Interleukin-2 receptor in the pathogenesis of diabetic complications and Sjögren's syndrome

Magdalena Keindl

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

The study presented in this doctoral thesis was conducted at the Center for Diabetes Research and the Broegelmann Research Laboratory at the Department of Clinical Science, Faculty of Medicine, University of Bergen during the period from October 2017 to September 2021, within the framework of the Bergen Research School of Inflammation (http://www.uib.no/en/rs/brsi). This work was supervised by Valeriya Lyssenko, Silke Appel, and Ruchi Subhash Chandra Jain.

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Abbreviations

ATP	Adenosine triphosphate
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CKD	Chronic kidney disease
C-peptide	Connecting peptide
CPT	Cell preparation tubes
CTLA-4	Cytotoxic T lymphocyte antigen-4
CVD	Cardiovascular disease
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOLCE	Diagnostic optimization and treatment of diabetes and its complications in the Chernihiv region
eGFR	Estimated glomerular filtration rate
FOXP3	Forkhead box P3
FSC	Forward scatter
GAD	Glutamic acid decarboxylase
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HOMA-B	Homeostatic model assessment of beta cell function
HOMA-IR	Homeostatic model assessment of insulin resistance
HOMA-S	Homeostatic model assessment of insulin sensitivity
ICA	Islet cell autoantibodies
IL	Interleukin
IL-2R	Interleukin-2 receptor
JAK	Janus kinase
LADA	Latent autoimmune diabetes in adults
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MHC	Major-histocompatibility-complex
MMP	Matrix metalloproteinase
MODY	Maturity-onset diabetes of the young
MS	Multiple sclerosis
NK	Natural killer
NP	Non-progressors
P	Progressors
PBMC	Peripheral blood mononuclear cells
PI3K	Phosphoinositide 3-kinase
PDR	Proliferative diabetic retinopathy
PD-1	Programmed death-1

PPAR-γ	Peroxisome proliferator-activated receptor γ
PROLONG	Protective genetic and non-genetic factors in diabetic complications and Longevity
PRR	Pattern recognition receptor
PTPN2	Protein tyrosine phosphatase non-receptor type 2
pTreg	Peripherally derived regulatory T cell
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
sIL-2R	Soluble interleukin-2 receptor
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SS	Sjögren's syndrome
SSA	Sjögren's syndrome antigen A
SSB	Sjögren's syndrome antigen B
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TC-PTP	T cell protein tyrosine phosphatase
TCR	T cell receptor
TEDDY	The Environmental Determinants of Diabetes in the Young
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
Treg	Regulatory T cell
tTreg	Thymus-derived regulatory T cell
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Treg	Regulatory T cell
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZnT8	Zinc transporter 8

Abstract

The cytokine interleukin-2 (IL-2) acts as a double-edged sword by promoting both immunity and immune tolerance. Regulatory T cells (Tregs) are responsible for maintaining self-tolerance and constitutively express the high affinity form of the IL-2 receptor (IL-2R). IL-2/IL-2R signalling is crucial for the survival of Tregs and disruptions can promote the development of autoimmune diseases, such as type 1 diabetes (T1D) and Sjögren's syndrome (SS). Elevated plasma levels of the soluble form of IL-2R (sIL-2R) have been associated with different autoimmune diseases including T1D and SS. Autoimmune diseases are chronic and frequently lead to complications of various forms. In people with diabetes, the risk of developing complications is modified by numerous factors including duration of disease and level of glycaemic control and can affect vital organs including eyes, kidneys and blood vessels leading to retinopathy, nephropathy and cardiovascular disease (CVD). The role of immune factors and its complex architecture in diabetic complications has not been entirely established. The overall aim of this thesis was therefore to identify and study immune markers associated with complications in diabetes. Furthermore, we aimed to functionally investigate IL-2/IL-2R signalling in Tregs from patients with different severities of SS.

In our first study (paper I) we found that elevated levels of sIL-2R associated with the presence of vascular complications in patients with long-term T1D. Furthermore, we identified single nucleotide polymorphisms (SNP) in the *IL2RA* gene to be associated with sIL-2R. Finally, we observed a shift from naïve to effector T cells in patients with vascular complications compared with those without, indicating a breakage of immune tolerance, thus allowing an increase in pro-inflammatory T cells. We concluded that *IL2RA* SNPs may not only affect susceptibility to T1D, but also promote the development of vascular complications possibly by regulating sIL-2R plasma levels and lowering T cell responsiveness.

In paper II, we expanded our investigations on the link between sIL-2R and diabetic complications and included patients with different subtypes of diabetes such as type 2

diabetes (T2D). T2D is traditionally considered a non-autoimmune form of diabetes, however there is increasing evidence on immune-related factors involved in the pathogenesis of insulin resistance and T2D. Interestingly, we found that increased plasma sIL-2R was consistently associated with vascular complications, irrespective of the diabetes subtype, suggesting that high sIL-2R can be indicative of disease severity. This notion was further supported by our finding that insulin-dependent patients with T2D had significantly elevated sIL-2R compared with those without insulin treatment. Furthermore, we identified SNPs in the *IL2RA* gene to be associated with sIL-2R and a vascular complication phenotype in individuals with T2D. Together, these results support a role for sIL-2R and its association with *IL2RA* SNPs and diabetic complications, not only in T1D, but in other types of diabetes as well.

In our third project on SS (paper III), we investigated if IL-2/IL-2R signalling could be compromised in patients with elevated plasma levels of sIL-2R. For this we studied patients with SS and found that particularly patients with a pathologically low salivary flow exhibited highest levels of sIL-2R, which was also associated with seropositivity. Functional studies on Tregs revealed that patients with SS had a reduced IL-2/IL-2R signalling capacity indicating an impaired suppressive activity in those cells. Our data indicated that Tregs have a weakened immunosuppressive function in patients with SS, which could mediate an aggressive lymphocyte infiltration into salivary glands. This in turn induces sicca symptoms, which are associated with higher plasma sIL-2R.

Taken together, our findings highlight an association of elevated sIL-2R with disease severity in the autoimmune diseases T1D and SS reflected by the presence of severe vascular complications and pathologic sicca symptoms, respectively. Furthermore, increased sIL-2R levels are also associated with insulin-treated T2D and with vascular complications in general. We believe that circulating sIL-2R can potentially be used as an indicator of diabetes severity and assist in disease monitoring and treatment of complications in autoimmune diseases including diabetes.

List of Publications

Paper I

Keindl M, Fedotkina O, du Plessis E, Jain R, Bergum B, Jensen TM, Laustrup Møller C, Falhammar H, Nyström T, Catrina SB, Jörneskog G, Groop L, Eliasson M, Eliasson B, Brismar K, Nilsson PM, Berg TJ, Appel S, Lyssenko V. Increased Plasma Soluble Interleukin-2 Receptor Alpha levels in Patients with Long-term Type 1 Diabetes with Vascular Complications associated with IL2RA and PTPN2 Gene Polymorphisms. *Front Endocrinol.* 2020;11:575469

Paper II

Keindl M, Fedotkina O, Cherviakova L, Khalimon N, Svietleisha T, Buldenko T, Nilsson PM, Appel S, Lyssenko V. Soluble IL-2 Receptor Alpha as a Biomarker of Diabetes Severity and Vascular Complications. *Manuscript*

Paper III

Keindl M, Davies R, Bergum B, Brun JG, Hammenfors D, Jonsson R, Lyssenko V, Appel S. Impaired activation of STAT5 upon IL-2 stimulation in Tregs and elevated sIL-2R in Sjögren's syndrome. *Manuscript submitted*

Contents

1. Introduction

1.1 The human immune system

The main role of the human immune system is to keep pathogens, such as bacteria, viruses, parasites, fungi and other infectious proteins, out of our bodies. When pathogens manage to penetrate natural barriers of the human body, including the skin, mucosal membranes or the stomach acid, the immune system reacts to prevent infections. To protect an individual against disease, the immune system must perform the following main tasks. It needs to detect the infection (immune recognition), contain the infection (immune effector function), self-regulate to avoid a hyperreaction (immune regulation) and lastly protect against recurring disease caused by the same pathogen (immunologic memory) (1, 2).

