriers were not accompanied with decreased IL-7 serum levels. Our findings shed novel light on the complex mechanisms of IL-7 availability and consumption in the context of autoimmunity-associated haplotypes.

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Conflict of interest

No potential conflicts of interest relevant to this article were reported.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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3.3 Paper III: *IL7RA* genetic variants are associated with impaired IL-7Rα expression and protection against tuberculosis

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- Genotyping
- Gene expression analysis
- Cloning and transfection
- Analysis of transfected cell lines
- Data analysis
- Writing of the manuscript

IL7RA genetic variants are associated with impaired IL-7Rα expression and protection against tuberculosis

Short title: Functional *IL7RA* SNPs associated with TB

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Abstract

Interleukin (IL)-7 dependent T-cell functions are impaired in chronic infections like tuberculosis. Functional IL-7 receptor α-chain (IL-7Rα) genetic variants are associated with susceptibility to autoimmune diseases and immune recovery of HIV/AIDS patients. We investigated the role of IL-7Rα genetic variants in human tuberculosis and analysed their effects on IL-7Rα regulation.

In a candidate gene association study, we genotyped six exonic IL-7Rα polymorphisms in tuberculosis patients and healthy household contacts from Ghana. Functional effects of identified IL-7Rα variants were characterized in primary T-cells from tuberculosis patients and contacts as well as in transfected cell lines.

Two minor alleles (i.e. rs11567764, rs1494558) were detected at lower frequencies in tuberculosis patients ($p = 0.002$, $p = 0.01$, respectively), indicating protective effects against progression to active tuberculosis. Together these protection-associated polymorphisms tag an IL-7Rα haplotype almost exclusively found in African populations. Analyses of functional effects revealed reduced mRNA expression of an IL-7Rα variant lacking exon 5-6 in T-cells from carriers of the protective rs11567764 allele. The protection-associated non-synonymous rs1494558 polymorphisms causes a Thr66Ile exchange in the exon 2 of the IL-7Rα and tags a previously described autoimmunity risk-associated haplotype. We detected lower soluble IL-7Rα secretion and membrane IL-7Rα expression in HEK293T cells transfected with the rs1494558-66Ile variant. These results were in accordance with recently published data showing lower soluble IL-7Rα serum concentrations in type 1 diabetes patients carrying the rs1494558-66Ile variant. We concluded that functional IL-7Rα polymorphisms, with potential impact on IL-7-mediated T-cell functions, mediate contrary effects on susceptibility to tuberculosis and autoimmune diseases.

Abstract importance

Interleukin-7 (IL-7)-mediated T-cell functions and impaired regulation of the Interleukin-7 receptor are involved in pathogenesis of autoimmune and chronic infectious diseases. *IL7RA* gene single nucleotide polymorphisms (SNPs) are associated with susceptibility to development of autoimmunity and a protection associated haplotype diminishes soluble IL-7Rα expression. In contrast no function of the autoimmunity risk-associated *IL7RA* haplotype has been described. We found association of two SNP alleles with protection against active tuberculosis. The protective rs1494558 allele tags the autoimmunity risk-associated haplotype in Caucasians. Functionally, we demonstrated impaired IL-7Rα trafficking and soluble IL-7Rα secretion of the rs1494558 variant. For the first time our study identified functional effects of the autoimmunity-associated risk and tuberculosis protection-associated *IL7RA* variant.

Keywords: Tuberculosis, T cells, IL-7, soluble IL-7 receptor α-chain, CD127, rs1494558, rs1494555, rs11567764, rs6897932

Background

Tuberculosis is a life-threatening mycobacterial disease of global relevance. The vast majority of *Mycobacterium tuberculosis*-infected individuals, however, does not develop tuberculosis but rather controls infection by immune-mediated protection(1). An important role of genetic factors for immune protection against tuberculosis has been demonstrated in animal models (2) and in humans (3-6). Further, previous studies identified an important role of IL-7 and IL-7-promoted T-cell response for protection against tuberculosis (7, 8). IL-7 promotes several functions of T-cell immunity including proliferation, cell survival, and cytokine expression (9).

The T-cell response to IL-7 is critically regulated on the IL-7Rα (CD127) level, and mIL-7Rα as well as a sIL-7Rα expression are of central relevance for IL-7-response and availability (10). The sIL-7Rα variant is generated by alternative splicing and has been described to exert IL-7 reservoir functions for T cells (11). An SNP (rs6897932C>T) located in *IL7RA* exon 6 affects alternative splicing, thereby altering the sIL-7Rα plasma concentration(12, 13). Alternative splicing events also generate *IL7RA* variants lacking exon 5-6, but the biological relevance hereof remains elusive (14-16). *IL7RA* exonic SNPs were previously found to be associated with susceptibility to develop autoimmune diseases, and in multiple sclerosis, a haplotype containing the rs6897932T SNP is associated with protection, whereas a rs1494555G-tagged haplotype is associated with increased risk to develop disease (12). The mechanisms underlying rs1494555G-mediated protection have not been defined.

Own previous studies revealed reduced mIL-7Rα and sIL-7Rα expression as well as impaired T-cell response to IL-7 during tuberculosis. In addition, minor differences in the frequency of the rs6897932T *IL7RA* allele between the study groups contributed to decreased sIL-7Rα serum concentrations in tuberculosis patients (8). Here, we investigated a possible role of exonic *IL7RA* SNPs in tuberculosis and evaluated functional effects of the polymorphisms.

Methods

Study participants

Samples for the genetic association analysis were collected from 2011 to 2016 within hospital-based observational studies. Patients with clinically confirmed tuberculosis (n = 114) and exposed, healthy contacts (n = 170) were recruited at Komfo Anokye Teaching Hospital (KATH), Kumasi South Hospital (KSH), Suntreso Government Hospital (SGH), Kwame Nkrumah University of Science and Technology (KNUST) Hospital, Tafo Government Hospital, Manhyia District Hospital (all Kumasi area, Ghana), and at Agogo Presbyterian Hospital in the Asante Akim North District (Ghana). Diagnosis of tuberculosis was based on patient's history, symptoms (persistent cough and fever, weight loss) and chest X-ray. For adults, tuberculosis was additionally confirmed by sputum smear test and, in case of a negative sputum test, by *Mycobacterium tuberculosis* sputum culture. Healthy contacts were recruited from households of patients with confirmed tuberculosis.

The study was approved by the Committee on Human Research, Publication and Ethics (CHRPE) at the School of Medical Sciences (SMS) at the Kwame Nkrumah University of Science and technology (KNUST) in Kumasi, Ghana (Ref.: CHRPE/AP/275/14, CHRPE/AP/221/14, CHPRE/AP/328/15). The aims and procedures of the study were explained to participants aged ≥18 years, and all adult participants gave written informed consent. For children, the aims and procedures of the study were explained to participants (if appropriate) and to their parents or legal guardians. Assent of children and written

informed consent was obtained from parents/guardians. In some cases, consent was confirmed by thumbprint, a procedure approved by the review board. All participants were free to drop out at any time of the study.
Venous blood was collected into heparinised Vacutainer Tubes (BD Biosciences). Anti-tuberculous medication according to the Ghanaian guidelines was initiated immediately after the first blood sample was taken. HIV testing was performed as a standard hospital-based procedure.
Analysis of mRNA from enriched $CD4^+$ T cells was performed on a subset of samples. Part of this patient group has been used previously for the analysis of IL-7 and IL-7Rα and results are published elsewhere (8).

Genotyping of IL7RA single nucleotide polymorphisms
DNA was isolated from peripheral blood mononuclear cells (Biocoll, Biochrom) or whole blood using QIAamp DNA Mini kit (Qiagen) or GenoType DNA Isolation Kit (Hain Lifescience) followed by genotyping with predesigned TaqMan SNP Genotyping Assays (Applied Biosystems) and analysed on a 7500 Real-Time PCR machine (Applied Biosystems). A call rate ≥ 97% was found for all SNPs, and none of the SNPs deviated from the Hardy–Weinberg equilibrium.

Real-Time PCR of IL-7Rα variants
$CD4^+$ cells from freshly isolated peripheral blood mononuclear cells (at least 1.5 x 10^7 cells) were isolated using anti-human CD4 magnetic particles (BD Biosciences) obtaining a cell purity generally >95% as evaluated by flow cytometry. Total RNA was isolated from at least 5 x 10^6 enriched $CD4^+$ cells using mirVana miRNA Isolation Kit (Life Technologies). cDNA was generated by Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific), while RT-PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) for full-length IL-7Rα (H20: forward 5'-AATAATAGCTCAGGGGAGATGG-3', reverse 5'-ATGACCAACAGAGCGACAGAG-3'), IL-7Rα lacking exon 6 (Δ6: forward 5'-GATCAATAATAGCTCAGGATTAAGC-3', reverse 5'-AAGATGTTCCAGAGTCTTCTTATG-3'), and IL-7Rα lacking exon 5-6 (Δ5-6: forward 5'-ATGAAAACAAATGGACGGATTAAGC-3', reverse 5'-AAGATGTTCCAGAGTCTTCTTATG-3'). The reaction in a final volume of 25 µl was run 2 min. at 50°C, 10 min. at 95°C, 45 cycles of 15 s at 95°C, 30 s at 53°C and 30 s at 72°C, followed by a melt curve sequence of 15 s at 95°C, 60 s at 60°C with a slow gradient to 95°C and finally 15 s at 60°C on a 7500 Real-Time PCR machine (Applied Biosystems). Data from duplicate reactions was evaluated by the $2^{-\Delta Ct}$ method.

Cloning of vectors
mIL-7Rα and sIL-7Rα corresponding to the NCBI Reference Sequences (NM_002185.4 and XM_005248299.3, respectively) were cloned from human mRNA and inserted into LeGO-iB2 vector (generated from LeGO-iG2(17) by exchanging eGFP for eBFP) using the EcoRI/NotI (mIL-7Rα) or BamHI-BglII/NotI (sIL-7Rα) restriction enzyme sites (all restriction enzymes from Thermo Fisher Scientific) applying standard molecular cloning techniques. A Kozak sequence (GCCACC) was introduced upstream of the start codon. *IL7RA* minigenes were generated as shown in Figure 2a by PCR of human DNA from exon 1 including 242 bp of the downstream intron, and exon 7 including 287 bp of the upstream intron. A complementary overhang containing XbaI/XhoI restriction enzyme sites was

included downstream and upstream of the introns, respectively. The two amplicons were combined in a second round of PCR using the forward and reverse primer for exon 1 and exon 7, respectively, and subsequently inserted into the LeGO iB2 vector using BamHI-BglII/NotI restriction enzyme sites. Exon 5 and exon 6 of *IL7RA* – including part of the upstream and downstream introns – were inserted into the minigene vector using XbaI/XhoI restriction enzyme sites. To generate mutants of mIL-7Rα, sIL-7Rα and minigene vectors, PCR was performed with Phusion High-Fidelity PCR Master Mix with GC Buffer (New England BioLabs) and appropriate primers. The reactions were run for 30s at 98°C followed by 18 cycles of 7s at 98°C, 30s at 60°C and 8 min. at 72°C, followed by 10 min. at 72°C. Plasmid of bacterial origin was removed by DpnI digestion prior to transformation. Successful mutation was verified by DNA sequencing. Primers for cloning and mutations are listed in Table S1.

Cell culture and transfection

HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (high glucose, GlutaMAX, pyruvate) supplemented with 10% heat-inactivated FCS, 25 mM HEPES (all Thermo Fisher Scientific), 100 units penicillin/ml and 100 µg streptomycin/ml (Sigma Aldrich). Transfections were performed in medium supplemented with 25 µM Chloroquine (Sigma Aldrich) using the Calcium Phosphate Transfection Kit (Sigma Aldrich).

Minigene

HEK293T cells were transfected with minigene vectors, and after 24h incubation, mRNA was isolated from cells using the NucleoSpin RNA kit (Macherey-Nagel) followed by generation of cDNA using Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific). PCR was performed with Phusion High-Fidelity PCR Master Mix with GC Buffer (New England BioLabs) and primers covering exon 1 to exon 7 (forward 5'- ATGACAATTCTAGGTACAACTTTTGG-3', reverse 5'-AAGATGTTCCAGAGTCTTCTTATG-3'). The reactions were run for 30s at 98°C followed by 30 cycles of 7s at 98°C, 15s at 60°C and 45s at 72°C, followed by 8 min. at 72°C. PCR products were mixed with Hi-Density TBE Sample Buffer and separated on a 10% Novex TBE gel in Novex TBE Running Buffer (Thermo Fisher Scientific) at 200V. Subsequently, the gel was stained with GelRed (Biotium) and analysed on a ChemiDoc Touch Imaging System (Bio-Rad).

Cell staining

Cells were harvested and stained with Viability Dye eFluor 780 (eBioscience) and mouse anti-human IL-7Rα AF647 (clone HIL-7R-M21, BD Biosciences), or polyclonal goat anti-human IL-7Rα (R&D Systems) followed by FITC-conjugated polyclonal donkey anti-goat IgG (R&D Systems). After staining, cells were fixed with Fixation Buffer (BioLegend) and analysed using an LSRFortessa flow cytometer (BD Biosciences). The two IL-7Rα antibodies (polyclonal and clone HIL-7R-M21) showed similar performance independent from the mutations of IL-7Rα (Figure S1).

Cytometric bead assay for sIL-7Rα

Measurement of sIL-7Rα concentrations from culture supernatants was performed as described earlier(8, 18) with minor modifications. Briefly, functional beads (Bead A4, BD Biosciences) were conjugated with polyclonal anti-human IL-7Rα (R&D Systems) according to manufacturer's protocol. Samples and

standard (rhIL-7Rα-Fc Chimera Protein, R&D Systems) were diluted in culture medium and incubated with anti-IL-7Rα-conjugated beads. Bound sIL-7Rα was detected using biotinylated mouse anti-human IL-7Rα (clone HIL-7R-M21, BD Biosciences) and Streptavidin PE (BioLegend). Beads were analysed on a BD LSRFortessa flow cytometer (BD Biosciences), and sIL-7Rα concentration was computed from median PE values by 4-parametric regression in GraphPad Prism version 6.07.

Protein preparation for western blot

Proteins from cell lysate was isolated using CelLytic M supplemented with Protease Inhibitor Cocktail (Sigma Aldrich), and protein concentration was determined with Protein Assay Dye Reagent (Bio-Rad) using BSA as standard (Sigma Aldrich). 20 µg protein was heated 10 min. at 100°C in Glycoprotein Denaturing buffer (New England BioLabs) and subsequently added PNGase F to remove N-glycans, while a combination of O-Glycosidase, α2-3,6,8,9 Neuraminidase A, β-N-acetylhexosaminidase f and β1-4 Galactosidase S was used in combination to trim and remove O-glycans according to manufacturer's protocol (New England BioLabs). After enzymatic treatment, samples were heated 10 min. at 80°C and loaded on a NuPAGE 4-12% Bis-Tris gels (all Thermo Fisher Scientific) together with Precision Plus Protein Standards All Blue (Bio-Rad). Run in NuPAGE MOPS Buffer (Thermo Fisher Scientific) at 100V. After separation, proteins were blotted to a 0.2 µm nitrocellulose membrane (Bio-Rad) in NuPAGE Transfer Buffer (Thermo Fisher Scientific) at 4°C for 2h at 240 mA. Membrane was blocked in 5% skim milk (Sigma Aldrich) in PBS/T (PBS, 0.1% Tween-20), incubated overnight at 4°C with polyclonal goat anti-human IL-7Rα (R&D Systems, 1:2000 in PBS/T/2% skim milk). HRP Rabbit anti-Goat IgG (H+L) (invitrogen, 1:2000 in PBS/T/2% skim milk) was used as secondary antibody, and membrane was developed using BM Chemiluminescence Western Blotting Substrate (POD) (Sigma Aldrich) on a ChemiDoc Touch Imaging System (Bio-Rad).

Statistical analysis

Haplotypes of selected SNPs in *IL7RA* were predicted from 1000 Genomes (19) data using HaploView version 4.2. Statistical analyses were performed in R version 3.3.0, applying Exact Mann-Whitney U test from the package *coin* for group comparisons or ANOVA of square root-transformed data. SNP associations were evaluated by logistic regression using the package *SNPassoc* (20). Plots were generated in GraphPad Prism version 6.07. Two-tailed p-values < 0.05 were considered significant.

Results

Two IL7RA SNPs are associated with protection against tuberculosis

We genotyped six exonic *IL7RA* SNPs (Figure 1a) in tuberculosis patients (n = 114) and healthy contacts (n = 170, Table S2). For two SNPs, i.e. rs1494558C>T and rs11567764G>A, minor alleles were present at higher frequencies among healthy contacts as compared to tuberculosis patients (Table 1). The major effects were seen for recessive models (rs11567764: OR 0.10, 95% CI: 0.01-0.75; rs1494558: OR 0.35, 95% CI: 0.15-0.84). A subgroup of tuberculosis patients (n = 16) and contacts (n = 2) had HIV co-infection but excluding these had only minor effects on the estimates (Table S3). rs11567764 is a synonymous SNP located in exon 5 (Figure 1a), and its minor allele is almost exclusively found in African populations (Table 2). rs1494558 is located in exon 2 of *IL7RA* and causes a nonsynonymous substitution (i.e. 197C>T, Thr66Ile). In combination, the associated minor alleles of both SNPs, i.e.

rs11567764A and rs1494558T tag a haplotype frequently present in African populations (Table 2). Another haplotype tagged by rs1494558T only is less frequent in African as compared to Caucasian populations (Table 2). Next, we aimed at characterising functional effects of the SNPs associated with protection against tuberculosis.