The human immune system is divided into the innate and the adaptive immune system, and its cellular component consists of leukocytes, which are also known as white blood cells. The first line of defence is represented by the innate immune system, which comprises immune cells that rapidly and non-specifically respond to and destroy foreign and potentially infectious agents. These immune cells include natural killer (NK) cells, granulocytes and phagocytes. Phagocytes typically engulf and digest foreign particles and are categorised into neutrophils, monocytes, macrophages and dendritic cells. The complement system is a highly conserved element of the innate immune system and involves acute phase proteins circulating in the blood, which mediate lysis and opsonisation of pathogens, in addition to activating the immune system (3). A specific immune response is achieved with the adaptive immune system and its main actors, the lymphocytes. Lymphocytes can be divided into T cells, which participate in the cell-mediated response, and B-cells, which carry out the humoral response. Dendritic cells (DCs) are involved in the antigen-presentation to activate the cell-mediated part of the immune system, thereby forming a bridge between innate and adaptive immunity. When an antigen is taken up by a DC, it gets proteolytically cleaved into short peptides, which are then presented on the cell surface by major-histocompatibility-complex (MHC) proteins. The human leukocyte antigen (HLA)

system encodes for the two classes of MHC proteins involved in antigen presentation: i) class I (HLA-A, HLA-B and HLA-C); ii) class II (HLA-DP, HLA-DQ and HLA-DR). Activated DCs upregulate the expression of B7 co-stimulatory molecules (CD80 and CD86) on their cell surface, which provide the signals necessary for naïve lymphocyte activation together with the antigen receptor (1).

1.1.1 Immune tolerance

A very important feature of the adaptive immune system is to discriminate self- from non-self-antigens to avoid an immune reaction against host tissues. Immune tolerance describes the ability to stay unresponsive to certain antigens, particularly to self-antigens. Self-tolerance is antigen-specific and results from the recognition of antigens by individual clones of lymphocytes. Immune tolerance can occur by two mechanisms: i) during lymphocyte maturation in the generative lymphoid organs (central tolerance); ii) during lymphocyte-antigen interactions in peripheral tissues (peripheral tolerance). Failure of self-tolerance may lead to immune reactions against healthy cells of the body resulting in autoimmunity (4).

When immature T lymphocytes enter the thymus, they each have a randomly generated specificity through their T cell receptor (TCR). During the maturation process in the thymic medulla, T cells undergo an elaborate screening process where they encounter widely distributed circulating and cell-associated peptides of the human body. If developing T cells bind to these self-antigens with high affinity, they are considered self-reactive and are eliminated by negative selection and undergo apoptosis. Alternatively, some of these self-reactive T cells differentiate into regulatory T cells (Tregs) specific for these antigens. These Tregs then leave the thymus and enter the periphery to inhibit reactions against self-antigens. However, some self-reactive T cells escape the negative selection process, which is why peripheral tolerance is critical to prevent their activation and survival. Peripheral tolerance can be achieved by three distinct mechanisms: i) anergy; ii) deletion; iii) immune suppression by Tregs. T cell anergy is a “sleeping” stage of the cell and can be induced when the TCR binds an antigen in the absence of co-stimulation. Inhibitory receptors or checkpoints, such as CTLA-4 and PD-1, can inhibit TCR-co-receptor signalling and thereby suppress the

activation of effector T cells. Blocking CTLA-4 and PD-1 with so-called checkpoint inhibitors is an efficient approach in cancer therapy (4, 5).

1.1.2 Regulatory T cells

Tregs are a subset of $CD4^+$ T cells whose function is to suppress immune responses and maintain self-tolerance. As mentioned in the previous chapter, Tregs are generated from self-reactive T cells in the thymus. However, Tregs can also develop from T cells recognising self or foreign antigens in the peripheral lymphoid organs (5).

A common characteristic of all Tregs is the expression of CD4 and CD25 on their surface, and their intracellular expression of the transcription factor FOXP3. Both Tregs and activated T cells express CD25 but can be distinguished from each other by the low expression IL-7 receptor (CD127) on Tregs (6). Furthermore, Tregs often express the inhibitory receptor CTLA-4 on their cell surface. The cytokine interleukin-2 (IL-2) is essential for the survival of Tregs, which is corresponded by their constitutive expression of the high-affinity IL-2 receptor (IL-2R) (7-9). IL-2 and IL-2R will be discussed in more detail in chapter 1.2.

Tregs have the ability to suppress immune reactions by several different modes of action (Figure 1). Most prominently, Tregs secrete anti-inflammatory cytokines, such as TGF-β, IL-10 and IL-35. Secondly, Tregs can also kill effector T cells by cytolysis via the release of cytotoxins granzyme A and/or granzyme B together with perforin, which allows granzymes to enter the target cell. Furthermore, Tregs disrupt the metabolism of effector T cells by reducing the bioavailability of IL-2. By expressing the high-affinity IL-2R, Tregs can outcompete effector T cells for IL-2 who are also dependent on IL-2 to remain activated. Those effector T cells will subsequently undergo cell death due to cytokine deprivation. Another mechanism by which Tregs cause metabolic disruption involves the production of adenosine. The ectoenzymes CD39 and CD73 are expressed on the surface of Tregs and catalyse the degradation of adenosine triphosphate (ATP) into adenosine. Adenosine can constrain the proliferation and production of pro-inflammatory cytokines by effector T cells, and depletion of ATP inhibits ATP-dependent DC maturation and ATP-dependent

secretion of pro-inflammatory IL-1β by monocytes. Lastly, Tregs can affect antigen-presenting cells and stop them from activating effector T cells via the inhibitory receptor CTLA-4. Whether every single Treg is able to exert all these different inhibitory mechanisms remains unknown. If so, it would be interesting to unravel under which circumstances which mechanism comes into play. Potentially any Treg response could be triggered by a heterogenous set of Tregs where each is specialised in one mechanism (10-12).

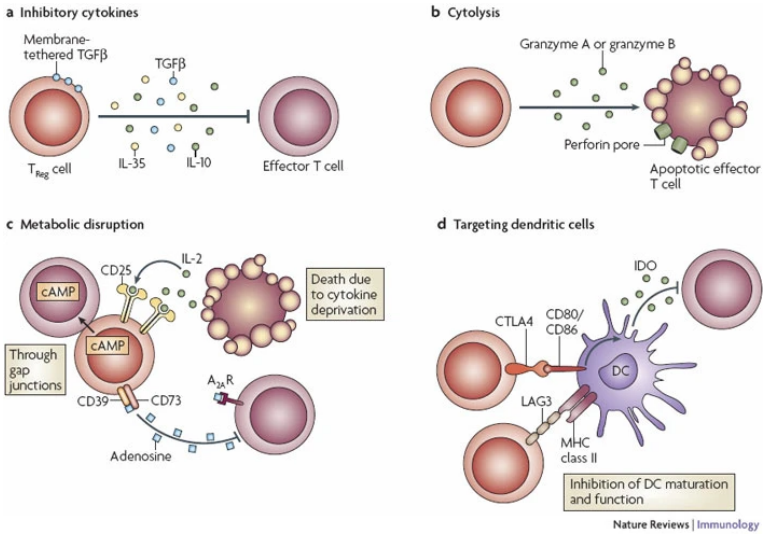

Figure 1. Modes of action used by Tregs. (a) Anti-inflammatory cytokines secreted by Tregs can suppress effector T cells. **(b)** The release of granzymes by Tregs can induce cytolysis in effector T cells. **(c)** By stably expressing CD25, Tregs can create a sink for IL-2 leading to cell death of effector T cells by cytokine deprivation. **(d)** Tregs express CTLA-4 thereby inhibiting DCs from activating effector T cells. Figure reprinted with permission from Springer Nature (10).

Tregs act as a natural barrier against autoimmunity and their importance has been underlined by their reduced function in various autoimmune diseases. Therefore, using Tregs to control autoimmune diseases or other inflammatory diseases has been a popular approach in research (8, 13).

1.1.3 Autoimmune disease

As described above, failure in self-tolerance and an immune response against self-antigens is defined as autoimmunity, which can further drive the development of autoimmune diseases. The responses to self-antigens or autoantigens resemble a typical

immune response to pathogens in that they induce specifically activated effector cells and production of autoantibodies against the self-antigen. Autoimmune diseases are chronic, often progressive and self-perpetuating due to a persistent and amplifying immune reaction. In principle, one can distinguish between two types of autoimmune diseases: i) organ-specific, where a particular organ is affected; ii) systemic with multiple tissues engaged. An example for an organ-specific disease is type 1 diabetes (T1D), which is characterised by an autoimmune destruction of the insulin-producing pancreatic β cells. Sjögren's syndrome (SS) is considered a systemic autoimmune disease affecting salivary and lacrimal glands (4). Both examples are described in more detail in chapters 1.3.1 and 1.4, respectively.

Incidence rates vary between different autoimmune diseases and currently affect approximately 5% of the world's population (14). For unknown reasons the general prevalence of several autoimmune diseases is on the rise and many autoimmune diseases disproportionately affect women (15).

So far, more than 80 autoimmune disorders have been found each affecting different tissues of the human body and the specific causes are in most cases unknown. However, genetic susceptibilities and environmental triggers have been identified to promote a failure of self-tolerance and are often shared in several autoimmune diseases. Despite many commonalities, the combination of genetic susceptibilities and environmental factors in each individual is specific, making autoimmune diseases heterogeneous and difficult to study. It is postulated that certain gene sets tend to promote an impaired self-tolerance and additional exposure to environmental stimuli can then trigger an activation of self-reactive lymphocytes inducing an autoimmune reaction (5).

Genetic factors

Most autoimmune diseases are polygenic meaning that many genes contribute to the development of autoimmunity at the same time. Genome-wide association studies (GWAS) have revealed a range of different gene variants or polymorphisms where each can make a small contribution by increasing the risk of developing a particular autoimmune disease. Healthy individuals typically have a lower frequency of these

polymorphisms. These genetic components may also increase disease severity or promote an earlier onset. Certain sets of alleles for different polymorphisms tend to be inherited together, which is called a haplotype. The strongest genetic associations with autoimmune diseases were found in the HLA locus encoding class I or II MHC molecules (5). For instance, the HLA-DR3-DQ2 or HLA-DR4-DQ8 haplotypes are strongly associated with T1D. In Scandinavia almost 90% of children diagnosed with T1D carry at least one of these haplotypes (16). The association between HLA alleles and autoimmune diseases is most likely related to their important role in antigen presentation. Certain HLA alleles may enhance antigen presentation to autoreactive T cells in the periphery, whereas others may affect self-antigen presentation in the thymus decreasing the negative selection of self-reactive T cells (17, 18). Besides the HLA locus, polymorphisms in various non-HLA genes are associated with autoimmune diseases and often encode for proteins involved in immune tolerance or lymphocyte activation (4). Polymorphisms in *IL2RA*, the gene encoding for the high affinity IL-2R constitutively expressed on Tregs, are associated with T1D, multiple sclerosis (MS) and rheumatoid arthritis (RA) (19). Nevertheless, genetic risk factors are less likely to cause the development of autoimmunity by themselves, but interactions with environmental factors can trigger the development of autoimmune disorders (4).

Environmental factors

Several environmental factors are associated with autoimmune diseases, including exposure to chemicals, hormones, cigarette smoke and most prominently infections by viruses, bacteria and other pathogens (20, 21). Infections may contribute to the development of autoimmunity in several ways, with molecular mimicry as one of the leading mechanisms. This occurs when an infectious microbe may contain antigens which share similarities with self-antigens and could thereby activate self-reactive T cells (14). Another proposed mechanism is termed epitope spreading, whereby the immune reaction against an infectious agent damages the host tissue leading to the release of self-antigens, which in turn are recognised by self-reactive T cells. Alternatively, a bystander activation can occur during an infection when self-reactive lymphocytes are activated in an indirect or non-specific manner (21).

Autoimmune diseases have a female predominance potentially due to the underlying differences in immune response between sexes. Sex hormones such as oestrogen have the potential to modulate lymphocyte development and function (15). Menopause has also been associated with autoimmunity, for instance SS occurs more frequently in postmenopausal women (22). Furthermore, the evolutionary differences in the reproductive function between male and female may explain the sex bias in autoimmunity. In pregnancy the mother acts as the host to a foetus with foreign antigens, and maternal immune tolerance is extremely important in preventing the rejection of the foetus (15). The innate immune system recognises molecular signatures through pattern recognition receptors (PRR), such as toll-like receptors (TLR), and inappropriate activation of TLRs can lead to the development of autoimmunity (23). Interestingly, TLRs may also contribute to the female predominance observed with autoimmune diseases as many TLRs, such as TLR-7 and TLR-8, are encoded by X-chromosomal genes (24). In general, several X chromosome defects involving processes such as X chromosome inactivation have been associated with the pathogenesis of autoimmune diseases (24-26). Altogether, it is of great importance that these striking sex differences are taken into consideration when studying autoimmune diseases.

1.2 IL-2 and its receptor

Interleukin-2 (IL-2) is a 15.5 kDa α-helical cytokine that was originally discovered as a T cell growth factor in 1976 (27, 28). Since then, IL-2 has been extensively studied and we now know that it acts as a double-edged sword by promoting both immunity and immune tolerance (7). IL-2 is predominantly secreted by activated $CD4^+$ T cells and to a lesser extent by $CD8^+$, DCs, NK cells and NKT cells (29-32). During T cell activation IL-2 is crucial to drive proliferation and differentiation thereby ensuring a strong immune response against a pathogen and promoting immunity (13, 28). Contrarily, Treg survival and function highly depends on IL-2, underscoring the importance of IL-2 in immune tolerance (33). Since Tregs do not produce their own IL-2, they are dependent on exogenous IL-2 from activated T cells reflecting a tight

connection between immunity and immune tolerance (34). Which cell type gets to use the available IL-2 is determined by the expressed receptor and its affinity to IL-2 (7).

1.2.1 IL-2 receptor

IL-2 receptor (IL-2R) consists of the three subunits IL-2Rα (CD25; encoded by *IL2RA*), IL-2Rβ (CD122; encoded by *IL2RB*) and IL-2Rγ (CD132; encoded by *IL2RG*), and IL-2 interacts with three classes of the IL-2R with different affinity (7, 13). IL-2Rγ is shared by several cytokines namely IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, which is why it is often referred to as the common γ chain or γc (35).

When the two transmembrane chains IL-2Rβ and γc interact, they form an intermediate affinity receptor ($K_d = 10^{-9}$ M), which is typically expressed on resting T cells and NK cells (13, 36, 37). The binding of IL-2 to the intermediate affinity receptor induces the transcription of IL-2Rα (38). When IL-2Rα is incorporated in the receptor complex, it attains high affinity for IL-2 ($K_d = 10^{-11}$ M), which results in a conformational change in IL-2 favouring its interaction with IL-2Rβ (7, 8, 39, 40). Together with γc a signalling cascade is induced through their cytoplasmic domains (41, 42). As already mentioned earlier, the high affinity tri-molecular receptor is constitutively expressed on the surface of Tregs (43, 44). High expression of the high affinity receptor can be only transiently observed on antigen-activated effector T cells (7). IL-2Rα on its own exhibits only low affinity for IL-2 ($K_d = 10^{-8}$) and has no signalling function (45, 46). Under certain conditions the monomeric IL-2Rα is expressed on DCs and it is theorised that it can present IL-2 *in trans* to cells expressing the intermediate affinity (13, 47). A "pseudo-high affinity" receptor is formed when IL-2Rα and IL-2Rβ are co-expressed ($K_d = 10^{-8}$) but does not result in signalling due to the lack of γc (48). To summarise, the intermediate and high affinity IL-2R are functional and have a signalling capacity due to the heterodimerisation of IL-2Rβ and γc cytoplasmic domains (41, 42). Finally, different compositions of the IL-2R complex have also been reported in B cells, NKT cells, macrophages, endothelial cells and fibroblasts (46).

In 2006 the crystal structure of the IL-2R complex was resolved (Figure 2), which revealed that, despite forming the largest component, IL-2Rα makes no contact with

IL-2Rβ or γc. IL-2 itself wedges tightly between IL-2Rβ and γc which induces intracellular signalling (40, 49). After IL-2 engagement, the receptor complex is rapidly removed from the cell surface. The ectodomain of IL-2Rα is proteolytically cleaved and released into the extracellular space as soluble IL-2Rα (50), which is further referred to as sIL-2R and described in detail in chapter 1.2.3. IL-2Rβ and γc on the other hand are engulfed into $Rab7^{+}$ vesicles and targeted towards degradation (51).