***Table 1**. Association between IL7RA SNPs in tuberculosis patients and healthy household contacts*

		Frequency (%)		Codominant		Dominant		Recessive	
SNP	**Genotype**	**Contacts**	**TB**	**OR**	**p-value**	**OR**	**p-value**	**OR**	**p-value**
	CC	39.9	45.0	-		-		-	
rs1494558	CT	44.0	48.6	0.98 (0.59-1.62)	**0.04**	0.81 (0.50-1.31)	0.39		**0.01**
	TT	16.1	6.3	0.35 (0.14-0.86)				0.35 (0.15-0.84)	
	AA	79.2	77.7	-		-		-	
rs1494555	AG	17.9	21.4	1.22 (0.67-2.23)	0.37	1.09 (0.61-1.95)	0.77		0.21
	GG	3.0	0.9	0.31 (0.04-2.66)				0.29 (0.03-2.55)	
	CC	80.6	75.0	-		-		-	
rs2228141	CT	19.4	24.1	1.33 (0.75-2.38)	0.26	1.38 (0.78-2.45)	0.27		0.40
	TT	0.0	0.9	NA				NA	
	GG	56.5	64.0	-		-		-	
rs11567764	AG	35.1	35.1	0.88 (0.53-1.46)	**0.008**	0.73 (0.45-1.19)	0.21		**0.002**
	AA	8.3	0.9	0.09 (0.01-0.72)				0.10 (0.01-0.75)	
	CC	87.6	83.0	-		-		-	
rs6897932	CT	12.4	17.0	1.45 (0.74-2.84)	0.28	NA	NA		NA
	TT	0.0	0.0	NA				NA	
	AA	50.0	43.5	-		-		-	
rs3194051	AG	39.2	48.1	1.41 (0.85-2.35)	0.33	1.30 (0.80-2.11)	0.29		0.49
	GG	10.8	8.3	0.88 (0.37-2.12)				0.75 (0.32-1.73)	

Abbreviations: TB: tuberculosis patients, OR: odds ratio, NA: not applicable.

***Table 2**. IL7RA haplotypes and ethnic distribution*

						Frequency (%)	
rs1494558	**rs1494555**	**rs2228141**	**rs11567764**	**rs6897932**	**rs3194051**	**AFR**	**EUR**
C	A	C	G	C	G	34.6	28.2
C	A	C	G	C	A	21.7	-
T	A	C	**A**	C	A	16.9	-
C	A	T	G	C	A	12.8	14.4
T	G	C	G	C	A	6.7	29.7
C	A	C	G	T	A	6.6	27.1

Haplotype subsets tagged by 6 selected SNPs in the *IL7RA* gene based on genetic variant information from 1000 Genomes Project phase 3(19). AFR population (n = 661) was combined from the subpopulations YRI, LWK, GWD, MSL, ESN, ASW and ACB. EUR population (n = 503) was combined from CEU, TSI, FIN, GBR and IBS. Haplotypes > 1.0% are shown.

The rs11567764A allele is accompanied with decreased IL7RA Δ5-6 mRNA expression

IL7RA transcripts lacking exon 6 (Δ6, coding for sIL-7Rα) or exon 5-6 (Δ5-6) are generated by alternative splicing (15, 16). Since the SNP rs6897932 located in exon 6 has been shown to influence exon 6 splicing (12), we used HEXplorer (21) to predict the effect of the exon 5 SNP rs11567764 on alternative splicing. We detected marked differences in HEXplorer scores indicating potential negative effects of the rs11567764A allele on exon 5 splicing (Figure S2). Consequently, we measured IL-7Rα mRNA of enriched $CD4^+$ T cells from study group donors for Δ5-6, Δ6 (sIL-7Rα), and full-length (mIL-7Rα) and compared relative amounts of variants between rs11567764 alleles. Donors carrying the rs11567764A allele had a tendency of lower Δ5-6 mRNA levels in comparison to full-length IL-7Rα

mRNA (p = 0.07; Figure 1b, left graph) and significantly lower Δ5-6 levels as compared to Δ6 IL-7Rα mRNA (p = 0.004; Figure 1b, middle graph). No effect was seen when comparing Δ6 mRNA with full-length IL-7Rα mRNA (p = 0.92; Figure 1b, right graph). Because of a low number of homozygous rs11567764A carriers, subgroup analysis of tuberculosis patients and healthy contacts for the recessive effect of rs11567764A on Δ5-6 mRNA expression was not possible. To investigate if differential Δ5-6 mRNA expression could be ascribed to the rs11567764 SNP, we generated exon 5 minigenes containing either rs11567764A or rs11567764G (Figure 2a). The rs6897932 exon 6 variants were also included since these have been proven to affect IL-7Rα exon 6 alternative splicing (12) (Figure 2a). In accordance we detected reduced levels of exon 6 skipping for the minigene including the rs6897932T variant (Figure 2b, upper panel). However, no differences of exon 5 splicing were seen between rs11567764G and 11567764A variants (Figure 2b, lower panel). Therefore, we could not assign differential Δ5-6 mRNA expression specifically to the rs11567764 SNP. We concluded that rs11567764A was associated with reduced expression of *IL7RA* Δ5-6 mRNA of $CD4^+$ T cells, but the causative mechanism needs to be elucidated.

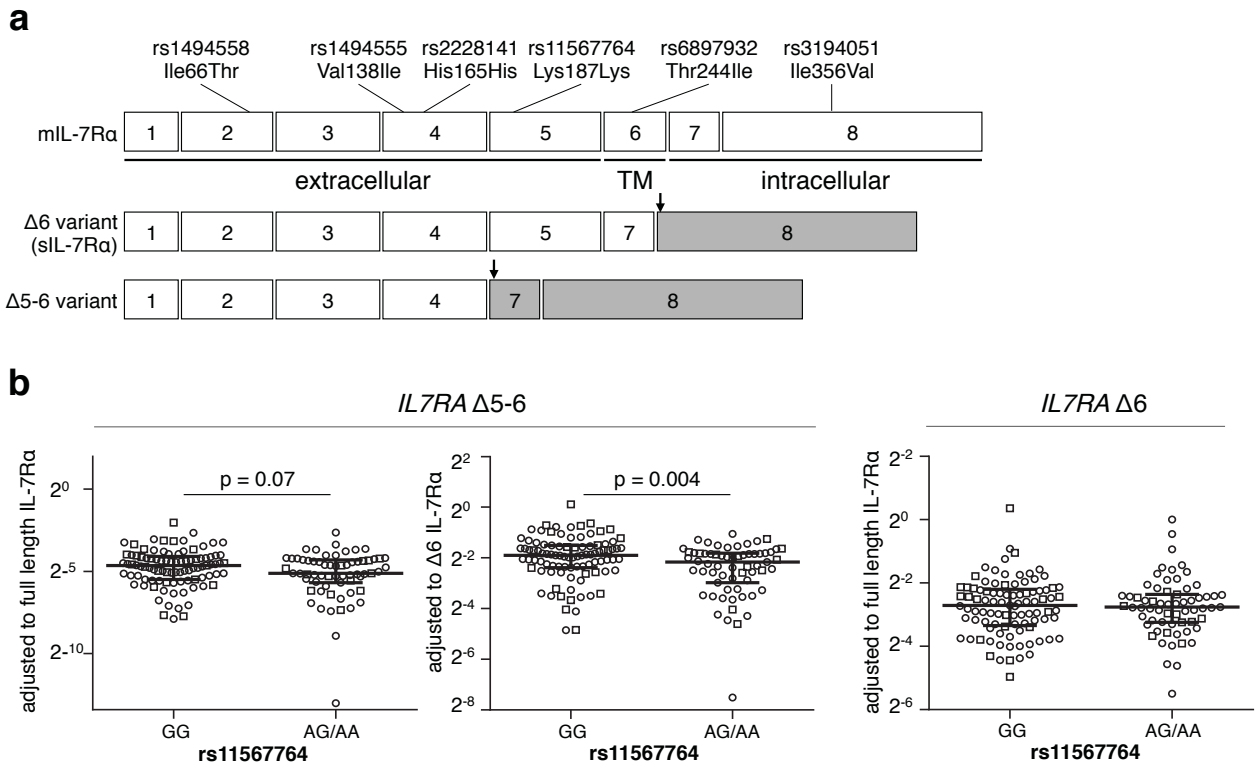

Figure 1: *IL7RA* single nucleotide polymorphisms (SNPs) and influence of rs11567764 alleles on *IL7RA* exon 5-6 splicing **(a)** Position of 6 selected SNPs in the exons of *IL7RA*. The full-length IL-7R including the transmembrane domain (TM) as well as two alternative splice variants lacking exon 6 (Δ6, coding for sIL-7Rα) or exon 5-6 (Δ5-6) are shown. Premature stop codons caused by a change in the reading frame are indicated by arrows for the Δ6 and the Δ5-6 variants. **(b)** Expression of *IL7RA* transcripts coding for IL-7Rα lacking exon 6 (Δ6) or exon 5-6 (Δ5-6) in $CD4^+$ cells from tuberculosis patients prior to treatment (squares) and households contacts (circles) carrying rs11567764GG (n = 93) or rs11567764AG/AA (n = 63). Expression level is normalised to full-length IL-7Rα (FL) or Δ6. Median and interquartile range for cycle threshold differences ($2^{-\Delta Ct}$) are indicated. Exact Mann-Whitney U-tests were used for comparison of groups. rs11567764AG and rs11567764AA were combined as one group due to a low number of rs11567764AA carriers.

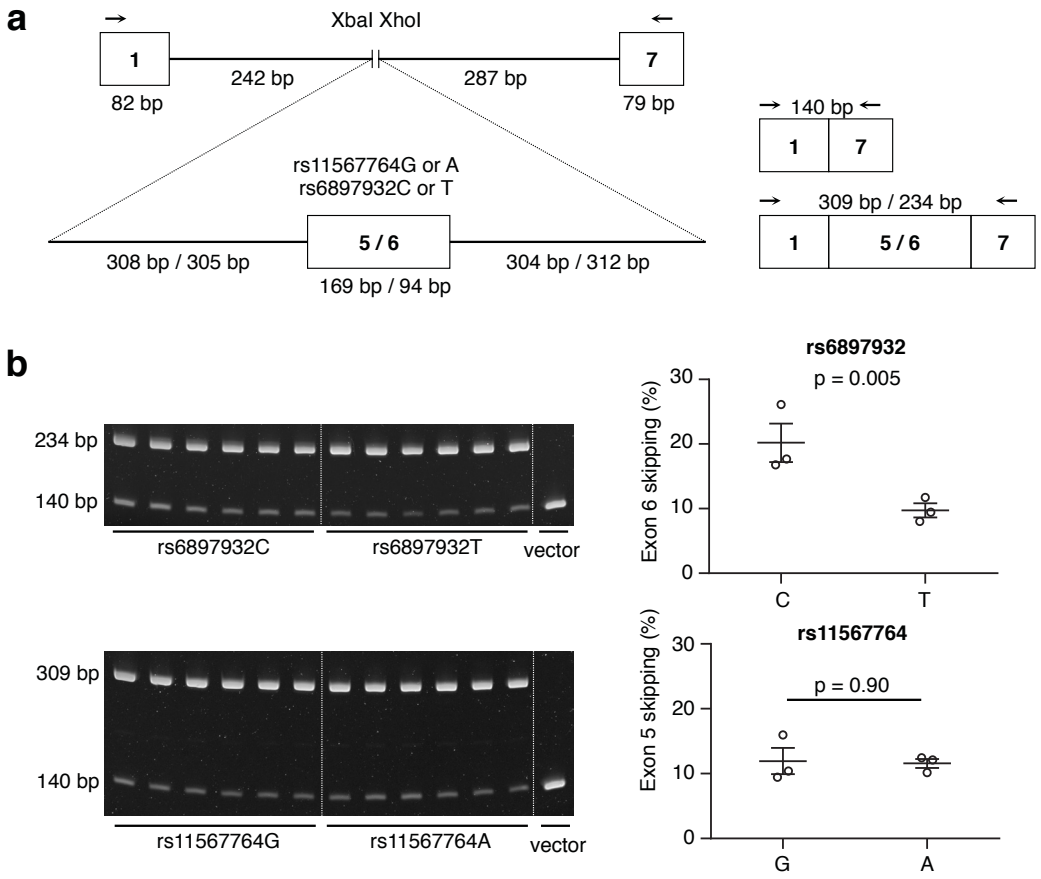

Figure 2: Effects of *IL7RA* rs11567764 alleles on alternative splicing determined using a minigene-based approach. **(a)** Schematic depiction of *IL7RA* minigene generation. Exon 5 or exon 6 of the *IL7RA* (including ~300 bp of the introns upstream and downstream of the exons) were inserted into a construct generated from *IL7RA* exon 1 and exon 7 including part of the downstream and upstream introns, respectively. Exon 5 and exon 6 were mutated to obtain rs11567764G/A and rs6897932C/T variants. On the right side, primer positions for PCR are indicated by arrows and expected product lengths are shown. **(b)** HEK293T cells were transfected in triplicate with minigene constructs. PCR was performed in duplicate on RNA from cell lysate, and products were separated on a 10% TBE gel stained with GelRed. The proportion of transcript with exon5/exon6 skipped is indicated in the plots with mean ± SEM. One representative experiment of four (rs11567764G/A) and two (rs6897932C/T) is shown. Data analysed by Exact Mann-Whitney test stratifying for experiment, and p-values are based on data from all experiments.

The rs1494558T-coded Ile66 variant affected IL-7Rα gel mobility

The nonsynonymous rs1494558 variants code for different amino acids (i.e. Thr66Ile) in the ectodomain of the IL-7Rα chain in a region distant from the IL-7-binding site (Figure 3a). First, we determined if this exchange affected the conformation of the IL-7Rα chain leading to changes in the gel mobility. Indeed, Thr66 and Ile66 sIL-7Rα variants expressed in HEK293T cells were detected at different positions in western blots (Figure 3b). Located next to the Asn65 N-glycosylation site (22, 23), we investigated the influence on N-glycosylation as a potential cause for differential gel mobility. Differences in gel positions, however, were still present after removal of N-glycans from cell lysate (Figure 3c). To exclude that N-glycosylated Asn65 could be resistant to enzymatic deglycosylation, we generated N65Q variants incapable of N-glycosylation at position 65. As expected, a reduction in molecular weights was seen for the N-glycosylation-defective N65Q mutants, but the differences between rs1494558 variants were still apparent (Figure 3c). In addition, we excluded that possible O-glycosylation of Thr66 was the reason for

different motility (Figure S3). Overall, Ile66/Thr66 variants differed in gel mobility, but differential glycosylation did not appear causative.

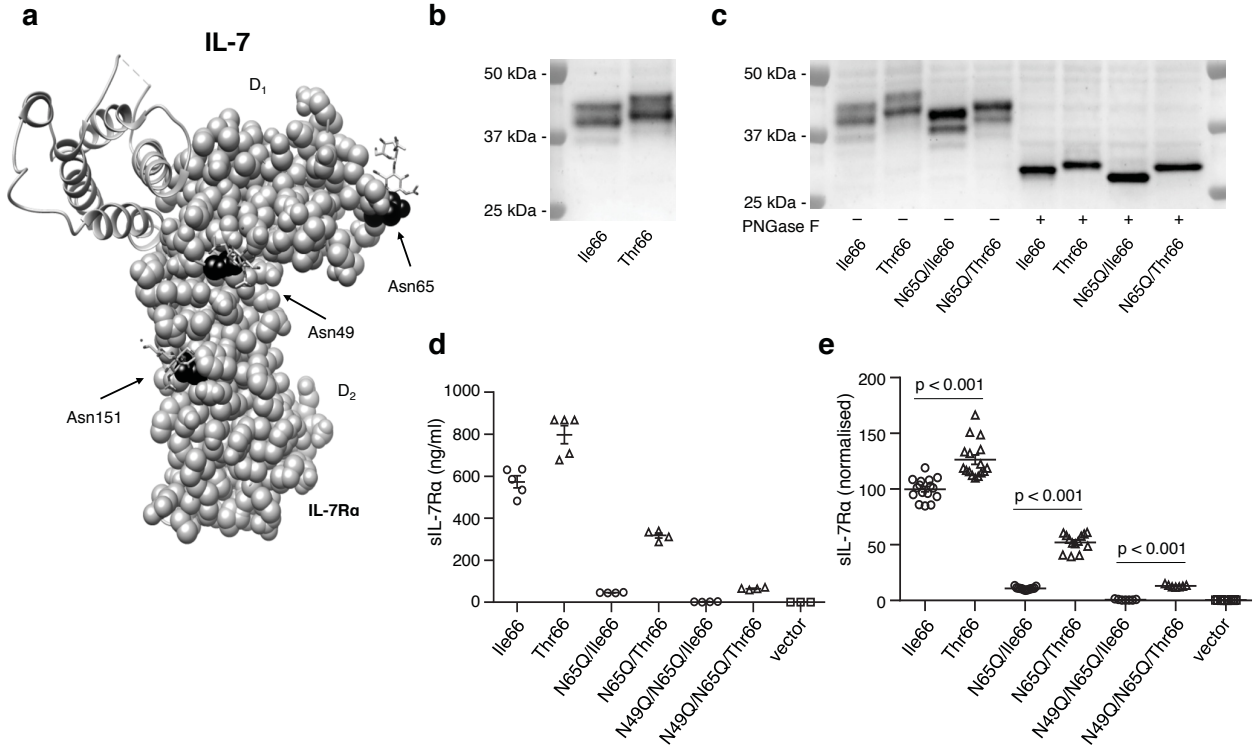

Figure 3: Soluble (s)IL-7Rα conformational changes of rs1494558 alleles coding for isoleucine (Ile) or threonine (Thr) in position 66 and effects on N-glycosylation-dependent secretion **(a)** Crystal structure of the ectodomain of IL-7Rα (sphere view) and IL-7 (ribbon diagram). Three N-glycosylation sites identified in the crystal structure are shown in black for the D_1 and D_2 domains. Structure generated in Chimera version 1.10.2 from PDB id 3DI3(22). **(b, c)** Western blots of cell lysate from HEK293T cells transfected with sIL-7Rα containing Ile or Thr in position 66. **(c)** Two variants mutated in *IL7RA* position 65 to prevent N-glycosylation were included and all samples were analysed with or without PNGase F-treatment. **(d, e)** sIL-7Rα concentrations of culture supernatants from HEK293T cells transfected with different sIL-7Rα variants measured by cytometric bead array are shown. In addition to the variant mutated in position 65 (N65Q), a second N-glycosylation-deficient variant in position 49 (N49Q) and vector control transduced HEK cells have been included. **(d)** sIL-7Rα concentrations from a representative experiment are shown. **(e)** Normalised concentrations of sIL-7Rα variants according to transfection efficacy (i.e. geometric mean of eBFP expression) are shown. The average sIL-7Rα in Ile66 samples was set to 100. Combined results from four (Ile66, Thr66, N65Q/Ile66 and N65Q/Thr66) or two (N49Q/N65Q/Ile66 and N49Q/N65Q/Thr66) experiments are shown. Square root-transformed data were analysed by ANOVA. Two-tailed p-values are given.