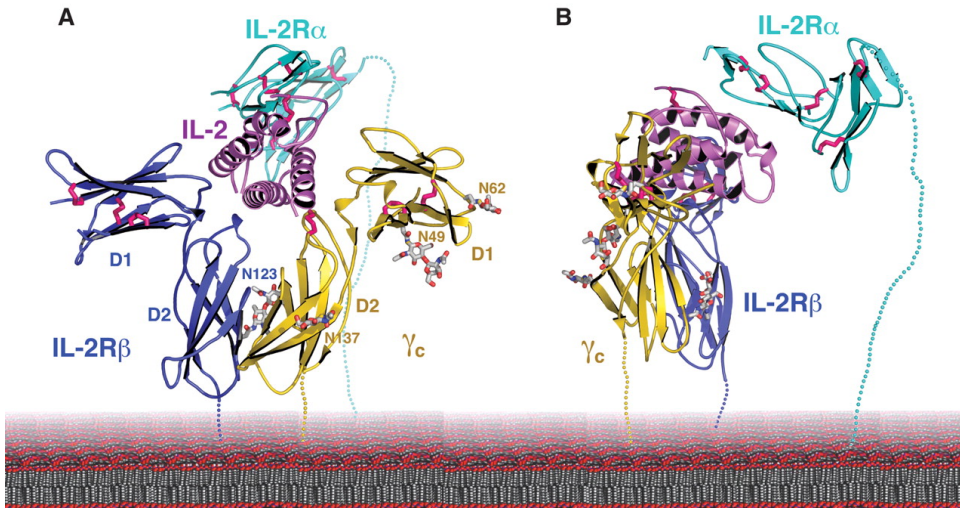

Figure 2. Crystal structure of IL-2R. Two views related by a ~90° rotation are shown (A and B). IL-2 is depicted in violet, whereas receptor chains are illustrated in cyan (IL-2Rα), blue (IL-2Rβ), and gold (γc). Disulphide bonds are shown in red. Dotted lines represent the connection of the respective C terminus to the cell membrane. Figure reprinted with permission from AAAS (40).

1.2.2 IL-2/IL-2R signalling

Following receptor binding, IL-2 activates various signalling pathways. Heterodimerisation of IL-2Rβ and γc induces the signalling cascade by activating the Janus family tyrosine kinases, JAK1 and JAK3. JAK1 is associated with IL-2Rβ, whereas JAK3 is bound to γc (52). These kinases phosphorylate each other at key residues on IL-2Rβ. Depending on the position of the IL-2Rβ phosphorylation, different intracellular pathways are activated, i.e. the phosphoinositide 3-kinase (PI3K)-AKT pathway, the mitogen-activated protein kinase (MAPK) pathway (Y338), and predominantly the signal transducer and activator of transcription 5 (STAT5) pathway (Y392, Y510) (7, 13, 53) (Figure 3). In Tregs only the STAT5 pathway is

activated and chemical or genetic inhibition of the PI3K pathway promotes Treg production (7). Due to the central role of STAT5 in Treg function and consequent control of autoimmune disease development, this chapter will only focus on IL-2/IL-2R signalling via STAT5.

It is important to mention that IL-2 can activate several STAT family members, including STAT1, STAT3 and STAT5, however STAT5 is the predominant IL-2 signalling molecule (54, 55). Upon IL-2Rβ phosphorylation, STAT5A and STAT5B dock on and are in turn phosphorylated by activated JAKs at position Y694 and Y699, respectively. This results in the formation of STAT5 dimers and tetramers which translocate into the nucleus where they act as a transcription factor on multiple target genes by directly binding the DNA. In Tregs those target genes include *IL2RA*, *FOXP3*, *PRDM1* (54). The STAT5-dependent regulation of *IL2RA* transcription represents a mechanisms to maintain expression of the high affinity IL-2R and signalling through IL-2 (7). Interestingly, IL-2 induced STAT5 signalling impacts immune metabolism by driving glycolysis, amino acid synthesis and oxidative phosphorylation (unpublished data) (56).

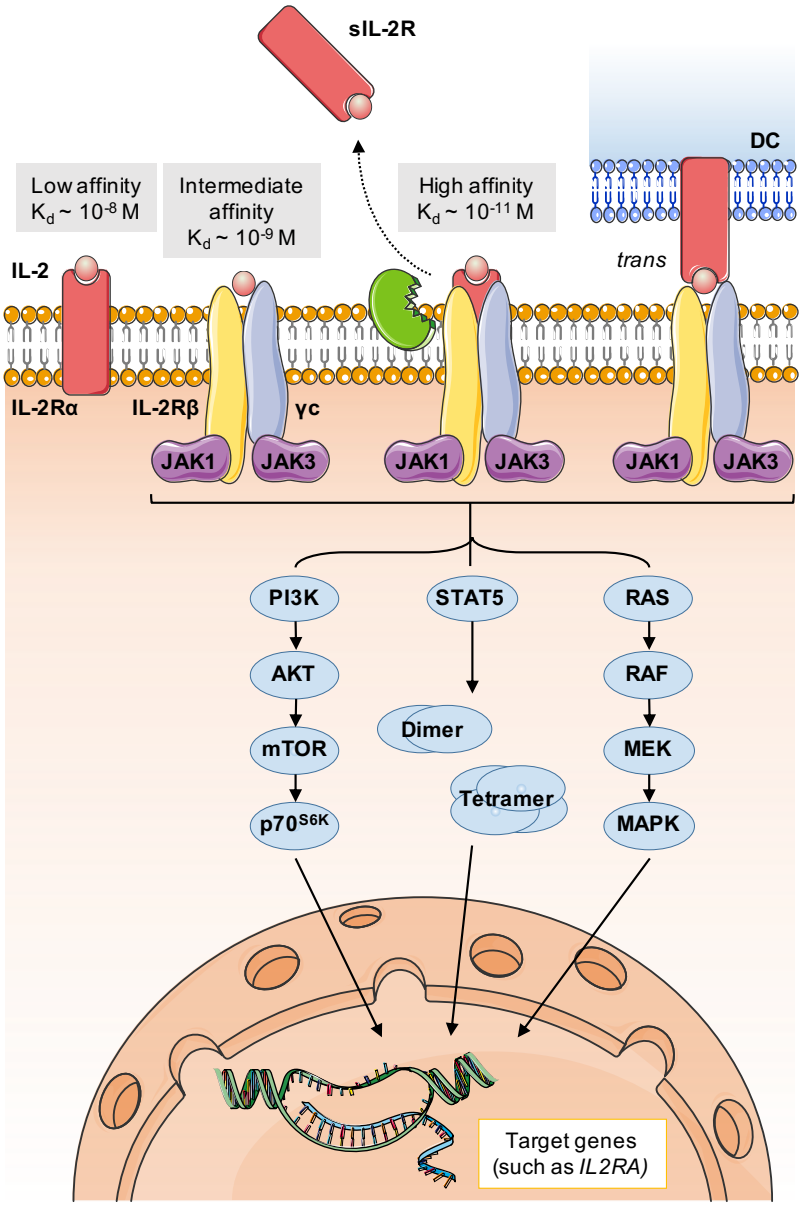

Figure 3. The IL-2 receptor system and IL-2/IL-2R signalling. The IL-2R can consist in different compositions (low affinity, intermediate affinity and high affinity). The highest affinity is achieved when all three receptor chains come together, IL-2Rα, IL-2Rβ and γc. Proteolytic cleavage of IL-2Rα leads to the release of soluble IL-2R (sIL-2R) into the extracellular space. DCs typically express the low affinity IL-2R, but together with neighbouring cells expressing the intermediate affinity, they can form a high affinity IL-2R complex through in trans presentation of IL-2. IL-2Rβ and γc can induce the IL-2/IL-2R signalling cascade by activating JAK1 and JAK3. Different signalling pathways can be induced, i.e. PI3K-AKT, MAPK and STAT5 pathway, which lead to the transcription of different target genes including *IL2RA*. Figure produced using Medical Servier Art.

1.2.3 Soluble IL-2R

Proteolytic cleavage of the ectodomain of the IL-2Rα results in the release of sIL-2R that is detectable in the circulation of healthy individuals and elevated in patients with infections, inflammation and autoimmune disease (45, 50). Even though an alternative splice variant cannot be ruled out as a contributing factor in the production of sIL-2R (57, 58), it is more commonly believed that sIL-2R occurs from proteolytic cleavage by different enzymes. Different candidates with the capability to cleave IL-2Rα have already been recognised, namely matrix metalloproteinase-9 (MMP-9), MMP-2, elastase, proteinase 3 and the environmental house dust mite allergen DerP1 (59-61). However, sIL-2R formation can still occur in the presence of enzymatic inhibitors suggesting that additional unidentified enzymes may be involved in the cleavage of sIL-2R (62, 63). Shedding of sIL-2R can occur on both activated T cells and Tregs and the biological role of sIL-2R remains unknown (45, 62, 64).

Brusko *et al.* performed an extensive *in vitro* study on sIL-2R and found that even though both Tregs and effector T cells shed sIL-2R, the surface expression of IL-2Rα remains stable on Tregs, whereas effector T cells only express IL-2Rα upon activation. Interestingly, they observed that higher sIL-2R in the supernatant correlated with increased effector T cell proliferation, but also with subsequent activation induced cell death. These findings imply that Tregs may obtain improved IL-2 signalling compared with effector T cells due to their increased membrane IL-2Rα stability and thereby affinity for IL-2. Overall, they concluded that the role of sIL-2R in immune modulation is presumably dependent on the local microenvironment controlling immune responses (62). However, additional studies have also examined the effect of sIL-2R *in vitro* but collectively the results and conclusions are controversial and various discrepancies were reported (45).

As mentioned above, IL-2Rα has low affinity for IL-2 (46). In fact, cleaved sIL-2R also retains the ability to bind IL-2 with low affinity, which can have opposing effects depending on the affected cell type (65). Due to this binding capacity, sIL-2R could function as a decoy-receptor for IL-2 thereby reducing the bioavailability of IL-2 in the microenvironment. This scenario would favour Tregs who constitutively express high

affinity IL-2R allowing them to maintain efficient IL-2 signalling when low levels of IL-2 are available. Thus, sIL-2R could act as a negative feedback mechanisms to restore the balance between immunity and immune tolerance (45). Alternatively, binding of sIL-2R to IL-2 in the extracellular space allows for an *in trans* presentation of IL-2 to cells expressing the intermediate affinity receptor, similarly to what has been postulated for DCs (13). Introducing IL-2Rα to the IL-2R receptor complex would enhance IL-2 signalling and depending on the cell type involved it could either benefit effector T cells and immunity, or Tregs and immune tolerance. Altogether, it is likely that sIL-2R interferes with IL-2/IL-2R signalling, but whether elevated sIL-2R level in immune diseases is a friend or foe is unclear (45).

1.2.4 IL-2R in autoimmune diseases

Considering the importance of IL-2/IL-2R signalling in Tregs and immune tolerance, it is not surprising that multiple genetic and immunologic associations of IL-2R with autoimmune diseases have been identified in the past decades.

Most prominently, elevated sIL-2R has been observed in serum/plasma from people with different autoimmune diseases including T1D, SS, MS, RA, systemic lupus erythematosus (SLE), idiopathic juvenile arthritis, polymyositis, myasthenia gravis, Graves' disease, Crohn's disease, ulcerative colitis, coeliac disease, sarcoidosis, IgA nephropathy, atopic dermatitis and psoriasis (66, 67). In many of those levels of sIL-2R correlate with disease severity as defined by typical clinical manifestations (66, 68-70). In the case of T1D, for instance, elevated sIL-2R was reported to correlate with reduced C-peptide – a preform of insulin – in young patients (71). Despite the fact that higher levels of sIL-2R are not disease specific, together with the respective clinical parameters, determination of sIL-2R levels may assist in the diagnosis of autoimmune diseases, particularly those difficult to diagnose. Considering the observed associations between sIL-2R and disease severity, sIL-2R may even be helpful in monitoring disease activity and prognosis to ensure an improved patient care (45). Furthermore, several associations between single nucleotide polymorphisms (SNPs) in the *IL2RA* gene and circulating sIL-2R have also been demonstrated in MS and T1D (72, 73). Whether elevated sIL-2R is the cause or consequence of autoimmune diseases remains

disputed, however gene associations point towards a genetic predisposition. Indeed, certain *IL2RA* SNPs cause defects in the maintenance of immune tolerance due to impaired IL-2/IL-2R signalling in Tregs (73-75). Interestingly, several genes involved in the IL-2/IL-2R signalling cascade, namely *IL2*, *IL2RA*, *PTPN2*, have been associated with different autoimmune diseases (19, 76). *PTPN2* (protein tyrosine phosphatase non-receptor type 2) encodes the T cell protein tyrosine phosphatase TC-PTP, which attenuates IL-2/IL-2R signalling by dephosphorylating JAK1, JAK3 and STAT5 (77).

Further support highlighting the central role of IL-2 in autoimmunity was provided by various animal studies. Mice deficient in IL-2, IL-2Rα or IL-2Rβ develop lethal autoimmunity or exhibit an impaired Treg function (30). In a non-obese diabetic mouse model researchers found intra-islet Tregs expressing less IL-2Rα, which led to an imbalance of Treg and effector T cell populations and the development of T1D in non-obese diabetic mice (78). IL-2/IL-2R signalling mainly activates STAT5 known to be critical for Treg function, and STAT5-deficiency leads to an impaired immune tolerance in mice (30).

Altogether, there is consistent data accentuating that disturbances in the IL-2/IL-2R signalling cascade are often accompanied by a compromised Treg function and autoimmunity (7).

1.3 Diabetes mellitus

Diabetes mellitus, or simply diabetes, is a group of metabolic disorders characterised by high blood sugar levels (hyperglycaemia) resulting from defects in insulin secretion, insulin action or both. Insulin, a hormone secreted from pancreatic β cells, has the ability to reduce blood glucose level by promoting glucose uptake, glucose transport and glycogen synthesis in peripheral tissues such as liver, muscle and fat. Chronic hyperglycaemia in individuals with diabetes can further lead to serious damage of different organs, primarily the eyes, kidneys, nerves and the cardiovascular system (79).

It is estimated that over 460 million people currently suffer from diabetes, which corresponds to 1 in 11 adults with diabetes worldwide. The prevalence of diabetes is increasing globally at epidemic rates with the number expected to reach 700 million by the year 2045. This rapid increase is most likely driven by a complex interplay of socioeconomic, demographic, environmental and genetic factors. Due to the immense incidence rates and associated diabetic complications, diabetes is considered a major driver of mortality and public health problem in our societies (80).

Diabetes is presently classified into the following general subtypes: type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes, and other types of diabetes (81). However, there are no clear criteria for these subgroups. T1D and latent autoimmune diabetes in adults (LADA) are mainly distinguished from T2D by the presence of autoantibodies against islet β cell antigens, most commonly against glutamic acid decarboxylase (GAD). With this classification, the majority of diabetes patients have T2D, which is often a diagnosis of exclusion, where those who do not meet the set criteria for a specified type of diabetes are called T2D. T2D is in reality a highly heterogeneous disorder with diverse clinical presentations due to different aetiology and factors affecting comorbidities. Today, there are several efforts in the diabetes research community aiming to refine diabetes classification into new categories based on pathophysiology. This is an important and necessary step towards guidance to individualised treatment and early identification of patients at risk of complication (82, 83).

1.3.1 Type 1 diabetes

T1D is caused by a T cell-mediated autoimmune destruction of insulin-producing pancreatic β cells leading to an absolute insulin deficiency. Therefore, people with T1D are dependent on exogenous insulin administration on a daily basis. The complex crosstalk between immune cells driving the development or the prevention of T1D is summarised in Figure 4. T1D typically manifests early in life, often during childhood, and accounts for ~5-10% of all diabetes. It is common that T1D develops suddenly and primarily affects children and adolescent individuals, but it can occur at any age. Autoimmune markers associated with T1D include islet cell autoantibodies (ICA) and

autoantibodies to GAD, insulin, the tyrosine phosphatases IA-2 and IA-2β, and zinc transporter 8 (ZnT8). The presence of these autoantibodies together with low or undetectable plasma C-peptide levels are important clinical hallmarks during the diagnosis of T1D (80, 81). As is the case for many autoimmune diseases, people with T1D are also prone to other autoimmune diseases including celiac disease, Graves disease, Addison disease, vitiligo, Hashimoto thyroiditis, autoimmune hepatitis, myasthenia gravis, and pernicious anaemia (81).