The rs1494558T-coded Ile66 variant impaired sIL-7Rα secretion and mIL-7Rα expression

Next, we investigated if Ile66/Thr66 differences affected IL-7Rα secretion. Since N-glycosylation of the D_1 domain of type I cytokine receptors has proven important for receptor trafficking (24, 25), we included mutants defective for N-glycosylation in the D_1 domain of IL-7Rα (i.e. N49Q and N65Q) as positive controls. sIL-7Rα-transfected HEK293T cells showed markedly reduced sIL-7Rα secretion for Ile66 compared to Thr66 (Figure 3d). The difference persisted also in the absence of glycosylation, although sIL-7Rα secretion was almost completely abrogated under these conditions (Figure 3d). Normalisation of sIL-7Rα concentrations and comparisons between repetitive experiments revealed significant impaired sIL-7Rα secretion of HEK293T containing the Ile66 variant ($p<0.001$ for all comparisons; Figure 3e).

To determine a potential effect on mIL-7Rα expression, HEK293T cells were transfected with full-length IL-7Rα containing both variants. We compared transfected HEK293T cells (eBFP-positive) for mIL-7Rα expression (i.e. geometric mean fluorescence of IL-7Rα specific antibody binding) (Figure 4a). Although less pronounced than for sIL-7Rα, lower surface mIL-7Rα expression was seen for Ile66 as compared to Thr66 (Figure 4b). Notably, variants lacking N-glycosylation showed even more pronounced differences and impaired mIL-7Rα expression of rs1494558 Ile66 containing HEK293T cells was significant, when evaluating multiple normalized experiments (Figure 4c). We concluded that the rs1494558 Ile66 variant caused diminished secretion of sIL-7Rα and impaired mIL-7Rα trafficking to the cell surface.

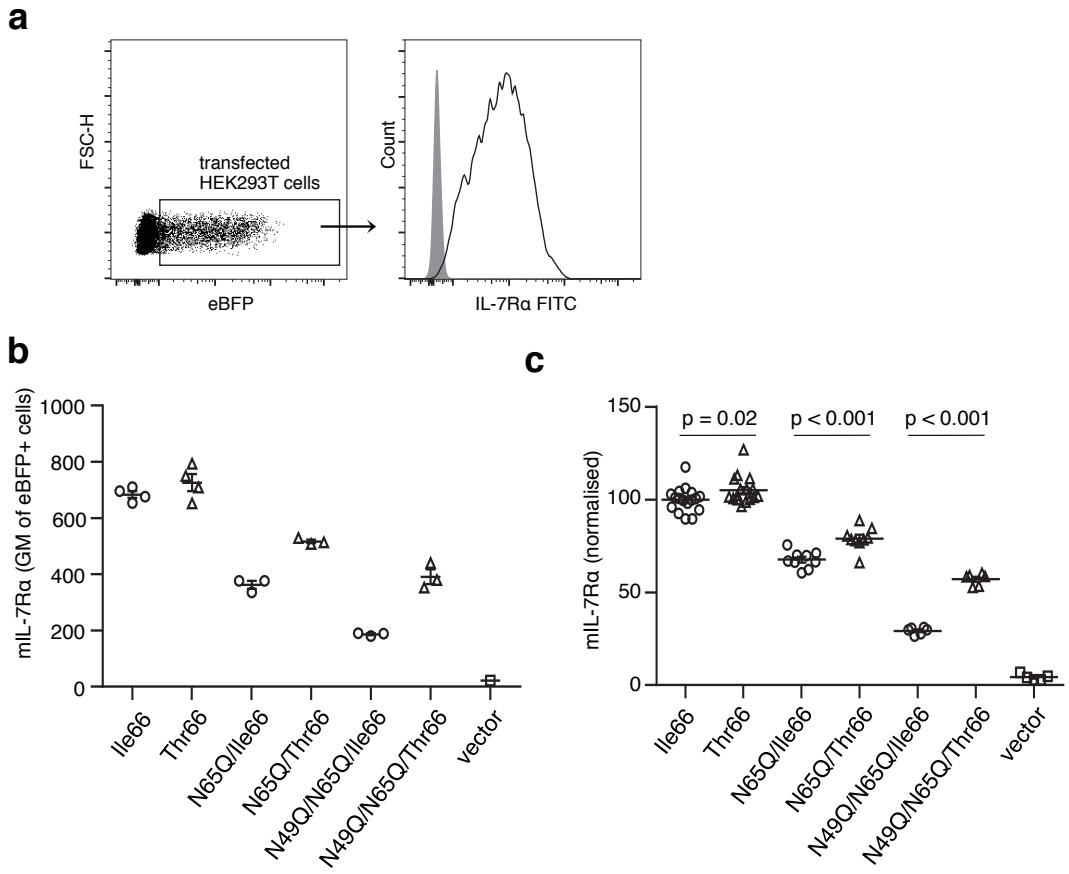

Figure 4: Membrane-associated (m)IL-7Rα expression of HEK293T cells transfected with different *IL7RA* rs1494558 alleles (i.e. Ile66, Thr66) and N-glycosylation-deficient mutants. **(a)** Gating procedure of mIL-7Rα transfected (i.e. eBFP-positive) HEK293T cells and mIL-7Rα specific antibody staining (right graph; Ile66: black-line open curve; vector control; grey curve)**. (b, c)** Surface expression of mIL-7Rα on eBFP-positive HEK293T cells after transfection (for 24h) with different *IL7RA* variants measured by fluorescence levels (geometric mean (GM) of IL-7Rα expression) are shown. In addition to the variant mutated in position 65 (N65Q), a second N-glycosylation-deficient variant in position 49 (N49Q), and vector control transduced HEK cells have been included. **(b)** mIL-7Rα expression levels from a representative experiment are shown. **(c)** Normalised mIL-7Rα expression of variants according to transfection efficacy (i.e. GM of IL-7Rα / GM of eBFP) are shown. The average mIL-7Rα in Ile66 samples was set to 100. Combined results from five (Ile66e and Thr66), three (N65Q/Ile66, N65Q/Thr66) or two (N49Q/N65Q/Ile66 and N49Q/N65Q/Thr66) representative experiments are shown. Square root-transformed data were analysed by ANOVA. Two-tailed p-values are given.

Discussion

We identified two SNPs with lower frequencies of *IL7RA* minor alleles (i.e. rs1494558T and rs11567764A) in tuberculosis patients as compared to healthy contacts. Impaired alternative splicing predicted for the rs11567764A allele was confirmed by reduced Δ5-6 IL-7Rα mRNA levels of $CD4^+$ T cells from study group donors. The IL-7Rα Ile66 variant coded by the rs1494558T allele was found to cause differences in the trafficking and secretion of IL-7Rα.

These results indicated protective effects of *IL7RA* rs1494558T/rs11567764A alleles against development of tuberculosis. There is strong evidence that IL-7 has a decisive role in the generation of protective T-cell memory against chronic infections (26, 27), and the protective capacity of IL-7-mediated effects on T cells against tuberculosis has also been shown (7). IL-7 can revert T-cell exhaustion (28) and is considered an important candidate for immune-modulatory therapy against chronic infectious diseases (29). Recently, we demonstrated impaired IL-7 responses of antigen-specific T-cell in acute tuberculosis accompanied by aberrant IL-7 and sIL-7Rα plasma levels (8). The described *IL7RA* SNP rs6897932T contributed to sIL-7Rα differences (8) and, therefore, we assumed general effects of *IL7RA* genetic variants on IL-7 mediated T-cell response against tuberculosis. Potential *IL7RA* SNP associations with susceptibility against tuberculosis was indicated in the present study. Genome wide association studies (GWAS) in tuberculosis patients and healthy controls have been performed previously (3, 4). However, both associated SNPs were not included in these studies of tuberculosis patients. This instance, and the restricted sensitivity of applied statistical methods for detection of recessive allele effects (30), may explain that a role of the *IL7RA* genomic region on chromosome 5 was not suggested (4).

The exact role of IL-7Rα variants for IL-7 availability and T-cell functions has been a matter of intensive investigations during past years. Several studies in the fields of autoimmunity were performed, inspired by strong genetic association of *IL7RA* SNP with autoimmune disease susceptibility (12, 31, 32). These studies demonstrated that increased IL-7 availability due to higher sIL-7Rα levels (i.e. IL-7 reservoir) promoted auto-reactive T cells (11), whereas lower sIL-7Rα levels (e.g. caused by the rs6897932T allele) were associated with protection against development of autoimmune diseases (31, 33, 34). We found lower sIL-7Rα secretion for the rs1494558T allele associated with protection against tuberculosis. Notably, own recent studies of type 1 diabetes patients detected reduced sIL-7Rα serum concentration for rs1494555G allele carriers (35). Based on the strong linkage disequilibrium between rs1494558 and rs1494555 in Caucasian populations ($r^2 = 0.97$, Table 2), we hypothesise that this effect is caused by the rs1494558T allele coding Ile66. These findings are contrary to the assumption that lower sIL-7Rα levels would generally lead to increased protection against development of autoimmune diseases (i.e. IL-7 reservoir theory) (10, 11). In tuberculosis, protective T-cell immunity and generation of effective memory T-cell response depended on increased IL-7 availability (7, 36). In accordance, differences of IL-7 serum levels in type 1 diabetes patients were not found for *IL7RA* rs1494555G allele carriers whereas autoimmunity protection-associated rs6897932T allele carriers had both, lower IL-7 and sIL-7Rα serum levels (35). On this basis, we speculate that sIL-7Rα/IL-7 reservoir independent mechanisms account for differential susceptibility of rs1494558T allele carriers to develop tuberculosis. In this regard, we provide initial evidence that the rs1494558T allele also affected mIL-7Rα expression. This suggested a possible role of *IL7RA* alleles for mIL-7Rα regulation. We detected marginal but significant mIL-7Rα differences between rs1494558 alleles, and this may explain why a previous publication found lower mIL-7Rα expression of NK cells but not T cells from multiple sclerosis patients carrying the *IL7RA* risk haplotypes

(13). However, under extreme conditions (i.e. impaired trafficking caused by glycosylation defects), differences in mIL-7Rα expression specific for the rs1494558T allele were apparent. This argued for regulatory mechanisms, which stabilise mIL-7Rα levels. How impaired mIL-7Rα trafficking contributes to immune protection against tuberculosis and increased risk for development of autoimmunity needs further clarification.

The rs11567764A allele was associated with lower Δ5-6 IL-7Rα mRNA levels of $CD4^+$ T cells, although the effect could not specifically be ascribed this SNP. However, linkage of rs11567764 to another functional SNP or regulatory elements not covered by the minigene could potentially account for this. Further, the function of the Δ5-6 variant remains unclear. It is predicted to be a target for nonsense-mediated decay due to a premature stop codon located upstream of the exon 7/exon 8 junction (37) (Figure 1a). In addition, the Δ5-6 variant lacks several structural parts of the D_2 domain coded by exon 5 (including the WSXWS motif important for folding of type I receptors (38)). Therefore, further studies are needed to elucidate the role of the Δ5-6 variant.

We identified two SNPs protective for tuberculosis that were associated with functional changes in the expression of *IL7RA* and with differences in the trafficking and secretion of IL-7Rα. Furthermore, the importance of N-glycosylation of IL-7Rα for optimal trafficking and secretion of IL-7Rα was highlighted. These results emphasise potential roles of IL-7Rα and genetic variants on T-cell response against tuberculosis. Beyond the field of tuberculosis, this study may generally add to our understanding of crucial processes involving IL-7 in T-cell response of autoimmune and infectious diseases.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CL contributed to conceptualization of the study, performed experiments, analysed and interpreted data and wrote the manuscript. AA recruited patients, performed experiments and edited/reviewed the manuscript. AG discussed the results and edited/reviewed the manuscript. KH performed experiments, analysed data and edited/reviewed the manuscript. HS contributed to prediction of alternative splicing, initiated helpful discussions and edited/reviewed the manuscript. EM and NN discussed results and reviewed/edited the manuscript. RP and EOD supervised patient recruitment, performed clinical characterization, and reviewed/edited the manuscript MJ designed and supervised the study, interpreted data and wrote the manuscript. All authors approved the final version of the manuscript.

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Legends to Supplements

Supplementary Figure 1: Similar detection of mIL-7Rα proteins by different antibodies. HEK293T cells were transfected with different sIL-7Rα variants (N49Q, N65Q, Ile66, Thr66 and combinations hereof) or empty vector and incubated for 24h. Subsequently, cells were harvested and stained with monoclonal (clone HIL-7R-M21) or polyclonal antibody against IL-7Rα. Subsequently, cells were fixed and analysed by flow cytometry. Spearman correlation coefficient (rho) is shown.

Supplementary Figure 2: HEXplorer analysis of rs11567764 and rs6897932**.** HEXplorer analysis (39) was performed to predict potential enhancing and silencing properties of splicing events for the rs11567764G/A allele (upper part). rs6897932C/T is included for comparison. Arrows indicate site of mutation.

Supplementary Figure 3: O-glycosylation effects on sIL-7Rα gele mobility. Cell lysate from HEK293T cells transfected with different sIL-7Rα variants was treated with or without PNGase F and a mix of enzymes (O-Glycosidase, α2-3,6,8,9 Neuraminidase A, β-N-acetylhexosaminidase and β1-4 Galactosidase S) to trim and remove O-glycans. Gele mobility was determined by western blot analysis using a polyclonal antibody for IL-7Rα.

Supplementary Table 1: Primers used for sIL-7Rα, mIL-7Rα and minigene vectors as well as mutated variants.

Supplementary Table 2: Patients characteristics.

Supplementary Table 3: Association between *IL7RA* SNPs in tuberculosis patients and healthy household contacts**.** HIV-infected individuals (TB n = 16; Contacts n = 2) have been excluded from the analysis.

3.4 Paper IV: Novel methods for characterisation of autoimmunity-associated *IL7RA* haplotypes on soluble and membrane-associated IL-7Rα variants and IL-7-induced proliferation

Lundtoft, C., Jacobsen, M. (Preliminary manuscript). Novel methods for characterisation of autoimmunity-associated *IL7RA* haplotypes on soluble and membrane-associated IL-7Rα variants and IL-7-induced proliferation

Contribution to this manuscript: 90%

- Cloning and transfection/transduction
- Analysis of transfected/transduced cell lines
- Gene expression analysis
- Data analysis
- Writing of the manuscript

Novel methods for characterisation of autoimmunity-associated *IL7RA* haplotypes on soluble and membrane-associated IL-7Rα variants and IL-7-induced proliferation

Abstract

Signalling of IL-7 is critical for development and homeostasis of T cells, and *IL7RA* haplotypes have been associated with disease susceptibility. The *IL7RA* single-nucleotide polymorphisms (SNPs) rs1494558 and rs6897932 each tag a disease-associated haplotype, and functional effects of the SNPs in regard to alternative splicing, and to the levels of the soluble (s)IL-7Rα and membrane-associated (m)IL-7Rα have been described.

Here, two cell line-based assays were developed for evaluation of the combined effects of the two *IL7RA* SNPs on receptor expression and IL-7-signalling.

An IL-7Rα gene construct that included the complete introns surrounding exon 6 allowed analysis of mIL-7Rα and sIL-7Rα levels after alternative splicing, and functional effects on mIL-7Rα and sIL-7Rα levels were seen for both rs1494558 and rs6897932 when the construct was overexpressed in HEK293T cells. In a second assay, an IL-7-dependent cell line was established. Initial results demonstrated the functionality of the assay, which may prove useful for future analysis of the role of *IL7RA* SNPs on IL-7-signalling.

In conclusion, two cell line-based assays for analysing the role of *IL7RA* SNPs on IL-7Rα-expression and IL-7-signalling were developed. The assays may help highlighting the genetic relevance of *IL7RA* SNPs in IL-7-signalling.

Introduction

IL-7 is a cytokine central for development and homeostasis of T cells, which signals through a heterodimeric receptor complex formed by IL-7Rα (CD127) and the γ_c receptor (CD132) (Mazzucchelli and Durum, 2007). In addition to mIL-7Rα located on the cell membrane, a soluble receptor (sIL-7Rα) that is able to bind IL-7 is produced by alternative splicing (Goodwin et al., 1990, Rose et al., 2009). The transcript for sIL-7Rα lacks exon 6, which contains the transmembrane domain of the receptor. Due to a change in reading frame, an alternative 26-aa tail is formed followed by a premature stop codon (Rose et al., 2009).

The non-synonymous SNP rs6897932C>T (Thr244Ile) in exon 6 has been shown to affect splicing of *IL7RA*, and carrying the T-allele leads to reduced skipping of exon 6 (Gregory et al., 2007). Consequently, lower plasma levels of sIL-7Rα have been detected in individuals with the T-allele (Lundström et al., 2013). With reduced expression of the sIL-7Rα variant, increased expression of mIL-7Rα would be expected, but no effect of rs6897932 has been seen on the surface level of mIL-7Rα on T cells (Jäger et al., 2013, Willing et al., 2018).

The minor allele of the rs6897932 SNP has been associated with protection against autoimmune diseases such as type 1 diabetes (Todd et al., 2007) and multiple sclerosis (MS) (Gregory et al., 2007) in European populations. In addition to rs6897932, Gregory et al. (2007) identified an *IL7RA* haplotype associated with increased risk of MS. Recently, we demonstrated that the SNP rs1494558C>T (Thr66Ile) tagging the MS risk-haplotype affected the protein levels of mIL-7Rα and sIL-7Rα when overexpressed in

HEK293T cells (Lundtoft et al., In review). rs1494558 is a non-synonymous SNP located in exon 2 of *IL7RA*, and we observed higher levels of both mIL-7Rα and sIL-7Rα for the Thr66 variants as compared to the Ile66 variants (Lundtoft et al., In review).
Thereby two functional SNPs in the *IL7RA* gene with opposite effect on risk of disease are apparent. Still, the exact function of the individual SNPs on mIL-7Rα, sIL-7Rα and IL-7-signalling has only been explored to minor extent. Here, we aimed to develop cell line-based assays for combined evaluation of the *IL7RA* SNPs rs1494558 and rs6897932 on expression of IL-7Rα variants and on IL-7-signalling.

Methods

Cell culture and transfection

HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (high glucose, GlutaMAX, pyruvate) supplemented with 10% heat-inactivated FCS, 25 mM HEPES (all Thermo Fisher Scientific), 100 units penicillin/ml and 100 µg streptomycin/ml (Sigma Aldrich). Ba/F3 cells (an IL-3-dependent murine pro-B cell line, kindly provided by Prof. Dr. Jürgen Scheller) were cultured in complete (c)RPMI medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 mM HEPES, 2 mM L-Glutamine (all Thermo Fisher Scientific), 100 units penicillin/ml and 100 µg streptomycin/ml) and added 0.2% WEHI supernatant (i.e. supernatant from IL-3-producing WEHI-3 cell line, kindly provided by Prof. Dr. Jürgen Scheller). Transfections were performed in medium supplemented with 25 µM Chloroquine (Sigma Aldrich) using the Calcium Phosphate Transfection Kit (Sigma Aldrich).