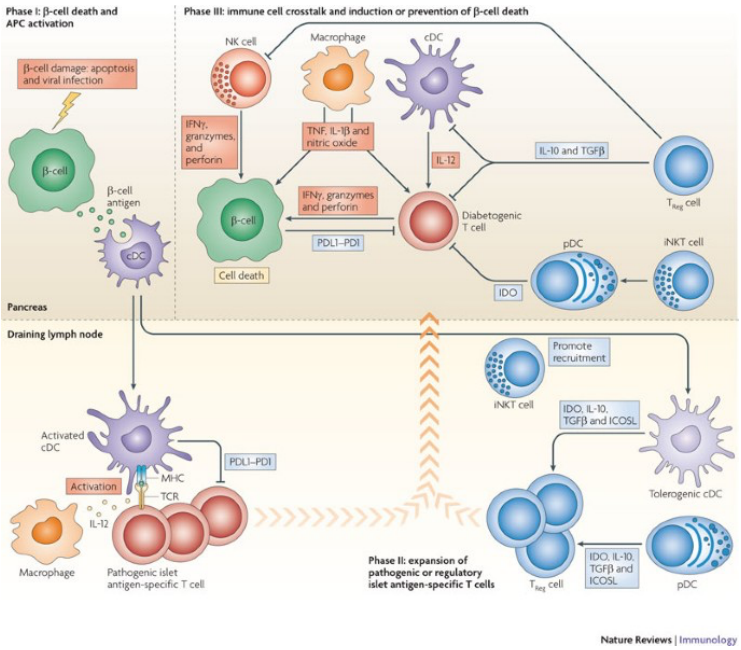

Figure 4. Immune cell crosstalk in T1D. During the initiation phase of T1D, β-cell damage leads to the release of β-cell antigens which are captured by conventional dendritic cells (cDC). Activated cDCs prime pathogenic islet antigen-specific T cells in the draining lymph node, which is further promoted by macrophages secreting IL-12. The activation of islet antigen-specific T cells can be inhibited by cDCs through PD-1. Invariant natural killer T (iNKT) cells can promote the recruitment of tolerogenic cDC and plasmacytoid DCs (pDC), which could expand Tregs by releasing indoleamine 2,3-dioxygenase (IDO), IL-10, TGF-β and inducible T cell co-stimulator ligand (ICOSL). Pancreatic β-cells can be killed by diabetogenic T cells, NK cells and macrophages through the release of various cytokines. Damage to β-cells and diabetogenic T cells can be controlled by Tregs and tolerogenic pDCs stimulated by iNKT cells. The β-cells themselves can also inhibit diabetogenic T cells through PD-1. Figure reprinted with permission from Springer Nature (84).

The incidence of T1D is on the rise with some regions affected more than others likely due to environmental factors that are still poorly defined. Multiple genetic

predispositions have been identified for T1D with HLA associations being the strongest one (81). Over the past decades, numerous studies have highlighted IL-2R as central player in the pathogenesis of T1D reporting elevated sIL-2R in the circulation and *IL2RA* SNPs affecting disease susceptibility (71, 72, 85-87). Administration of low-dose IL-2 has also shown promising effects in the treatment of T1D (88-90).

The development of autoimmune diseases cannot be solely caused by a genetic predisposition but depends on environmental factors interacting with predisposing genes. Infections with certain viruses, particularly enteroviruses, have been identified as a potential environmental trigger for the development of T1D (91). An international study called The Environmental Determinants of Diabetes in the Young (TEDDY) is an ongoing effort to determine risk factors for the development of T1D and follows children from USA, Finland, Germany and Sweden carrying risk HLA-DR-DQ genotypes from birth on. They reported that certain genotypes, including certain HLA alleles, can predict the presence of different autoantibodies (92) and that 21% of children with autoantibodies at the age of 3 developed T1D by age 6 (93). Furthermore, in TEDDY it was shown that the number of respiratory infections within any 9 month period was associated with the subsequent onset of islet autoimmunity within the following 3 months (94). A prospective virome analysis in TEDDY conveyed that prolonged enterovirus B infections may be linked with islet autoimmunity but not necessarily with T1D. Furthermore, early-life mastadenovirus C infections associated with islet autoimmunity (95).

In the recent past a considerable heterogeneity within T1D has been appreciated and several efforts aim to dissect this heterogeneity and stratify patients by different endotypes (96, 97). Endotypes reflect subtypes of T1D based on underlying pathobiological mechanisms, which are also tractable therapeutically, instead of focusing only on observable phenotypes. The definition of endotypes can be led by observations and hypotheses, data-driven methodologies and response to therapy (non-responders vs. responders). For instance, patients with T1D can be categorised depending on the timing of emergence of specific autoantibodies. An early incidence

of insulin autoantibodies is strongly linked to the HLA-DR4 haplotype, whereas GAD autoantibodies are associated with the HLA-DR3 haplotype (96).

1.3.2 Type 2 diabetes

A progressive loss of insulin secretion and development of insulin resistance are characteristic for people with T2D, which accounts for 90-95% of all diabetes (81). Although T2D usually manifest in people over 40 years of age, it is becoming increasingly common in children and adolescents (98, 99). Often T2D is accompanied by overweight or obesity and symptoms may go unnoticed for many years since hyperglycaemia develops gradually. In addition, obesity itself causes insulin resistance to some degree (81). Both obesity and insulin resistance are parameters of a larger group of disorders known as the metabolic syndrome. The metabolic syndrome describes a combination of metabolic disturbances where increased blood pressure, high blood sugar, abdominal obesity, and abnormal cholesterol or triglyceride levels occur together. Collectively this leads to an increased risk of heart disease, stroke, and T2D. At least initially, and often throughout their lifetime, individuals with T2D do not need insulin treatment. T2D can in many cases be managed by the promotion of a healthy lifestyle including a low-glucose diet, regular physical activity, and maintenance of a healthy body weight. Otherwise, glycaemic control can be achieved by oral medication with metformin as the first-line medicine. Only in severe T2D cases oral medications may not be sufficient to control hyperglycaemia and insulin injections may be necessary (80). In general, the risk of developing T2D increases with age, obesity and lack of physical activity. Apart from lifestyle-associated environmental factors, a strong genetic predisposition has been identified for T2D despite the heterogenous phenotype (81).

A chronic low-grade inflammation has been observed in the adipose tissue from obese individuals, particularly due to the infiltration of proinflammatory macrophages (Figure 5). There is increasing evidence supporting the involvement of these adipose tissue macrophages in the development of insulin resistance. Furthermore, pancreatic β cells may also be damaged as a result of the chronic inflammatory state driving hyperglycaemia further, or by glucotoxicity stemming from hyperglycaemia. The

vicious circle is completed by the fact that constant high blood glucose stimulates macrophage infiltration into the expanding adipose tissue. The link between inflammatory signalling and metabolic signalling is termed immunometabolism and a better understanding of this connection is necessary to fully comprehend the complex traits of T2D (100).