Cloning of vectors

Human mIL-7Rα corresponding to the NCBI Reference Sequence (NM_002185.4) was cloned from human mRNA and inserted into the LeGO-iG2 vector (a vector that co-express eGFP downstream of an internal ribosome entry site (Weber et al., 2008)) using the EcoRI/NotI restriction enzyme sites. Human γ_c receptor corresponding to NCBI Reference Sequence (NM_000206.2) was cloned from human mRNA and inserted into LeGO-iBSD2 vector (eGFP replaced by blasticidin-S deaminase (BSD)) (Weber et al., 2008) using the BamHI/NotI restriction enzyme sites. Human splIL-7Rα, an mIL-7Rα construct containing the complete introns upstream and downstream of exon 6 (see Figure 1a) was generated from human mRNA and DNA by multiple PCR reactions and inserted into LeGO-iG2 using the BamHI-BglII/NotI restriction enzyme sites. The sequence corresponds to the NCBI Reference Sequences (NM_002185.4 for exons and NG_009567.1 for introns). A Kozak sequence (GCCACC) was introduced upstream of the start codon of the constructs. All restriction enzymes were from Thermo Fisher Scientific, and standard molecular cloning techniques were applied. For generation of rs1494558/rs6897932 mutants, PCR was performed with Phusion High-Fidelity PCR Master Mix with GC Buffer (New England BioLabs). The reactions were run for 30s at 98°C followed by 18 cycles of 7s at 98°C, 30s at 60°C and 8 min. at 72°C, followed by 10 min. at 72°C. Plasmid of bacterial origin was removed by DpnI digestion prior to transformation. Primers used for cloning and mutations are listed in Table 1, and successful cloning and mutation was verified by DNA sequencing.

Letiviral transduction of Ba/F3 cells

Lentivirus was produced by co-transfection of HEK293T cells with pMD2.G (addgene #12259, env, 1 µg), pMDLg/pRRE (addgene #12251, gag/pol, 2 µg), pRSV-Rev (addgene #12253, rev, 1 µg) and the

appropriate iG2 or iBSD2 transfer vectors (2.5 µg) in 6-well TC plates. Virus-containing supernatant was harvested on day 2, filtered and stored at -80°C. Lentiviral γ_c·iBSD2 or vector·iBSD2 supernatant was added to Ba/F3 cells in cRPMI supplemented with 8 µg/ml polybrene (Sigma Aldrich) and 0.2% WEHI supernatant. Cells were centrifuged at RT for 1h at 1,000g and subsequently transferred to 37°C, 5% CO_2. After 24h, medium was discarded and replaced with cRPMI supplemented with 0.2% WEHI supernatant, and successfully transduced cells were selected by 7d treatment with 5 µg/ml blasticidin S (Sigma Aldrich). Untransduced Ba/F3 cells were included as a negative control to verify the effect of blasticidin S.

Selected γ_c·iBSD2-transduced or vector·iBSD2-transduced cells were subsequently transduced with mIL-7Rα·iG2 or vector·iG2, and successfully transduced cells were sorted by fluorescence-activated cell sorting (MoFlo XDP, Beckman-Coulter, Core Flow Cytometry Facility at University Hospital Duesseldorf) based on their expression of eGFP, and subsequently incubated in cRPMI supplemented with 0.2% WEHI supernatant at 37°C, 5% CO_2.

Table 1 Primers used for cloning, mutation and PCR.

mIL-7Rα		**restriction enzyme site**	**sequence**	**bp**
mIL-7Rα	forward	EcoRI	GCGCGAATTCGCCACCATGACAATTCTAGGTACAACTTTTGGC	43
	reverse	NotI	GCGCGCGGCCGCTCACTGGTTTTGGTAGAAGCTGGA	36
γ_c (CD132)		**restriction enzyme site**	**sequence**	**bp**
γ_c (CD132)	forward	BamHI	GCGCGGATCCGCCACCATGTTGAAGCCATCATTACCATTC	40
	reverse	NotI	GCGCGCGGCCGCTCAGGTTTCAGGCTTTAGGGTG	34
splIL-7Rα		**restriction enzyme site**	**sequence**	**bp**
exon 1-5 (mRNA)	forward	BglII	GCGCAGATCTGCCACCATGACAATTCTAGGTACAACTTTTGGC	43
	reverse	-	CTGAGCTATTATTGATCTCTGG	22
exon 5-7 (DNA)	forward	-	CCAGAGATCAATAATAGCTCAG	22
	reverse	-	AAGATGTTCCAGAGTCTTCTTATG	24
exon 7-8 (mRNA)	forward	-	CATAAGAAGACTCTGGAACATCTT	24
	reverse	NotI	GCGCGCGGCCGCTCACTGGTTTTGGTAGAAGCTGGA	36
mutation		**restriction enzyme site**	**sequence**	**bp**
Ile66Thr	forward	-	GGACCCAGATGTCAACACCACCAATCTGGAATTTG	35
	reverse	-	CAAATTCCAGATTGGTGGTGTTGACATCTGGGTCC	35
Thr244Ile	forward	-	GGGGAGATGGATCCTATCTTACTAATCATCAGCATTTTGA	40
	reverse	-	TCAAAATGCTGATGATTAGTAAGATAGGATCCATCTCCCC	40
PCR IL-7Rα		**restriction enzyme site**	**sequence**	**bp**
exon 4-7	forward	-	GTGTCGTCTATCGGGAAGGAG	21
	reverse	-	AAGATGTTCCAGAGTCTTCTTATG	24

Counting of Ba/F3 cells

Ba/F3 cells were washed twice in cRPMI medium to remove residual IL-3 and subsequently the concentration of cells was determined using an LSRFortessa flow cytometer (BD Biosciences) by mixing 90 µl cell suspension with 10 µl 123count eBeads Counting Beads (eBioscience). Approximately 6,000 cells/well were added to a 96-well flat bottom plate in a final volume of 200 µl, and the surrounding wells were added PBS to avoid excessive evaporation. The exact number of cells added initially for the different variants was later used to calculate cell index. Human IL-7 (Sigma Aldrich) at 10 ng/ml was added to duplicate wells, one well was supplemented with 0.2% WEHI supernatant, and one well was left untreated. The plate was incubated at 37°C, 5% CO2. At day 3 and day 5, 100 µl cell suspension was

harvested for performing cell count, replacing the harvested cell suspension with 110 µl cRPMI containing the various stimuli on day 3.

Cell staining

Cells were harvested and stained with Viability Dye eFluor 780 (eBioscience) and mouse anti-human IL-7Rα AF647 (CD127, clone HIL-7R-M21, BD Biosciences) or rat anti-human CD132 (γ_c receptor, clone TUGh4, BD Biosciences). After staining, cells were fixed with Fixation Buffer (BioLegend) and analysed using an LSRFortessa flow cytometer (BD Biosciences). Normalisation of mIL-7Rα to transfection level has been described in Lundtoft et al. (In review).

Cytometric bead assay for sIL-7Rα

Measurement of sIL-7Rα concentrations from culture supernatants was performed as described earlier (Lundtoft et al., In review), using functional beads (Bead A4, BD Biosciences) conjugated with polyclonal anti-human IL-7Rα (R&D Systems). Standard (rhIL-7Rα-Fc Chimera Protein, R&D Systems) and samples were diluted in HEK medium and incubated with anti-IL-7Rα-conjugated beads. sIL-7Rα bound to the capture beads was detected using biotinylated mouse anti-human IL-7Rα (clone HIL-7R-M21, BD Biosciences) and Streptavidin PE (BioLegend). Beads were analysed on a BD LSRFortessa flow cytometer (BD Biosciences), and sIL-7Rα concentration was calculated from median PE values in GraphPad Prism version 6.07 using 4-parametric regression. Normalisation of sIL-7Rα to transfection level has been described in Lundtoft et al. (In review).

mRNA analysis of splIL-7Rα-transfected HEK cells

mRNA was isolated from HEK293T cells transfected with splIL-7Rα using the NucleoSpin RNA kit (Macherey-Nagel) with subsequent synthesis of cDNA using Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific). PCR was performed with Phusion High-Fidelity PCR Master Mix with GC Buffer (New England BioLabs) (see PCR primers in Table 1). The reactions were run for 30s at 98°C followed by 25 cycles of 7s at 98°C, 15s at 60°C and 45s at 72°C, followed by 8 min. at 72°C. PCR products were mixed with GelPilot 5x Loading dye (Qiagen) and separated on an 1% Agarose gel (BioBudget) in TBE buffer added 0.007% GelRed (Biotium) at 100V. Subsequently, the gel was analysed on a ChemiDoc Touch Imaging System (Bio-Rad).

Statistical analysis

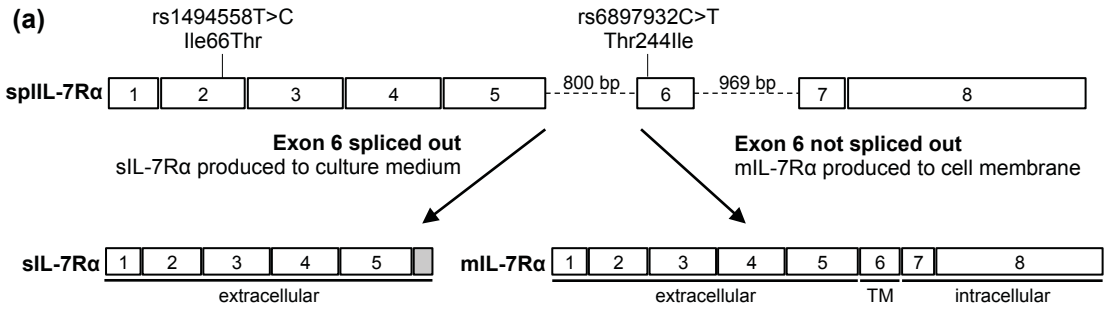
Data from flow cytometry was analysed in FlowJo version 10.1. Statistical analyses (ANOVA) were performed in R version 3.4.3. Plots were generated in GraphPad Prism version 6.07. Two-tailed p-values < 0.05 were considered significant.

Results

Generation of splIL-7Rα gene constructs

In order to evaluate the individual effects of rs1494558 and rs6897932 on production of mIL-7Rα and sIL-7Rα, a genetic construct containing the *IL7RA* exons and the entire introns surrounding exon 6 was generated (i.e. splIL-7Rα, Figure 1a). Inclusion of exon 6 would generate mIL-7Rα that can be detected on the cell surface, while skipping of exon 6 would lead to secretion of sIL-7Rα to the culture medium.

When introducing the genetic alleles into the construct by site-directed mutagenesis, four combinations representing simplified *IL7RA* haplotypes were obtained (Figure 1b).



(b)

	haplotype	rs1494558		rs6897932		EUR frequency (%)	MS association[1]
(1)	Ile66/Thr244	T	Ile66	C	Thr244	29.7	risk
(2)	Thr66/Thr244	C	Thr66	C	Thr244	42.6	reference
(3)	Ile66/Ile244	T	Ile66	T	Ile244	-	-
(4)	Thr66/Ile244	C	Thr66	T	Ile244	27.1	protective

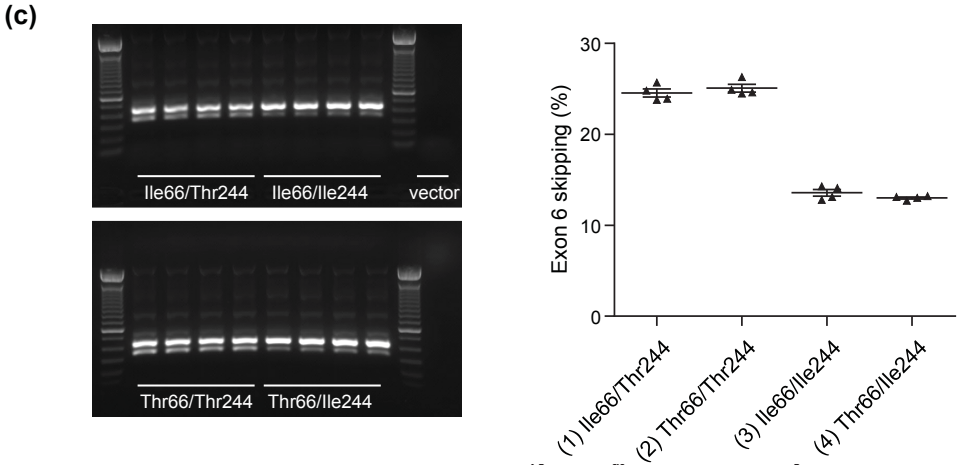


Figure 1 Generation of the splIL-7Rα construct

(a) An IL-7Rα construct containing the complete introns surrounding exon 6 was generated, adding rs1494558T>C and rs6897932C>T SNPs in exon 2 and exon 6, respectively, by site-directed mutagenesis. After transcription and splicing, inclusion of exon 6 generates the membrane-associated (m)IL-7Rα variant, whereas skipping of exon 6 leads to the soluble (s)IL-7Rα variant. Due to a change in reading frame, an alternative 26-aa C-terminal tail is generated on the sIL-7Rα variant (marked in grey). TM: Transmembrane.

(b) Four simplified 'haplotypes' are generated by introducing the rs1494558T>C and rs6897932C>T SNPs in the splIL-7Rα construct, in both cases leading to amino acid substitutions. Frequency of the haplotypes in EUR population combined from CEU, TSI, FIN, GBR and IBS populations (n = 503) is indicated (The Genomes Project, 2015). Note that haplotype (3) is presumably non-existing. [1]Association to multiple sclerosis (MS) based on Gregory et al. (2007).

(c) mRNA analysis of the splIL-7Rα construct expressed in HEK293T cells. *Left panel:* Two intense bands from PCR of exon 4-7 are seen, the upper band representing mIL-7Rα, the lower band representing sIL-7Rα. *Right panel:* Quantification of the expression of the sIL-7Rα variant (i.e. skipping of exon 6) relative to the total IL-7Rα expression. Mean ± SEM is indicated.

Analysis of mRNA from HEK293T cells transfected with splIL-7Rα constructs gave two intense bands representing mIL-7Rα and a 94 bp shorter sIL-7Rα transcript, respectively (Figure 1c). The lack of additional high-intensity bands indicated that splicing was complete, and that virtually no undesirable intron-retention took place. As expected, the relative expression of the transcript coding for sIL-7Rα was

reduced for the rs6897932T allele as compared to rs6897932C ($p < 0.001$), whereas no difference was seen for rs1494558 ($p = 0.95$) (Figure 1c). Overall, the splIL-7Rα construct appeared functional on the mRNA level when transfected into HEK293T cells.

Effect of IL7RA SNPs on IL-7Rα levels

Next, the secretion of sIL-7Rα to the culture medium from splIL-7Rα-transfected HEK293T cells was evaluated. Both SNPs were found to affect the level of sIL-7Rα secreted to the culture medium (rs1494558: $p < 0.001$; rs6897932: $p = 0.001$; test for interaction: $p = 0.55$) (Figure 2a). For rs1494558, higher levels were seen for the Thr66 variant, while for rs6897932, the C-allele (Thr244) associated with increased skipping of exon 6 also led to higher levels of sIL-7Rα (Figure 1c, Figure 2a).

For mIL-7Rα, a significant effect was seen for rs6897932, but not for rs1494558 (rs1494558: $p = 0.25$; rs6897932: $p < 0.001$; test for interaction: $p = 0.18$) (Figure 2b). For one of the two rs1494558 comparisons, however, a weak tendency towards increased mIL-7Rα was seen for the Thr66 variant ($p = 0.10$, Figure 2b). Again, the information on mRNA expression of rs6897932 was useful for prediction of the protein level of mIL-7Rα, since the rs6897932 T-allele (Ile244) – which showed reduced levels of sIL-7Rα – was associated with increased levels of mIL-7Rα on the cell surface (Figure 1c, Figure 2b). Overall, the minor allele of rs1494558 leads to increased sIL-7Rα and, in one case, a tendency towards an increased level of mIL-7Rα, while the minor allele of rs6897932 is associated with reduced sIL-7Rα and increased mIL-7Rα.

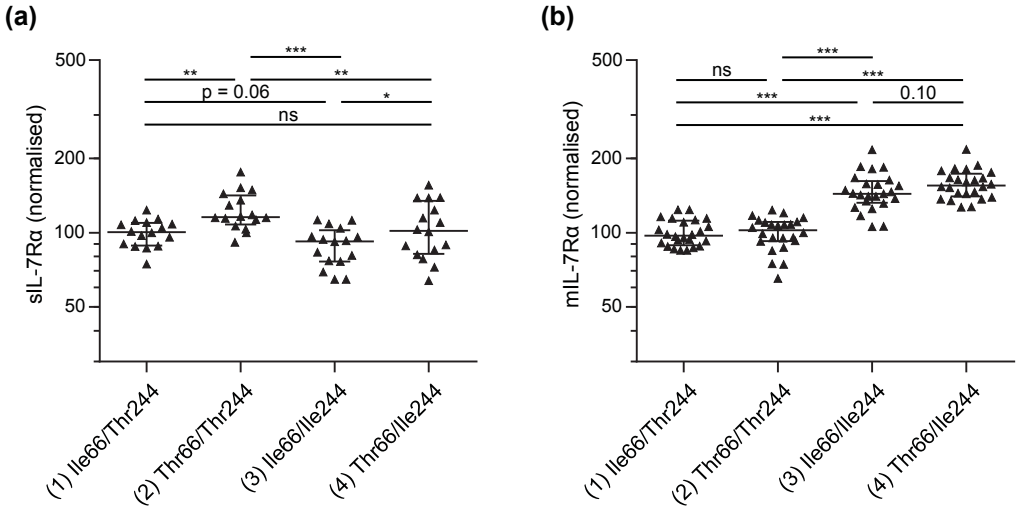


Figure 2 sIL-7Rα and mIL-7Rα protein levels from splIL-7Rα construct

(a) Level of sIL-7Rα in culture medium 24h after transfection of HEK293T cells with splIL-7Rα constructs. Concentration of sIL-7Rα was analysed by cytometric bead assay and has been normalised to the transfection level (geometric mean of cellular eGFP expression) and average sIL-7Rα in Ile66/Thr244 samples.

(b) Surface level of mIL-7Rα on $eGFP^+$ HEK293T cells 24h after transfection with splIL-7Rα constructs. mIL-7Rα level has been adjusted for transfection efficacy (geometric mean of mIL-7Rα / geometric mean of eGFP) and average mIL-7Rα in Ile66/Thr244 samples. Combined results from 4 (sIL-7Rα) and 6 (mIL-7Rα) independent experiments are shown, each point representing one individual transfection. Median and interquartile range is indicated, and log-transformed data has been analysed by ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant.

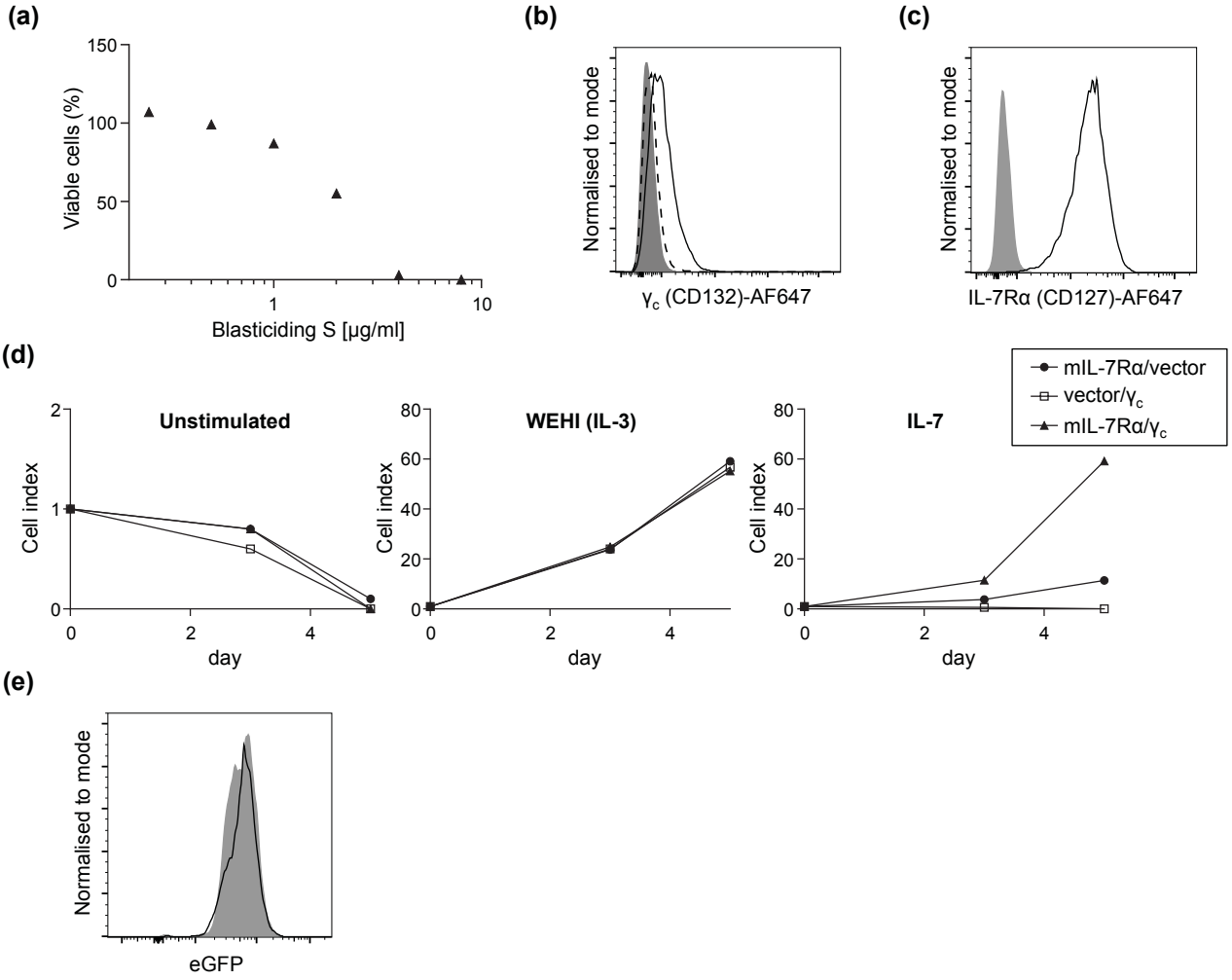


Figure 3 Generation of an IL-7-dependent cell line

(a) Titration of Ba/F3 cells with blasticidin S in a kill assay. Cells were counted by flow cytometry applying counting beads. Proportions of live cells relative to untreated sample are shown.

(b) Expression of γ_c on γ_c·iBSD2 (solid line) and vector·iBSD2 cells (dashed line) after 7d selection of cells with blasticidin S. An unstained γ_c·iBSD2 control is indicated in grey.

(c) Surface staining of IL-7Rα on mIL-7Rα-transduced cells (solid line) and vector-transduced cells (shaded grey).

(d) Proliferation of mIL-7Rα/γ_c, mIL-7Rα/vector, and vector/γ_c cells after no stimulation (left panel), or stimulation with WEHI supernatant (IL-3, middle panel) or IL-7 (right panel). Cell index relative to day 0 is shown. Cells were quantified by flow cytometry using counting beads.

(e) Expression of eGFP on mIL-7Rα/γ_c cells with the Thr66 variant (solid line) or Ile66 variant (shaded grey) after sorting of $eGFP^+$ cells. Geometric mean of eGFP: Thr66: 5,496; Ile66: 5,358.

Generation of an IL-7-dependent cell line

To analyse effects of *IL7RA* SNPs on IL-7-signalling, an IL-7-dependent cell line was established based on Ba/F3 cells, an IL-3-dependent murine pro-B cell line. First, the human γ_c receptor was inserted by lentiviral transduction. Ba/F3 cells that were successfully transduced with γ_c·iBSD2 or vector·iBSD2 were selected using blasticidin S (Figure 3a-b). Next, human mIL-7Rα·iG2 was inserted in the γ_c-expressing Ba/F3 cells, again by means of lentiviral transduction. $eGFP^+$ cells were sorted by fluorescence-activated cell sorting, and mIL-7Rα was detectable on the cell surface when compared to cells transduced with the empty vector (Figure 3c).

To verify the applicability of the generated Ba/F3 cell lines, the cells were incubated 5 days with IL-7, WEHI supernatant (IL-3) or without stimulation. In the absence of stimuli, all cells died, whereas all cell lines proliferated in the presence of WEHI supernatant (Figure 3d, left and middle panel). However, when stimulated with IL-7, only cells expressing human mIL-7Rα proliferated, whereas expression of human γ_c alone was no sufficient (Figure 3d, right panel).

Although both mIL-7Rα/γ_c and mIL-7Rα/vector cells proliferated in the presence of IL-7, a higher growth rate was seen for the former cell line. These results suggest that human IL-7 can signal through the murine γ_c receptor, which is likely expressed by Ba/F3 cells (Figure 3d).

It was noted, however, that sorting of the cells based on eGFP$^+$ expression led to slightly different levels of eGFP between the cell lines (Figure 3e). This would likely bias the results since expression of eGFP is expected to correlate with the expression of mIL-7Rα, and care should be taken in order to assure that generated cell lines are as similar as possible before comparison of cell growth. In summary, an IL-7-dependent cell line was generated, which may be useful in evaluating the role of exonic *IL7RA* SNPs in relation to IL-7-signalling.

Generation of splIL-7Rα-expressing Ba/F3 cells

In addition to an IL-7-dependent cell line expressing mIL-7Rα, a Ba/F3 cell line expressing splIL-7Rα was generated. sIL-7Rα from this cell line could be detected in the culture medium, and mIL-7Rα was expressed on the cell surface (data not shown). However, mRNA analysis of splIL-7Rα-transduced Ba/F3 cells with either rs6897932C or rs6897932T showed no difference in the level of exon 6 skipping ($p = 0.85$), suggesting that rs6897932 does not influence *IL7RA* exon 6 splicing in murine cells.

Discussion

Here, two assays were developed for evaluation of the impact of two *IL7RA* SNPs on alternative splicing and receptor expression. For the first assay, a splIL-7Rα gene construct was generated based on the *IL7RA* exons and further including the introns around exon 6. Overexpression of this construct in HEK293T cells permitted analysis of the effect of rs6897932 on exon 6 splicing. *IL7RA* minigenes including part of the introns surrounding exon 6 have already been applied for analysis of the effect of rs6897932C>T on exon 6-splicing at the mRNA level (Gregory et al., 2007, Lundtoft et al., In review), but the splIL-7Rα construct containing all exons allowed for concurrent analysis of sIL-7Rα and mIL-7Rα protein levels together with mRNA expression. Further, inclusion of rs1494558C>T in the sIL-7Rα construct made it possible also to evaluate the effect of simplified 'haplotypes' on both sIL-7Rα and mIL-7Rα.

As shown previously, the rs6897932 T-allele led to reduced secretion of sIL-7Rα, likely due to reduced skipping of exon 6 during splicing (Figure 2a) (Gregory et al., 2007, Lundström et al., 2013, Jäger et al., 2013). However, using the splIL-7Rα construct, it was demonstrated that expression of the rs6897932 T-allele led to increased levels of mIL-7Rα (Figure 2b), which may be explained by the reduced skipping of exon 6 during splicing, thereby leading to increased levels of mIL-7Rα transcript. In T cells, no effect has been seen of rs6897932 on mIL-7Rα (Jäger et al., 2013, Willing et al., 2018), although Jäger et al. noted a slightly increased mIL-7Rα in NK cells from donors homozygous for the T allele. Recently, however, Al-Mossawi et al. (2018) demonstrated that rs6897932T was associated with increased levels of mIL-7Rα on monocytes after lipopolysaccharide-induced upregulation of the receptor. Therefore, it is likely

that an effect of rs6897932 on mIL-7Rα may primarily be detectable after upregulation of IL-7Rα, but not in resting cells.

We have previously shown that the IL-7Rα Thr66 variant caused by the non-synonymous SNP rs1494558 led to increased sIL-7Rα and mIL-7Rα when the individual receptor constructs were overexpressed in HEK293T cells (Lundtoft et al., In review). Using the splIL-7Rα construct, the effect of Thr66 again led to increased sIL-7Rα relative to the Ile66 variant (Figure 2a), while the effect of the Thr66 variant was not found when evaluating mIL-7Rα (Figure 2b). Increased variation in the current study may account for the inconsistent results, but it is worth noting that the effect of rs1494558 on sIL-7Rα could be observed in serum samples from human donors (Seyfarth et al., 2018).

Additionally, an IL-7-dependent cell line was generated based on the murine Ba/F3 cells, which allowed analysis of IL-7-signalling in cell lines with a defined genotype or amino acid sequence of mIL-7Rα. Proliferation of the cell lines measured simply by counting the cells would likely reflect the level of IL-7Rα expression and binding affinity of IL-7, but more experiments are needed in order to verify these assumptions. However, initial results indicated a differential expression of eGFP between cell lines, which may bias the proliferation since the eGFP level is expected to correlate with mIL-7Rα expression. Therefore, care should be taken to ensure that results are not biased.

Finally, generation of IL-7-dependent cells expressing the splIL-7Rα construct may allow concurrent evaluation of the role of splicing and secretion of sIL-7Rα on IL-7-signalling. However, the lack of an effect of rs6897932 on exon 6 skipping – likely due to the murine origin of the cells – makes the cell line less suitable for evaluating the role of this specific SNP on IL-7-signalling.

In conclusion, two assays for analysis of *IL7RA* SNPs on receptor expression were developed, and these cell line-based assays may be a useful supplement to more specifically analyse the effect of *IL7RA* SNPs/haplotypes in human samples.

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4. Discussion

IL-7 is critical for balancing the immune response, and we showed that IL-7-signalling is impaired in patients with active tuberculosis (Lundtoft et al., 2017). Further, higher plasma IL-7 and lower sIL-7Rα was seen in tuberculosis patients as compared to contacts, and these levels normalised during treatment (Lundtoft et al., 2017). In addition, we identified two functional *IL7RA* SNPs that were associated with protection against tuberculosis (Lundtoft et al., In review). Analysis of a large group of patients with type 1 diabetes allowed for evaluation of the effect of haplotypes on the interplay between IL-7 and sIL-7Rα (Seyfarth et al., 2018). Together, these results add to the current knowledge on the role of IL-7 on immune regulation. Lastly, two cell line-based assays relevant for analysing the effect of *IL7RA* SNPs on receptor expression and IL-7-signalling were developed (Paper IV, Section 3.4), and these assays may become useful in future studies. Although the individual papers already included discussions on the findings, a few general points are highlighted and discussed collectively here.

4.1 Role of sIL-7Rα on availability of IL-7 and IL-7-signalling

Higher plasma concentration of IL-7 was detected in patients with active tuberculosis as compared to healthy contacts, while at the same time, IL-7-signalling was impaired in tuberculosis (Lundtoft et al., 2017). The increased level of IL-7 may either be explained by increased production of IL-7 from lymphoid tissues or due to lower consumption of IL-7. Plasma level of IL-7 has been shown to correlate negatively with $CD4^+$ cell count in HIV patients with low T cell number (i.e. < 270 $CD4^+$ cells/µl blood) (Napolitano et al., 2001), and although HIV-negative tuberculosis patients were found to have lower T cell numbers than healthy controls (Al-Aska et al., 2011, Jones et al., 1997), lymphopenia seen in tuberculosis is generally less severe than in HIV. Interestingly, increased plasma IL-7 and reduced plasma sIL-7Rα has been reported after haematopoietic stem cell transplantation with both levels normalising after successful engraftment, and where T cell number correlated negatively with plasma IL-7 (Kielsen et al., 2015, Kielsen et al., 2017). This suggests that low T cell numbers during lymphopenia can lead to lower consumption of IL-7 in plasma with a concurrent low production of sIL-7Rα from T cells. Further, a positive correlation has been seen between plasma sIL-7Rα and $CD8^+$ T cell counts after administration of IL-7 to rhesus macaques infected with simian immunodeficiency virus (Steele et al., 2017). However, since we did not analyse T cell number in blood, we were not able to show if lymphopenia was the causative factor for altered levels of IL-7 and sIL-7Rα in active tuberculosis (Lundtoft et al., 2017). An alternative explanation to the increased plasma levels of IL-7 may be a reduced consumption of the cytokine due to lower levels of mIL-7Rα on T cells found in tuberculosis patients (Lundtoft et al., 2017)

Two groups recently showed reduced levels of circulating IL-7 in tuberculosis patients from India (Kumar et al., 2017) and China (Bai et al., 2018), respectively, when compared to healthy controls, and for both patient groups, the level of IL-7 normalised during treatment. In addition, no differences were seen between patients with active tuberculosis and individuals with latent infection in the study by Kumar

et al. The reason for the contrasting results between these and own findings is unknown, but one explanation may be found in the definition of study groups, and also differential methods for detection of IL-7. Regional differences between hosts/pathogens and influence on IL-7 production could be another explanation (Mvubu et al., 2018).

Soluble receptors can add an extra layer of regulation in signalling, where some soluble receptors improve signalling, e.g. by avoiding clearance or degradation of a cytokine, while others may act as an antagonist and thereby inhibit signalling of its cognate cytokine (Heaney and Golde, 1996). Both roles have been shown for sIL-7Rα that – on the short term – can inhibit IL-7-signalling (Monti et al., 2013), while it prevents fast consumption or degradation of IL-7 and gives a more consistent signal on the longer term (Lundström et al., 2013). However, the lack of correlation between circulating IL-7 and sIL-7Rα suggests that sIL-7Rα has no or only a minor effect on the availability of IL-7 in plasma (Lundtoft et al., 2017, Seyfarth et al., 2018), and since sIL-7Rα is generally found in plasma in a >1000-fold molar excess relative to IL-7, the soluble receptor may still influence the ability of IL-7 to induce cellular signalling to some extent. The IL-7-dependent cell line developed in paper IV (Section 3.4) may be relevant for studying the effect of sIL-7Rα on IL-7-signalling, e.g. through addition of extrinsic sIL-7Rα to mIL-7Rα-expressing cells, or by using the splIL-7Rα cells that concurrently express intrinsic sIL-7Rα and mIL-7Rα.

4.2 rs6897932 and expression of sIL-7Rα and mIL-7Rα

The role of sIL-7Rα in immune regulation gained additional interest after the rs6897932 SNP associated with autoimmune diseases such as type 1 diabetes (Todd et al., 2007) and multiple sclerosis (Gregory et al., 2007) was shown to affect splicing of *IL7RA* and generation of sIL-7Rα (Gregory et al., 2007, Lundström et al., 2013). Here, the rs6897932 T allele associated with protection against autoimmune diseases led to reduced expression of sIL-7Rα, thereby resulting in lower plasma levels of sIL-7Rα (Jäger et al., 2013). Although we confirmed an effect of rs6897932 on sIL-7Rα in our studies (Lundtoft et al., 2017, Seyfarth et al., 2018), this SNP alone could not explain the differential plasma level of sIL-7Rα between tuberculosis patients and contacts. Further, the lack of an association between serum IL-7 levels and mIL-7Rα expression on T cells, as well as *in vitro* T cell-response to IL-7 or T cell-activation, also questions whether the only functional change of the rs6897932 SNP is related only to sIL-7Rα (Seyfarth et al., 2018).

Since changes in the total mRNA expression of IL-7Rα does not affect the ratio between expression of sIL-7Rα and mIL-7Rα (Rose et al., 2009), higher surface levels of mIL-7Rα would be expected on T cells from individuals carrying the rs6897932 T-allele. However, no effect of rs6897932 has been observed on the surface expression of mIL-7Rα on T cells until now (Jäger et al., 2013, Willing et al., 2018). Further, due to a low number of donors analysed for surface expression of mIL-7Rα on T cells in our studies, we were not able to evaluate the effect of rs6897932 on mIL-7Rα (Lundtoft et al., 2017, Seyfarth et al., 2018). Nevertheless, a recent study showed a higher upregulation of mIL-7Rα on

monocytes after *in vitro* stimulation with LPS for donors carrying the rs6897932 T allele (Al-Mossawi et al., 2018), suggesting that an effect of rs6897932 may only be seen during upregulation of mIL-7Rα and not when analysing cells in a resting state. Overexpression of the splIL-7Rα construct in paper IV (Section 3.4) also showed that rs6897932 affects the level of mIL-7Rα. Therefore, future studies should not only focus on the role of rs6897932 on sIL-7Rα, but also analyse the effect of the SNP on mIL-7Rα.