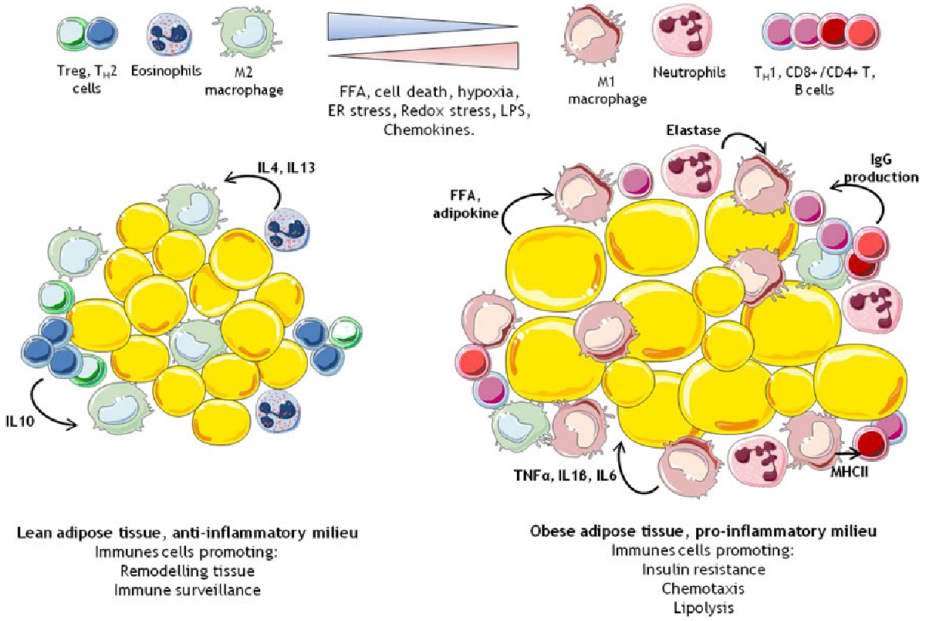

Figure 5. Adipose tissue inflammation during obesity. In lean adipose tissue, eosinophils, Tregs and Th2 cells promote an anti-inflammatory milieu through the production of IL-10, IL-4 an IL-13. This in turn stimulates the anti-inflammatory M2-like phenotype of the adipose tissue macrophages, which promote tissue remodelling. In an obese state, expanding adipocytes and cellular stress lead to the tissue infiltration of various immune cells and the development of a pro-inflammatory milieu. Furthermore, resident M2 macrophages polarise towards a pro-inflammatory M1-like phenotype. FFA, free fatty acid; ER, endoplasmic reticulum; LPS, lipopolysaccharide; Ig, immunoglobulin. Figure reprinted with permission from Springer Nature (101).

There is increasing recognition of the assumption that T2D pathogenesis also encompasses autoimmune aspects. The vicious circle mentioned above is sometimes termed “accelerator hypothesis” and speculates that an autoimmune-mediated β-cell destruction occurs in both T1D and T2D but at a different tempo (102, 103). Polly Matzinger elaborated the so-called “danger model” which moves away from the classical image of self and non-self and suggests that the immune system rather focuses

on entities that do damage than those that are foreign (104). Any molecule resulting from cellular stress which can bind to PRRs can act as a danger signal and are often referred to as damage-associated molecular patterns (DAMP) (105). Danger signals occur during persistent inflammation, and it is theorised that obesity-associated adipose tissue inflammation and β-cell stress due to gluco- and lipotoxicity continuously supply danger signals activating the immune system. Danger signals released upon glucotoxicity-induced β-cell apoptosis can lead to the activation of T cells reactive to β-cell antigens, which could promote an autoimmune response (106). In fact, expression of β-cell antigens is increased when β-cells are stimulated by glucose (107). From the perspective of the self/non-self model, inflammation-induced tissue destruction can lead to the release of cryptic β-cell antigens, which can activate self-reactive T cells in the periphery and drive a T cell-mediated β-cell destruction (106, 108). Chronic adipose tissue inflammation is not only associated with the infiltration of pro-inflammatory macrophages, but also T cells are critically involved (109). Tregs are central in the prevention of autoimmune responses, nonetheless the relevance of Tregs in the pathogenesis of T2D is poorly understood. Interestingly, the number of Tregs in adipose tissue from obese patients correlated with the degree of inflammation (110). Furthermore, obese patients with insulin resistance were reported to have reduced thymus-derived Tregs (tTreg) but increased peripherally derived Tregs (pTreg) in the visceral adipose tissue (VAT). Using the db/db mouse model, Treg depletion by anti-CD25 antibodies enhanced insulin resistance and remarkably, adoptive transfer of Tregs into these mice improved insulin sensitivity (111). Despite IL-10 being a crucial anti-inflammatory cytokine, Treg-specific loss of IL-10 in mice led to improved insulin sensitivity and protection from diet-induced obesity by increasing browning of white adipose tissue accentuating multiple functions of adipose tissue Tregs (112).

Taken together, these data confirm that inflammation is not only central in the development of autoimmune T1D, but also in the pathogenesis of insulin resistance and T2D. More emphasis should be taken on targeting the immune system by anti-inflammatory and immunomodulatory drugs in T2D monitoring, as they have proven effective in improving the metabolic profile of many patients with T2D (106).

1.3.3 Latent autoimmune diabetes in adults

While T1D is manifested by a fast progression of β-cell destruction, autoimmune diabetes can also occur after a slow progression of β-cell destruction leading to a later disease onset. This is characteristic for the so-called latent autoimmune diabetes in adults (LADA). To this day, it is still debated whether LADA classify as a separate diabetes category, which is why it is often included under the T1D rubric (81). However, this may not be the correct approach as LADA genetically differs from classical and late-onset T1D (113, 114). Furthermore, the classification of LADA includes arbitrary criteria that LADA patients do not require insulin treatment for at least 6 months after diagnosis (115). Despite the presence of autoantibodies, clinically LADA initially resembles T2D due to the late onset and slow disease progression often leading to a misdiagnosis of patients with LADA (116). LADA is a very heterogenous disease and clinical diagnosis criteria can vary between countries with GAD autoantibody positivity being common among them. A more detailed classification of LADA is necessary to allow for an improved assignment of prognosis and therapy (117). An increased risk of developing LADA has been associated with overweight and obesity, similar to T2D, but also with a family history of diabetes, as observed in T1D (118). Contrarily to T1D and T2D, the long-term consequences of LADA in terms of diabetic complications and mortality are scarcely studied (119). Data from the Norwegian HUNT study have reported a lower prevalence of the metabolic syndrome in LADA yet accompanied with poorer glycaemic control and increased likelihood of developing myocardial infarction compared with T2D (120). A UK-based study found that compared with T2D, patients with LADA have a lower frequencies of microvascular complications at diabetes onset, which is followed by an increased risk of complications beyond 9 years after diagnosis (121).

Altogether, a heterogeneity of LADA has been widely recognised, but the broad concept of LADA as an intermediate form of autoimmune diabetes has persisted (117). Interestingly, an altered Treg phenotype and reduced Treg count has been identified in patients with LADA thereby suggesting a breakage of immune tolerance (116, 122).

1.3.4 Other diabetes forms

Other forms of diabetes include gestational diabetes, monogenic diabetes and diseases of the exocrine pancreas, and drug- or chemical-induced diabetes. Gestational diabetes is typically diagnosed in the second or third trimester of pregnancy. Mothers with a history of gestational diabetes bear an increased risk of developing T2D later in life, which is why lifelong screening for the development of diabetes is recommended (81). Monogenic diabetes includes maturity-onset diabetes of the young (MODY) and neonatal diabetes. Neonatal diabetes occurs within the first 6 months of life and has a strong underlying monogenic cause often characterised by a dominant inheritance (80). MODY is the most common of the monogenetic forms and to date, 11 forms of MODY are reported, caused by mutations in individual genes (123). Patients with MODY are often misdiagnosed with T1D, but the exact diagnosis of the monogenic forms of diabetes is of importance because in some instances therapy can be tailored to the specific genetic defect (80, 124). Diseases of the exocrine pancreas include syndromes such as pancreatitis and cystic fibrosis, whereas drug- or chemical-induced diabetes refers to diabetes induced for example after organ transplantation (81).

1.3.5 Diabetic complications

Prolonged periods of hyperglycaemia may lead to dysfunction of many vital organs including eyes, kidneys, heart, blood vessels and the nervous system. In all diabetes subtypes, patients are at risk for developing the same chronic complications, however the rates of progression can differ from person to person (81). Diabetic complications can be broadly divided into microvascular (damage to small blood vessels) and macrovascular complications (damage to arteries). Microvascular complications affect the body's most intricately vascularised organs, namely the retina, the kidneys and the nervous system leading to retinopathy, nephropathy and neuropathy, respectively. Macrovascular complications include disorders of the large blood vessels and the heart causing cardiovascular disease (CVD) and are arguably more concerning as they confer a high mortality risk (125, 126). A schematic illustration of micro- and macrovascular complications in diabetes is provided in Figure 6 and will be described in more detail in the following chapters. Other chronic complications associated with diabetes include

depression (127), cognitive decline and dementia (128), and sexual dysfunction (129, 130).