4.3 Genetic association studies on tuberculosis

We found two *IL7RA* SNPs (i.e. rs1494558 and rs11567764) that were associated with protection against active tuberculosis, and the fact that functional effects on expression and secretion of IL-7Rα could be linked to the two SNPs supports the relevance of the findings (Lundtoft et al., In review). Further, the association of rs1494558 – in part through the linkage disequilibrium with rs1494555 in European populations – with autoimmune disease (Gregory et al., 2007), graft-versus-host disease (Shamim et al., 2013), and plasma levels of sIL-7Rα (Seyfarth et al., 2018) further supports that rs1494558 can be a functional SNP that is associated with risk of active tuberculosis.

Despite indications for a genetic basis for active tuberculosis based on studies on monozygotic and dizygotic twins, only few disease-associated genetic regions have been identified in genome-wide association studies (Abel et al., 2018), and no association between *IL7RA* SNPs and active tuberculosis has been reported previously.

Generally, larger genetic diversity in African populations with lower linkage disequilibrium and shorter blocks complicates the identification of risk loci (Hill, 2012), leaving only three reports on tuberculosis-associated SNPs/genetic regions in populations from Ghana and The Gambia (Thye et al., 2012, Thye et al., 2010, Curtis et al., 2015). In addition, genome-wide association studies are generally less successful in infectious diseases (Hill, 2012), which in the case of tuberculosis may be even more challenging due to the different mycobacterial species and strains that vary between regions and continents (Intemann et al., 2009, Abel et al., 2018), potentially with different interaction between pathogen and host. Since only one of the six *IL7RA* SNPs genotyped in our study (i.e. rs6897932) (Lundtoft et al., In review) was included in the commercial genotyping chips applied in genome-wide association studies for tuberculosis, this genetic region may not have been covered thoroughly, even in case of genotype imputation (Thye et al., 2012).

Inclusion and exclusion criteria are of central importance in genetic case-control studies, and for studies on tuberculosis, patients with active tuberculosis can be compared to either healthy donors or individuals with latent *M. tuberculosis* infection. In our study, we included healthy household contacts as the control group (Lundtoft et al., In review), and the majority of the contacts showed a positive IFN-γ-response upon stimulation of PBMCs with protein-derivative from *M. tuberculosis* as performed for a subset of the contacts (Afum-Adjei Awuah et al., 2014). Further, the genetic variants associated with active disease may be more easily detectable if cases and controls are related, which is also the rationale used in the transmission disequilibrium test for family-based association studies. In previous genome-wide

association studies performed in African populations, the control groups were composed of unrelated neighbours, community members and infants from birth clinics (Intemann et al., 2009, Thye et al., 2012), and although identification of disease-associated regions possible in these studies, there is a general requirement for a high number of cases and controls in order to obtain statistically strong associations. From other infectious diseases, much has been learned from selecting infected hosts that show no disease and compare the genetic basis of these to that of patients with active disease rather than selecting non-infected donors as controls (Hill, 2012).

An alternative but more time-consuming approach for identification of risk-associated genes has been to perform a more focused and thorough analysis of disease-associated genes found in smaller studies or in other populations, or genes shown to be important in animal models of tuberculosis. This has in general been a more successful method for identification of tuberculosis-associated genes and genetic variants (Thye et al., 2006, Khor et al., 2007, Nejentsev et al., 2008, Cooke et al., 2008, Herb et al., 2008, Intemann et al., 2009, Thye et al., 2009). Therefore, the approach used here with initial analysis of the IL-7 signalling pathway that was found to be altered in active tuberculosis (Lundtoft et al., 2017) followed by a more detailed analysis of SNPs in *IL7RA* (Lundtoft et al., In review) may be a useful method for analysis of other relevant genetic associations with tuberculosis.

4.4 Downregulation of mIL-7Rα in active tuberculosis

mIL-7Rα was downregulated on $CD4^+$ and $CD8^+$ T cells in two independent groups of patients with active tuberculosis when compared to healthy contacts, but since no differences of IL-7Rα variants were detected on the mRNA level, the results indicated regulation of mIL-7Rα on the post-transcriptional level (Lundtoft et al., 2017). A similar pattern with downregulated mIL-7Rα has been seen for murine antigen-specific $CD8^+$ T cells in chronic LCMV infection as compared to acute infection with LCMV (Wherry et al., 2004), and also in HIV-infected patients (Rethi et al., 2005). Further, $CD8^+$ T cells showed an impaired response to IL-7-stimulation and a compromised induction and homeostasis of memory T cells in chronic LCMV infection (Wherry et al., 2004). Therefore, the reduced level of mIL-7Rα with impaired IL-7-response in active tuberculosis may resemble the situation seen in chronic viral infections, although some degree of chronic infection may also persist in individuals with latent *M. tuberculosis* infection.

Lang et al. (2005) described a permanent downregulation of mIL-7Rα on antigen-specific $CD8^+$ T cells with reduced viability during persistent antigen stimulation, whereas stimulation with short-lived antigen led to a temporary downregulation of mIL-7Rα and generation of memory response. In tuberculosis, the proportion of mIL-7Rα^+ cells among $CD4^+$IFN-γ^+ cells (Pollock et al., 2013) and $CD8^+$IFN-γ^+ cells (Day et al., 2014) after stimulation with *M. tuberculosis*–proteins was lower for patients with active tuberculosis than for individuals with latent infection. Although the mRNA expression of the exhaustion markers programmed cell death protein 1 (PD-1) and suppressor of cytokine signalling 3 (SOCS3) did not differ between $CD4^+$ cells in patients with active tuberculosis and healthy contacts, the differential

surface level of mIL-7Rα on T cells may be due to chronic infection with increased and persistent antigen stimulation in the case of active tuberculosis (Lundtoft et al., 2017, Wherry and Kurachi, 2015).

4.5 Effect of IL-7Rα glycosylation

Expression of IL-7Rα deficient in N-glycosylation on position Asn49 and Asn65 showed that glycosylation of these two positions improve secretion of sIL-7Rα and trafficking of mIL-7Rα to the membrane (Lundtoft et al., In review). The importance of optimal N-glycosylation for receptor trafficking has also been shown for other members of the type I receptor family, i.e. IL-21 receptor, γ_c receptor, IL-6 receptor β (gp130), and thrombopoietin receptor (Hamming et al., 2012, Siupka et al., 2015, Waetzig et al., 2010, Albu and Constantinescu, 2011).

In addition to N-glycosylation of IL-7Rα at Asn49 and Asn65, a Thr66Ile substitution caused by rs1494558 also led to altered amounts of sIL-7Rα and mIL-7Rα, with higher levels seen for the Thr66 variant (Lundtoft et al., In review). As neither N-glycosylation of Asn65 nor O-glycosylation of Thr66 were the causative factors for the changes in the receptor levels, the physical properties of the amino acid substitution itself may be the reason for higher IL-7Rα levels for the receptor with the hydrophilic Thr66 as compared to the hydrophobic Ile66 (Lundtoft et al., In review). This explanation is also in line with a need for a hydrophilic force in the area facing away from the IL-7-binding site in the D_1 domain, which is added by N-glycosylation of Asn65.

In addition to a potential local hydrophilic effect, the crystal structure of the IL-21 receptor produced in HEK293 cells revealed a 'sugar bridge' between the N-glycosylation site in the D_1 domain and a C-mannosylated WSXWS motif in the D_2 domain, which is thought to sustain proper folding and trafficking of the receptor (Siupka et al., 2015). It is tempting to speculate if such a 'sugar bridge' is also present in IL-7Rα and other members of the type I cytokine receptor family, which will be an interesting aspect of future studies.

Not only does glycosylation affect trafficking of IL-7Rα, but McElroy et al. (2009) also reported a ~300-fold stronger binding of IL-7 to glycosylated IL-7Rα (K_d ~60 nM) than to non-glycosylated IL-7Rα (K_d ~18 μM). It was shown that neither the size nor the complexity of the glycans was important for the binding, since treatment of the glycosylated receptor with the enzyme Endo H that removes all but one sugar unit from the glycosylation sites had no effect on the binding affinity. On the other hand, glycosylated IL-7Rα treated with PNGase F to remove the entire N-glycans had a similar binding affinity to IL-7 as had non-glycosylated IL-7Rα produced in *E. coli*, indicating that the altered binding was not due to different protein expression systems (McElroy et al., 2009). However, the contribution of individual N-glycosylation sites to the affinity between IL-7 and IL-7Rα remain unknown, but the crystal structure of glycosylated IL-7Rα bound to IL-7 did not show any interaction between the cytokine and N-glycans on the receptor, suggesting that the effect of glycosylation is due to an intrinsic effect on IL-7Rα conformation or stability (McElroy et al., 2009). We did not evaluate the binding affinities of the IL-7Rα variants deficient in N-glycosylation in our study (Lundtoft et al., In review), but this would be a relevant

objective for a future study. Further, it would be interesting to see whether the binding affinity of IL-7 to IL-7Rα is changed by the Thr66Ile substitution caused by rs1494558. The IL-7-depending cell line assay developed in paper IV (Section 3.4) may also be a useful approach for evaluating these questions.

4.6 Outlook

It was here shown that IL-7-signalling was impaired in patients with active tuberculosis. Further, two functional *IL7RA* SNPs were associated with protection against tuberculosis, and the interplay between *IL7RA* SNPs, IL-7Rα levels and circulating IL-7 was evaluated. Together, these results highlight aspects of the role of IL-7 in autoimmune and infectious diseases. In addition, two cell line-based assays were developed, and these may become useful in future evaluation of the effect of *IL7RA* SNPs on alternative splicing, receptor expression and IL-7-signalling.

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7. Statutory declaration

I declare under oath that I have compiled my dissertation independently and without any undue assistance by third parties under consideration of the 'Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf'.

The dissertation has not been presented to other faculties, nor do I have any record of previous successful or unsuccessful attempts to obtain a doctorate.

_____

Name

_____

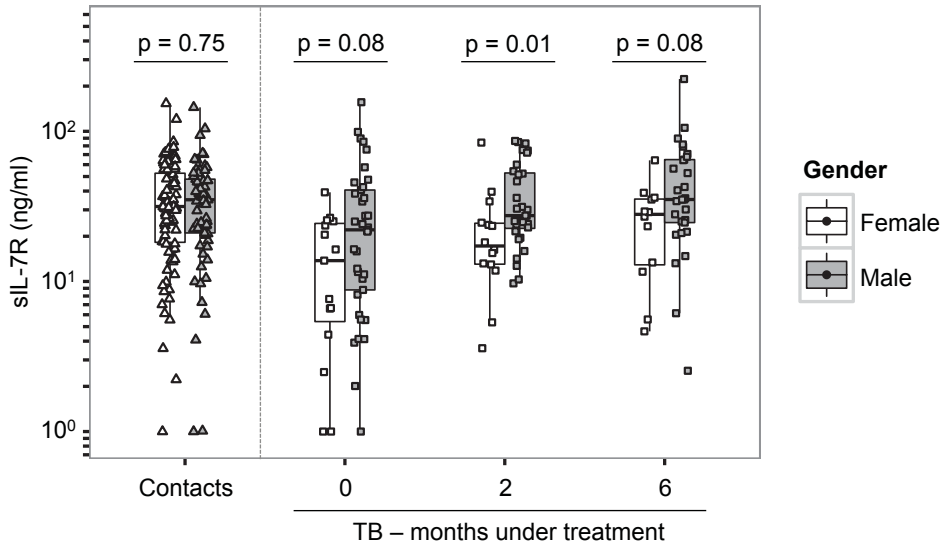
Date / Signature

S1 Table

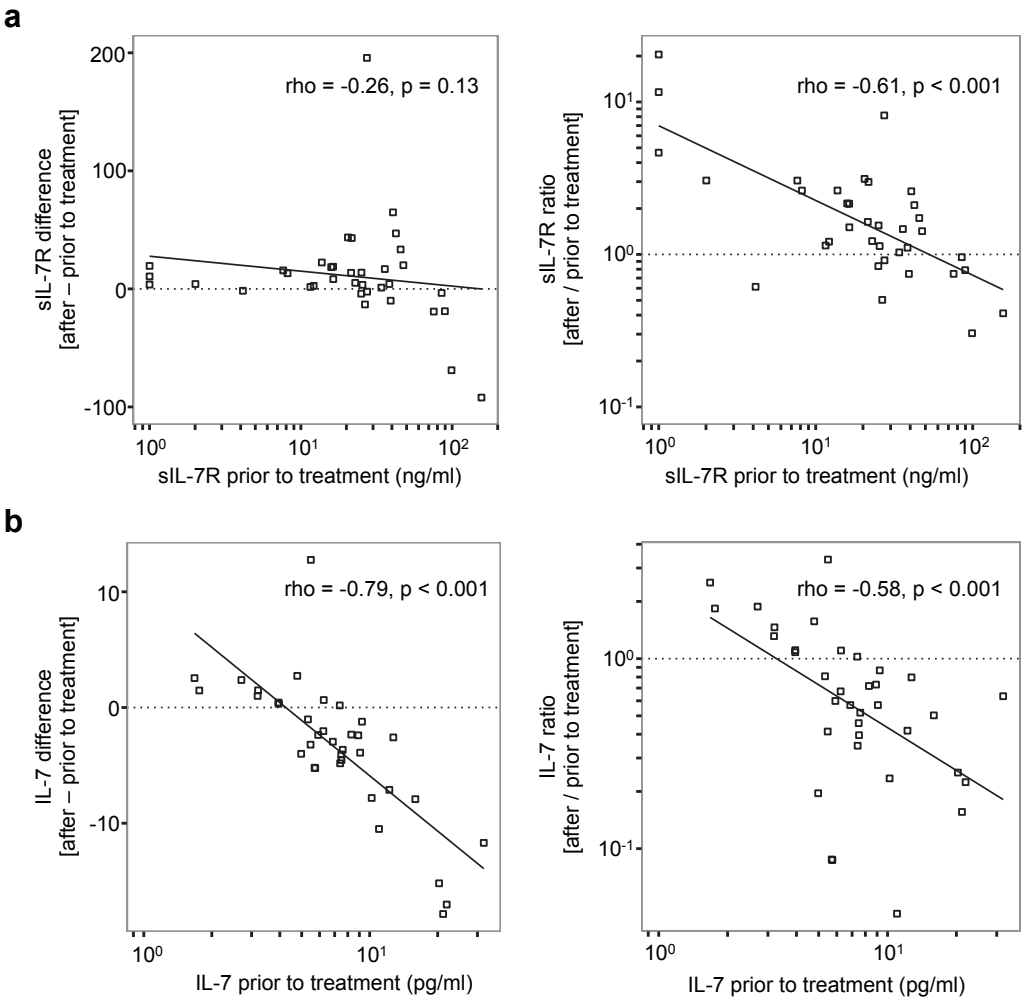
			Contacts		TB	
			rho	p-value	rho	p-value
$CD4^+$	IL-7	MFI IL-7R	-0.38	0.10	-0.01	0.97
		$IL\text{-}7R_{low}$ cells	0.27	0.25	-0.14	0.59
	sIL-7R	MFI IL-7R	0.42	0.06	-0.04	0.88
		$IL\text{-}7R_{low}$ cells	-0.26	0.26	-0.19	0.46
$CD8^+$	IL-7	MFI IL-7R	-0.17	0.46	0.30	0.25
		$IL\text{-}7R_{low}$ cells	0.17	0.47	-0.13	0.62
	sIL-7R	MFI IL-7R	0.19	0.40	0.11	0.67
		$IL\text{-}7R_{low}$ cells	-0.32	0.15	0.00	0.99

S1 Table: Correlation between mIL-7R expression on T cells and plasma IL-7 or sIL-7R concentrations. Association between mIL-7R in $CD4^+$ and $CD8^+$ cells from tuberculosis patients (n = 17) and healthy contacts (n = 21) and plasma IL-7 or plasma sIL-7R concentrations. Mean fluorescence intensity (MFI) analyses of IL-7R and proportions of $IL\text{-}7R_{low}$-expressing cells were evaluated by flow cytometry. Spearman rank correlation analysis was performed.

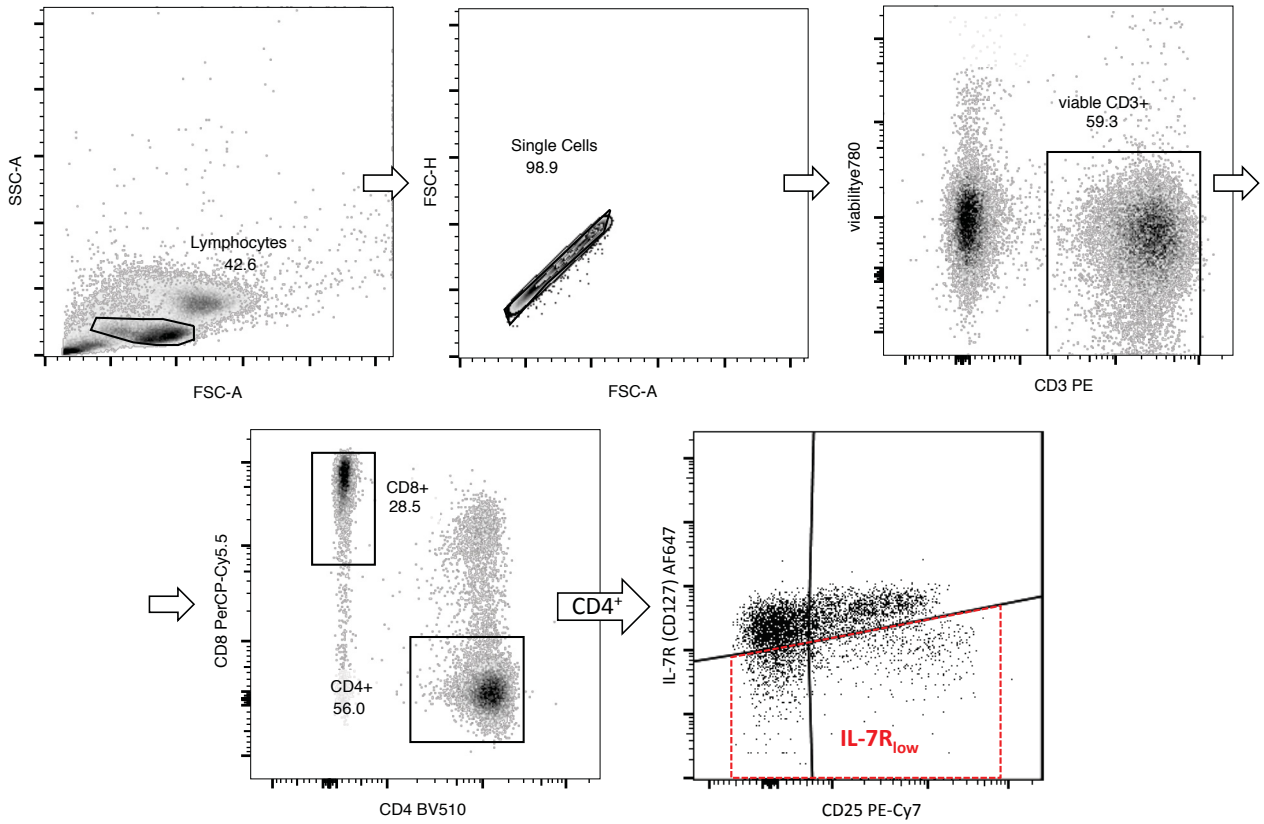
S1 Fig



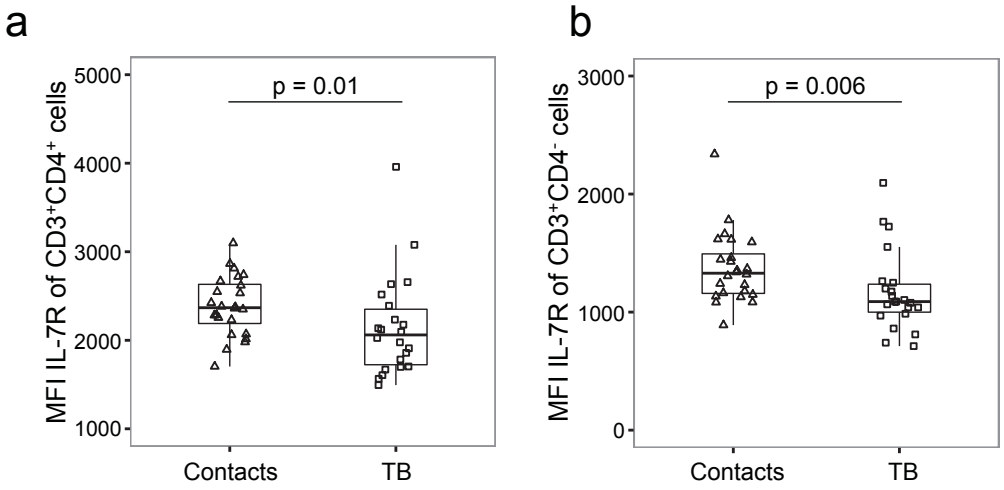
S2 Fig


a
rho = -0.26, p = 0.13
sIL-7R difference [after – prior to treatment]
sIL-7R prior to treatment (ng/ml)
rho = -0.61, p < 0.001
sIL-7R ratio [after / prior to treatment]
sIL-7R prior to treatment (ng/ml)
b
rho = -0.79, p < 0.001
IL-7 difference [after – prior to treatment]
IL-7 prior to treatment (pg/ml)
rho = -0.58, p < 0.001
IL-7 ratio [after / prior to treatment]
IL-7 prior to treatment (pg/ml)

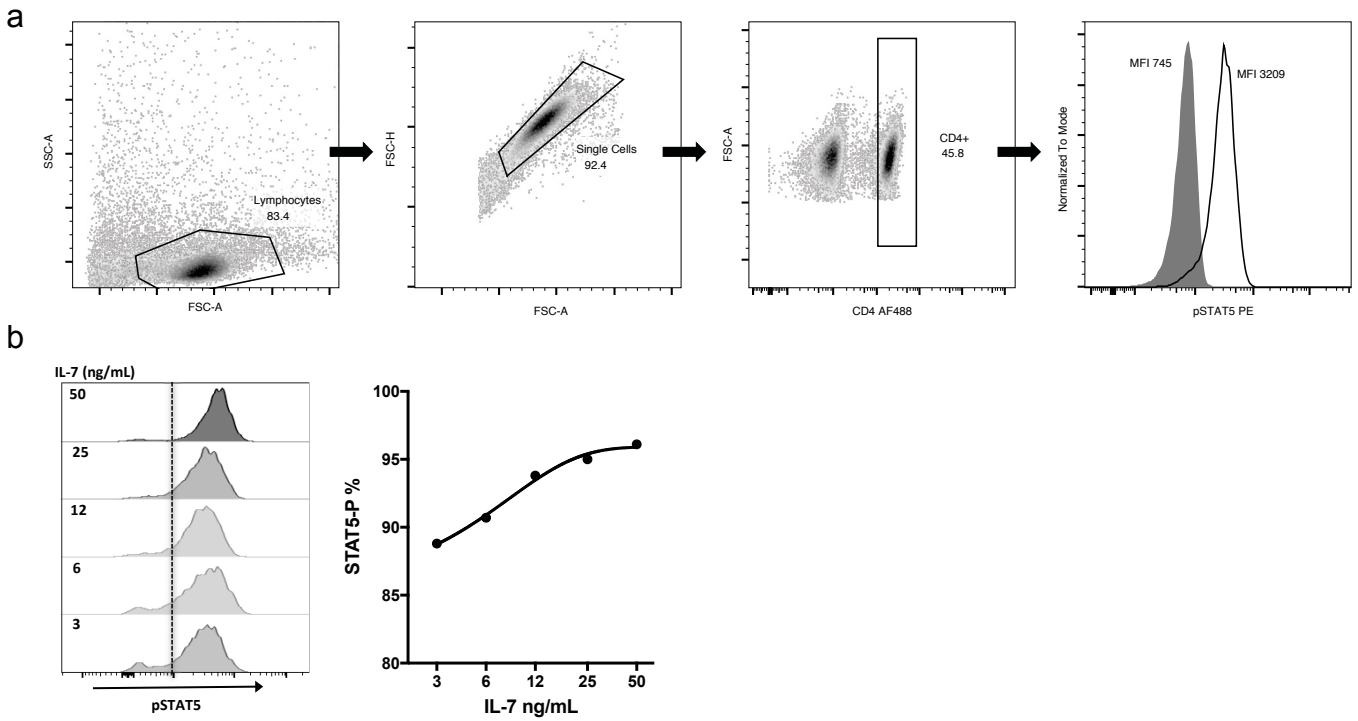
S3 Figure



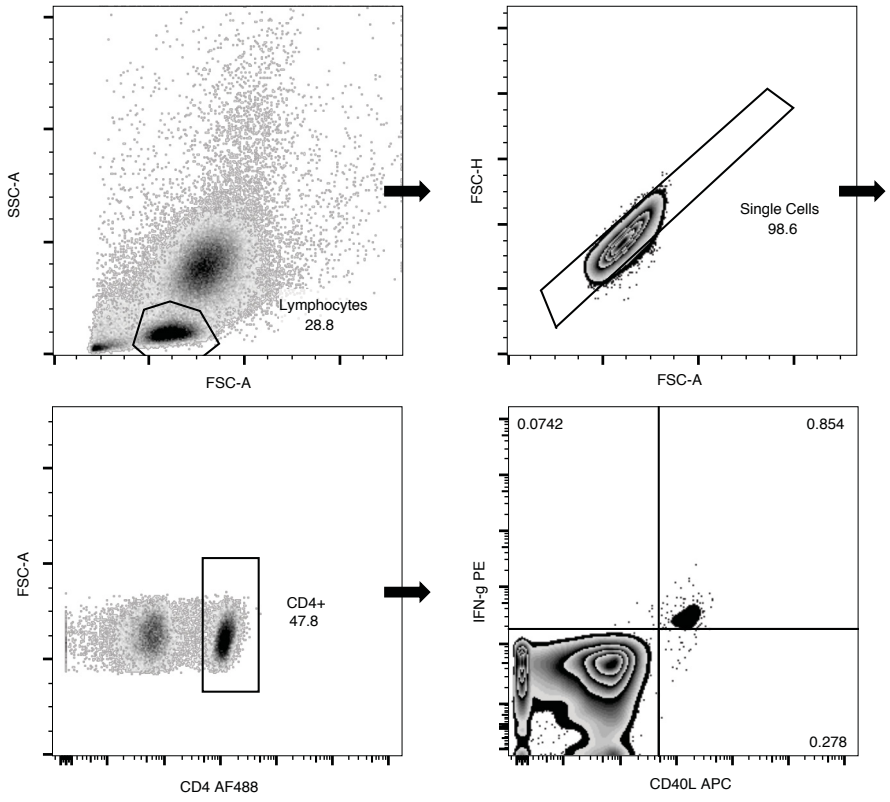
S4 Fig



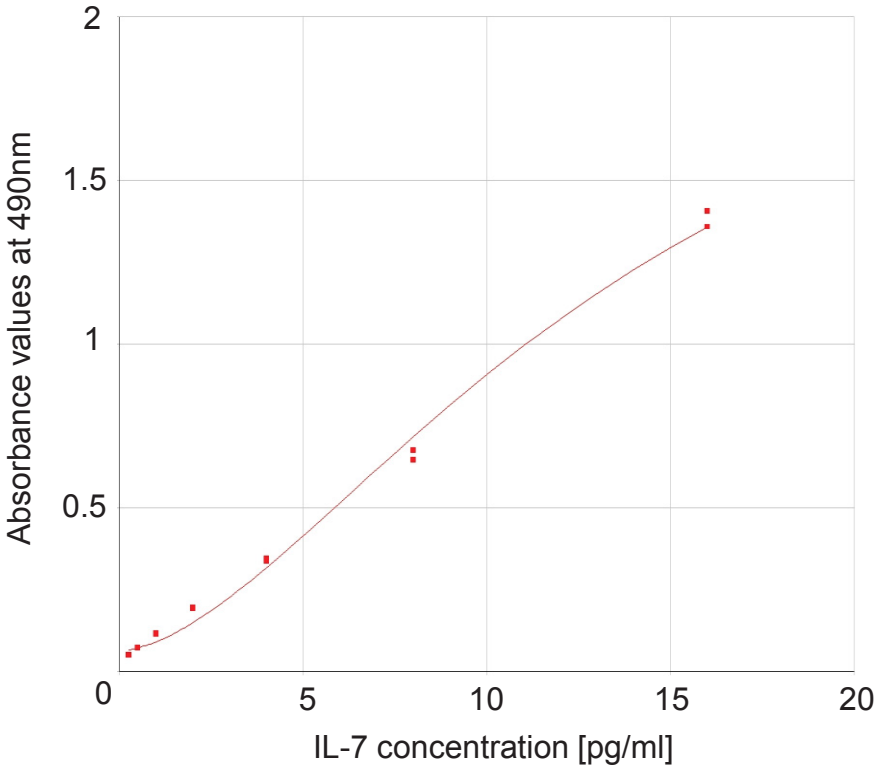
S5 Fig


a
SSC-A
FSC-A
Lymphocytes
83.4
FSC-H
FSC-A
Single Cells
92.4
FSC-A
CD4 AF488
CD4+
45.8
Normalized To Mode
pSTAT5 PE
MFI 745
MFI 3209
b
IL-7 (ng/mL)
50
25
12
6
3
pSTAT5
STAT5-P %
100
95
90
85
80
3
6
12
25
50
IL-7 ng/mL

S6 Fig

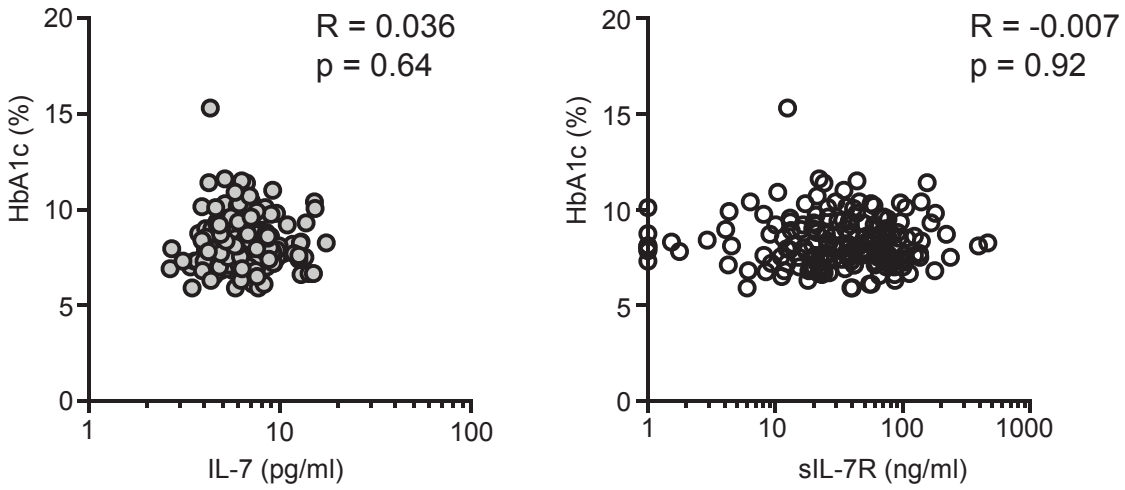

SSC-A
FSC-A
Lymphocytes
28.8
FSC-H
FSC-A
Single Cells
98.6
FSC-A
CD4 AF488
CD4+
47.8
IFN-g PE
CD40L APC
0.0742
0.854
0.278

Supplementary Figure 1



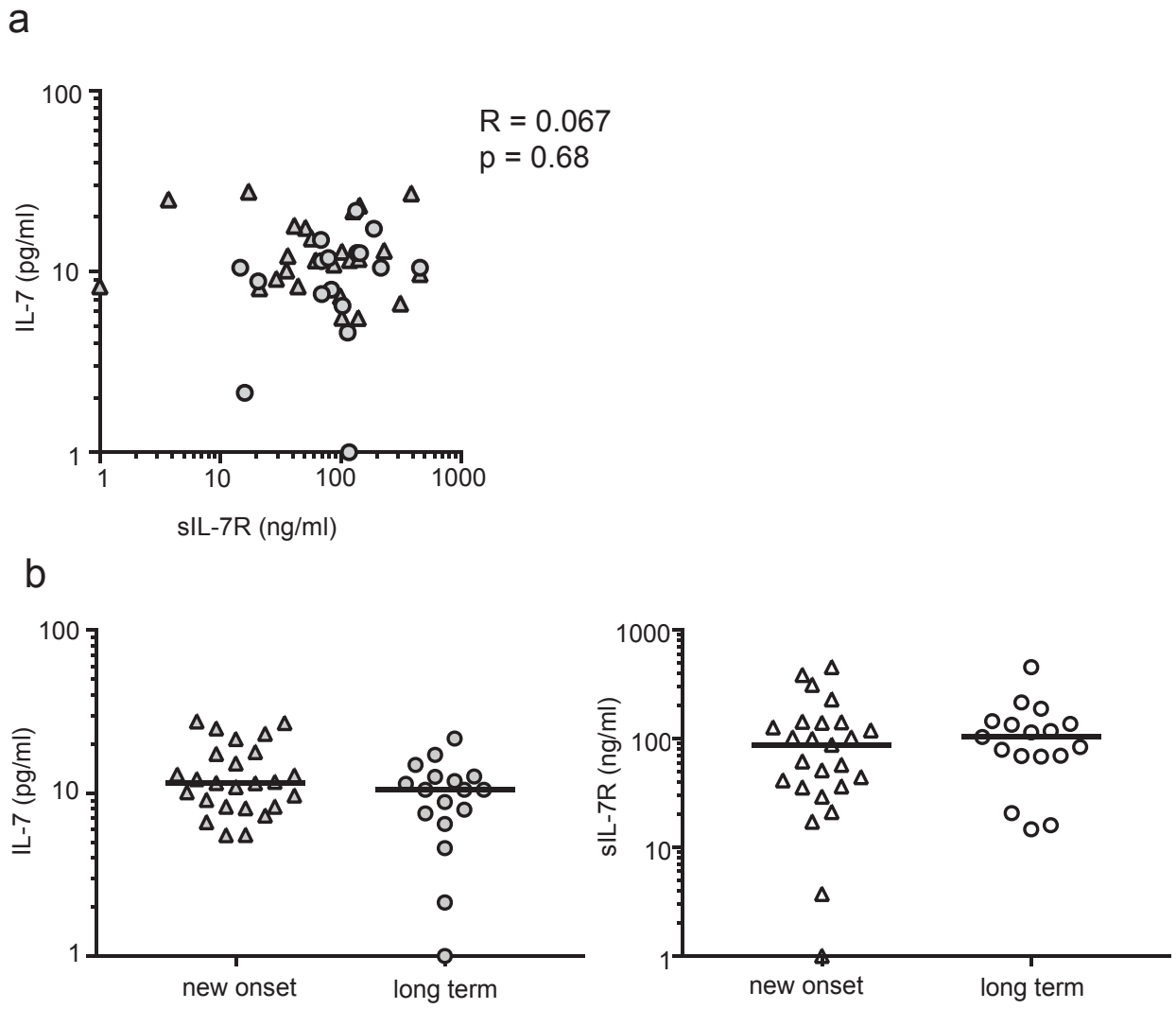
Representative human IL-7 Quantikine HS ELISA kit standard curve fitted by 4-parametric logistic regression as recommended by the manufacturer.

Supplementary Figure 2



Correlation of HbA1c (%) with serum IL7 (left graph, n=173) and s-IL7R (right graph, n=181)
Every symbol represents data of an individual donor. Spearman`s rank correlation coefficients R and the respective p-values are indicated.

Supplementary Figure 3



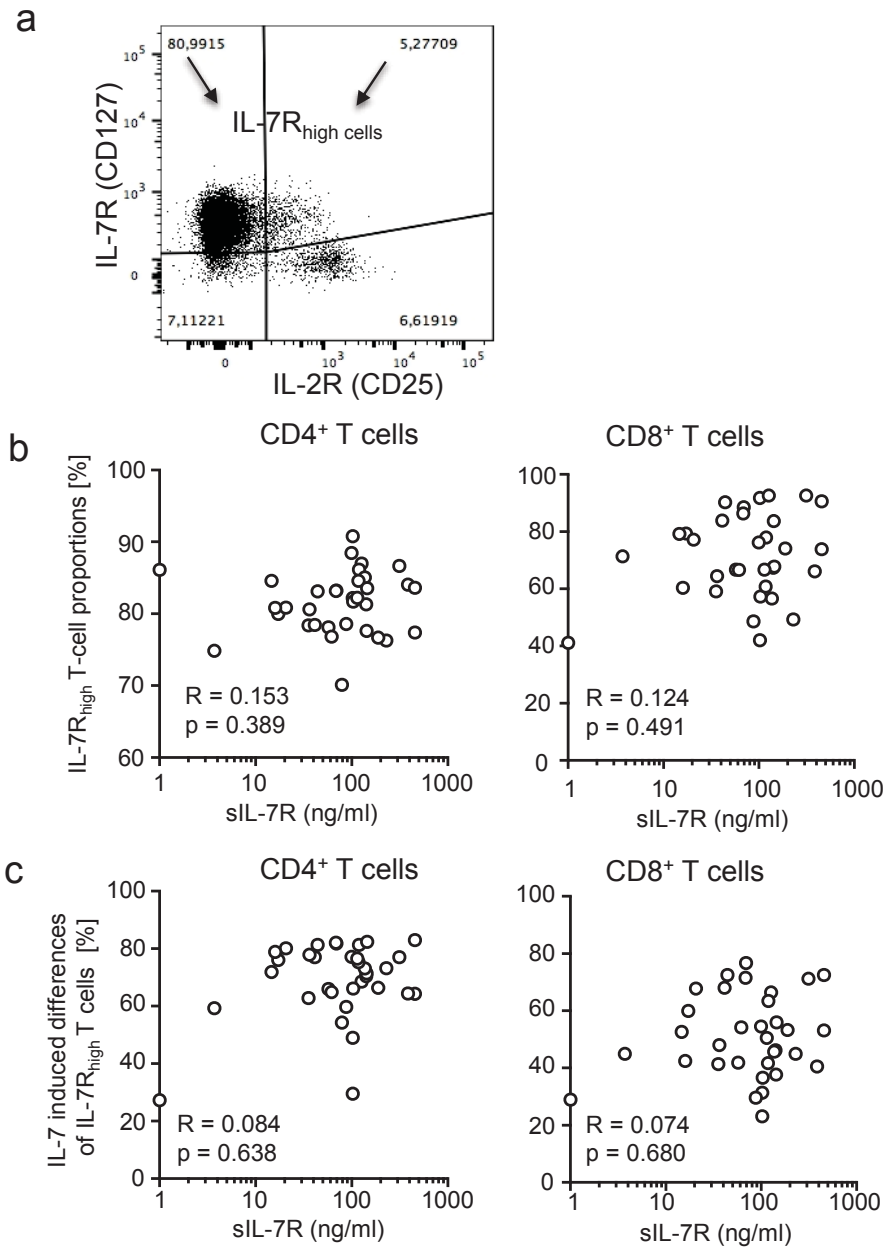
(a) Correlation of serum IL-7 and sIL-7R in the second cohort of T1D patients (n = 42).

Every symbol represents data of an individual donor (new onset patients: triangle, long term patients: circle). Spearman`s rank correlation coefficient R and the respective p-value are indicated.

(b) Comparison of serum IL-7 (left graph) and sIL-7R (right graph) between patients at onset of disease (new onset, n=25) and at least 6 months after clinical onset of disease (long term, n=17).

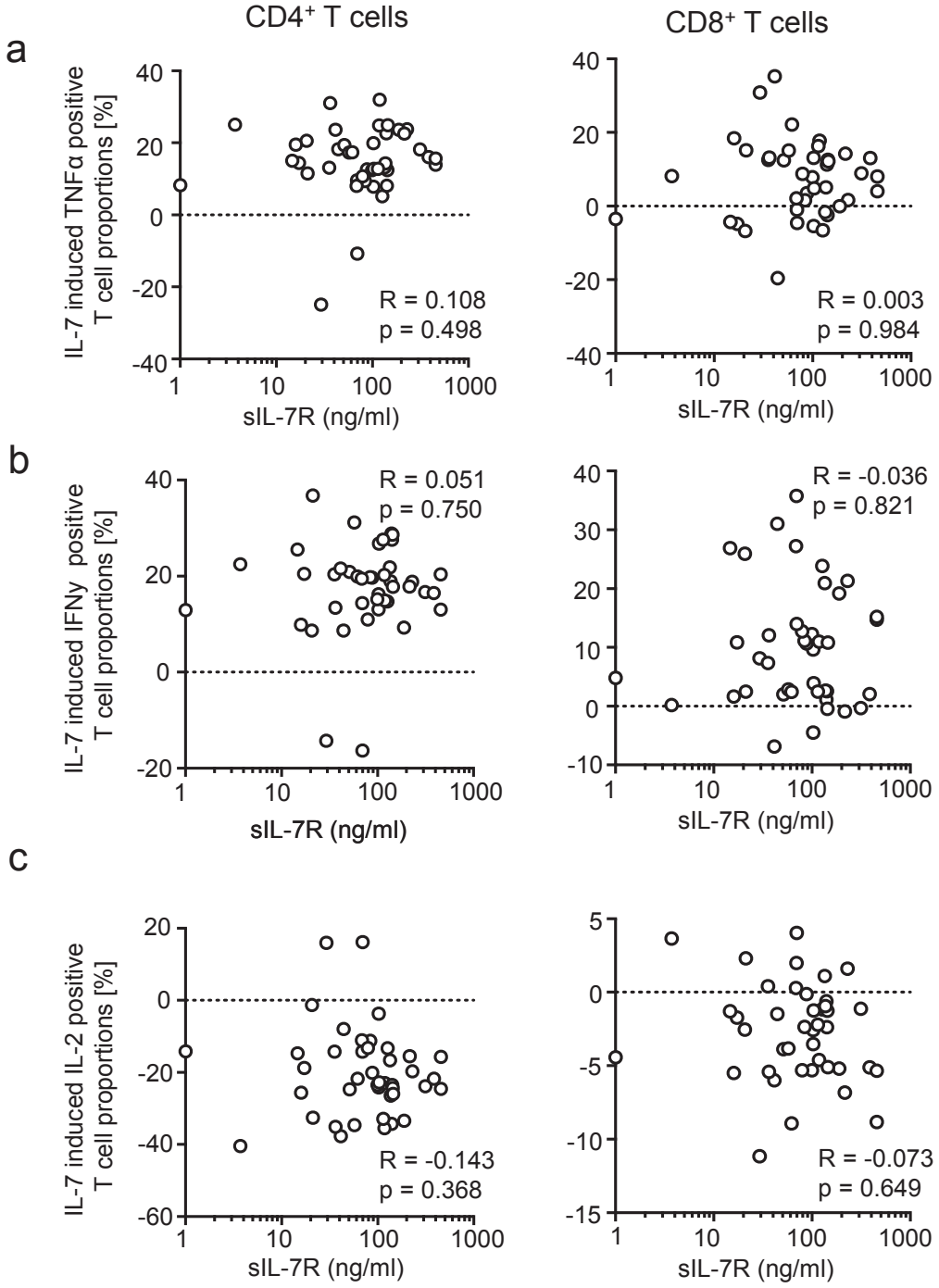
Each symbol represents data of an individual donor. Median values for groups are indicated.

Supplementary Figure 4



(a) Representative example of a flow cytometry dot plot for gating of IL-7R$_{high}$ on CD4 T cells. (b) Proportion of IL-7R$_{high}$ expressing CD4$^+$ (left graph) and CD8$^+$ T cells (right graph) in the second cohort of T1D patients (n = 33) were correlated with sIL-7R. Spearman`s rank correlation coefficient R and the respective p-values are indicated. (c) Differences of IL-7R$_{high}$ cells between IL-7 non-treated and IL-7 treated samples were correlated with sIL-7R in CD4$^+$ (left graph) and CD8$^+$ T cells (right graph) . Spearman´s rank correlation coefficient R and the respective p-values are indicated.

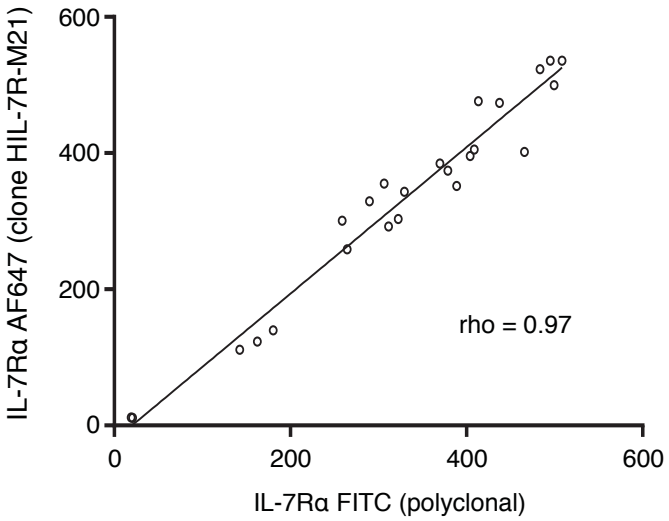
Supplementary Figure 5



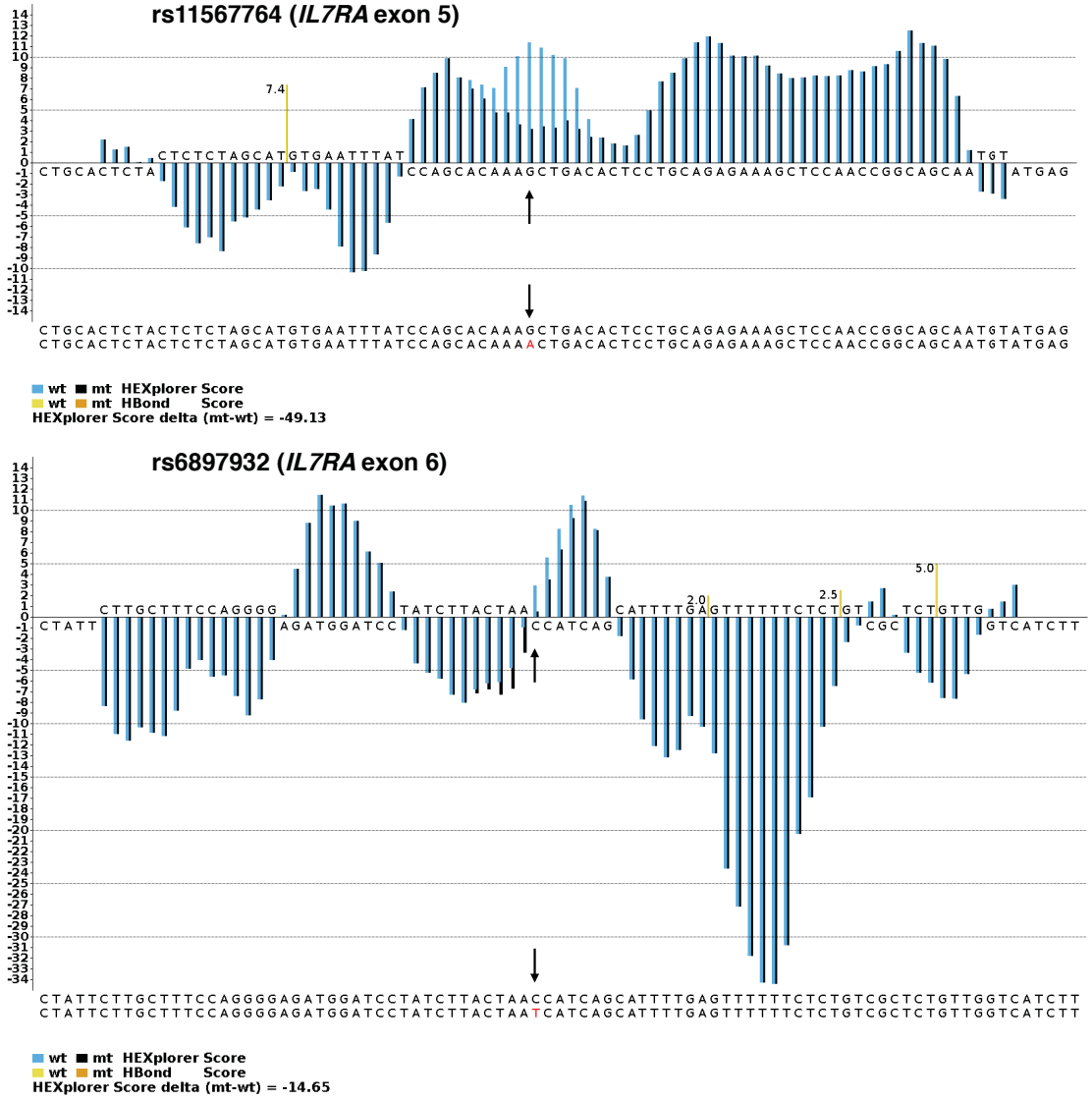
Correlations of IL-7 induced proportions of TNFα (a), IFNγ (b) and IL-2 (c) producing memory CD4+ (left graphs) and CD8+ (right graphs) T cells in T1D patients (n = 41) with sIL-7R. Spearman´s rank correlation coefficient R and the respective p-values are indicated.

Supplementary information | Paper III

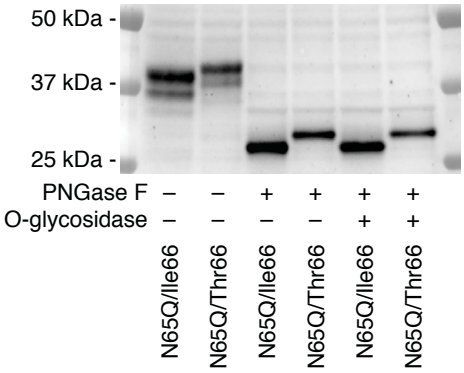
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Table 1

IL-7Rα		restriction enzyme site	sequence	bp
sIL-7Rα	forward	BglII	GCGCAGATCTGCCACCATGACAATTCTAGGTACAACTTTTGGC	43
	reverse	NotI	GCGCGCGGCCGCTCACTGGTTTTGGTAGAAGCTGGA	36
mIL-7Rα	forward	EcoRI	GCGCGAATTCGCCACCATGACAATTCTAGGTACAACTTTTGGC	43
	reverse	NotI	GCGCGCGGCCGCTCACTGGTTTTGGTAGAAGCTGGA	36

minigene		restriction enzyme site	sequence	bp
IL7RA exon 1	forward	BglII	GCGCAGATCTGCCACCATGACAATTCTAGGTACAACTTTTGGC	43
	reverse	XbaI/XhoI	CTCGAGGTTGTCTCTAGACTACACTTGGGAGTGAAATGC	39
IL7RA exon 7	forward	XbaI/XhoI	TCTAGAGACAACCTCGAGCCTTGGTGTTCCTTCCCTC	37
	reverse	NotI	GCGCGCGGCCGCTTATTTTCTTGGTTTCTTACAAAGATG	39
IL7RA exon 5	forward	XbaI	GCGCTCTAGAGGATAGAGAGAAAAGAAGAAG	31
	reverse	XhoI	GCGCCTCGAGCCAGGCTAAATTGGTTAACTGGACG	35
IL7RA exon 6	forward	XbaI	GCGCTCTAGACATCATCTCCTGCTCTGGCTTC	32
	reverse	XhoI	GCGCCTCGAGCAAGTACATTGTGCATTTGCC	31

IL-7Rα mutation		restriction enzyme site	sequence	bp
N49Q	forward	-	TATAGCCAGTTGGAAGTGCAGGGATCGCAGCACTCACTG	39
	reverse	-	CAGTGAGTGCTGCGATCCCTGCACTTCCAACTGGCTATA	39
N65Q	forward	-	CTTTTGAGGACCCAGATGTCCAGATCACCAATCTGGAATTTGA	43
	reverse	-	TCAAATTCCAGATTGGTGATCTGGACATCTGGGTCCTCAAAAG	43
N65Q/Ile66Thr	forward	-	CTTTTGAGGACCCAGATGTCCAGACCACCAATCTGGAATTTGA	43
	reverse	-	TCAAATTCCAGATTGGTGGTCTGGACATCTGGGTCCTCAAAAG	43
Ile66Thr	forward	-	GGACCCAGATGTCAACACCACCAATCTGGAATTTG	35
	reverse	-	CAAATTCCAGATTGGTGGTGTTGACATCTGGGTCC	35

Supplementary Table 2

	Household contacts	TB
Number of subjects	170	114
Age (y)	35 [18-68]	31 [1-72]
Gender		
Female	97 (57%)	41 (36%)
Male	73 (43%)	73 (64%)
HIV+	2 (1%)	16 (14%)

Median [range] or number (proportion) is shown.

Supplementary Table 3

		Frequency (%)		Codominant		Dominant		Recessive	
SNP	**Genotype**	**Contacts**	**TB**	**OR**	**p-value**	**OR**	**p-value**	**OR**	**p-value**
rs1494558	CC	39.8	44.2	-	0.10	-	0.48	-	**0.03**
	CT	44.0	48.4	0.99 (0.58-1.69)		0.83 (0.50-1.39)			
	TT	16.3	7.4	0.41 (0.16-1.02)				0.41 (0.17-0.98)	
rs1494555	AA	78.9	78.1	-	0.49	-	0.88	-	0.28
	AG	18.1	20.8	1.16 (0.62-2.19)		1.05 (0.57-1.93)			
	GG	3.0	1.0	0.35 (0.04-3.05)				0.34 (0.04-2.94)	
rs2228141	CC	81.0	79.2	-	0.53	-	0.73	-	0.36
	CT	19.0	19.8	1.06 (0.56-2.00)		1.12 (0.60-2.09)			
	TT	0.0	1.0	NA				NA	
rs11567764	GG	56.6	62.2	-	**0.02**	-	0.37	-	**0.005**
	AG	34.9	36.7	0.96 (0.57-1.62)		0.79 (0.48-1.32)			
	AA	8.4	1.0	0.11 (0.01-0.86)				0.11 (0.01-0.86)	
rs6897932	CC	87.5	83.3	-	0.35	-	NA	-	NA
	CT	12.5	16.7	1.40 (0.69-2.83)		NA			
	TT	0.0	0.0	NA				NA	
rs3194051	AA	50.3	43.5	-	0.37	-	0.29	-	0.57
	AG	38.8	47.8	1.43 (0.83-2.44)		1.32 (0.79-2.20)			
	GG	10.9	8.7	0.92 (0.37-2.30)				0.78 (0.32-1.87)	

Abbreviations: TB: tuberculosis patients, OR: odds ratio, NA: not applicable.