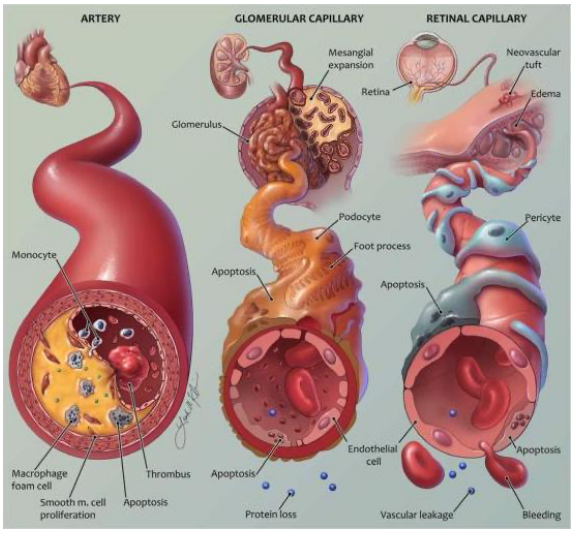

Figure 6. Micro- and macrovascular complications in diabetes. Pathological changes in a coronary artery (left), glomerular capillary (middle) and retinal capillary (right) during the development of diabetic complications are illustrated. Lipid overload in macrophages leads to their necrosis and an accumulation of foam cells forming an atherosclerotic plaque (CVD) (left). In diabetic nephropathy damage to the endothelial walls causes leakages, which break the filtration barrier in the renal glomeruli (middle). Retinopathy is characterised by vascular leakages and haemorrhages in the retina leading to retinal detachment and blindness (right). Figure reprinted with permission from Elsevier (131).

The development of diabetic complications is multifactorial, nevertheless the underlying mechanisms are complex and not fully understood. Risk factors encompass diabetes duration, chronic hyperglycaemia or excessive fluctuations of glucose and insulin, hypertension, generation of reactive oxygen species (ROS) and inflammation (125). However, clinical risk factors and glycaemic control cannot solely account for the development of diabetic complications and numerous studies have identified a clear genetic component to vascular complications (132). In fact, the development of diabetes and its associated vascular complications share multiple pathogenic pathways (125). Remarkably, some individuals with diabetes do not progress to vascular complications despite a long disease duration and it remains a mystery what protects these patients. There are several ongoing efforts investigating these so-called non-progressors and aim to get a clearer understanding of complication-free survival with

diabetes. These studies include the Joslin Medalist Study based in the USA, the Swedish and Danish PROLONG study (Protective genetic and non-genetic factors in diabetic complications and longevity) and the DIALONG study in Norway (125, 133-135).
Progression to diabetic complications can be delayed by early detection and treatment. Therefore, the identification of biomarkers is a prerequisite for enabling prediction, earlier diagnosis and better treatment of patients with diabetes.

Retinopathy

Diabetic retinopathy is the most common diabetic complication with about one third of all patients being affected and a wide variation among different populations (132, 136). Diabetic retinopathy is a progressive disease and is classified as mild-to-moderate non-proliferative, severe non-proliferative and proliferative diabetic retinopathy (PDR) (137). Both severe forms are vision threatening and diabetic retinopathy is considered as one of the leading causes of blindness among adults (126, 137). Disease progression occurs through a hyperglycaemia-mediated damage to the vascular system in the retina leading to haemorrhage, retinal detachment and blindness (132). PDR is characterised by a neovascularisation throughout the retina and into the posterior vitreous space (126, 132). While the concept of diabetic retinopathy as a microvascular disease is well-known, there is growing evidence on the involvement of neurodegeneration particularly in the early pathogenesis of diabetic retinopathy (136, 137). Therefore, diabetic retinopathy is nowadays often referred to as a neurovascular complication.

A neurovascular unit is formed around the retina where endothelial cells, neurons and glia are functionally coupled building a blood-retinal barrier. The disruption of the blood-retinal barrier is mediated by a low-grade inflammation, where proinflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1, CCL2) recruit proinflammatory M1 macrophages driving the progression of diabetic retinopathy further. In the early stages of diabetic retinopathy, anti-inflammatory M2 macrophages ameliorate inflammation and delay disease progression, but over time the immune balance is disturbed and favours polarisation of M1 macrophages. Whether the

breakdown of immune tolerance observed in T1D also plays a role in the development of diabetic retinopathy is unknown (136).

Nephropathy

Diabetic kidney disease, or diabetic nephropathy, is a progressive disease-specific complication largely impacting the dramatic increase in end-stage renal disease and chronic kidney disease (CKD) worldwide (138). Diagnosis is primarily based on two clinical markers, namely increased albuminuria and decreased estimated glomerular filtration rate (eGFR) reflecting a reduced renal function. Albuminuria, formerly known as microalbuminuria, is based on the albumin-to-creatinine ratio measured in urine and levels above these ranges define proteinuria (126). Apart from a genetic predisposition, risk factors for the development of diabetic nephropathy include hyperglycaemia, hypertension, insulin resistance, obesity, smoking and diabetes duration (139).

Even though diabetic nephropathy was originally not considered an inflammatory disease, it has become more and more evident that inflammation plays a central role in the development and progression to diabetic nephropathy. In the early stages of the disease, macrophages and T cells accumulate in the glomeruli and interstitium of the kidney promoting the release of proinflammatory cytokines and chemokines thereby recruiting additional leukocytes and generating a proinflammatory microenvironment (140). For instance, MCP-1 has been associated with the number of infiltrating interstitial macrophages and increased expression may contribute to renal tubular damage (141). The increased inflammation promotes ROS production, which can damage the endothelial walls causing leakages and driving kidney dysfunction further (139). Altogether, dampening of the kidney inflammation may have beneficial effects raising the question whether Tregs are important in the pathogenesis of diabetic nephropathy. Indeed, in the diabetes *db/db* mouse model a Treg depletion enhanced kidney inflammation and dysfunction, whereas adoptive transfer of Tregs reduced nephropathy (111). Furthermore, inhibiting Tregs in an ischaemia-reperfusion model worsened renal injury (142). To date, there is no cure for diabetic nephropathy and

treatment is focused on managing hyperglycaemia, proteinuria and kidney damage until late stages when dialysis or kidney transplants are necessary for survival (132).

Neuropathy

Diabetic neuropathy affects both somatic and autonomic parts of the peripheral nervous system and can be divided into autonomic neuropathy, atypical neuropathy, non-diabetic neuropathy and peripheral neuropathy, with the latter being the most common one (132, 143). Peripheral neuropathy affects the distal nerves of the limbs altering sensory function leading to numbness and pain (144). It increases the risk of developing ulcers, particularly in the feet (the so-called "diabetic foot"), and lower-extremity amputation (132). Due to the heterogeneity of the disease diabetic neuropathy is highly understudied and the causes are poorly understood (144). However, oxidative and inflammatory stress have been suggested to be predominant drivers (144-146). Furthermore, the inclusion of diabetic neuropathy as a microvascular complication has been criticised. Clinical characterisation of neuropathy was initially characterised by the development of vascular abnormalities, however, those are now considered to be a secondary effect of an underlying neuronal and glial disorder (143).

Cardiovascular disease

Diabetes is associated with an approximate doubling of CVD risk and can be reduced by lowering blood glucose and blood pressure, and by using lipid-lowering medication (147). In both T1D and T2D, microvascular complications have also been associated with increased CVD risk and often occur together (132, 143). CVD largely accounts for the increased rate of morbidity and mortality seen in the diabetic population (143).

CVD is associated with accelerated atherosclerosis leading to myocardial infarction and stroke. Atherosclerosis is a complex process involving several cell types and their interaction with each other. Disruptions in the endothelial walls disturbing its permeability are thought to be involved in the initiation of atherosclerosis. A damaged barrier function of the endothelium leads to the movement of low-density lipoprotein (LDL) into the subendothelial space forming a "fatty streak". Oxidation of LDL activates and recruits immune cells, in particular macrophages, which aim to remove

the formed LDL deposits by phagocytosis. Ultimately, macrophages overloaded with LDL die of necrosis inducing the formation of so-called “foam cells”. Further activation of the immune system and LDL deposition creates a snowball of foam cells, which combined with proliferating smooth muscle cells form an atherosclerotic plaque. Atherosclerotic plaques commonly occur at sites of turbulent flow and may occlude the blood vessels at the formation site (143). Diabetic cardiomyopathy describes a damage to the heart muscle in the absence of atherosclerosis and hypertension in patients with diabetes. A diastolic dysfunction is characteristic for cardiomyopathy and describes the inability of the heart to relax (148).

In recent years accumulating evidence supports a role of Tregs in promoting angiogenesis and the repair of blood vessels and heart muscle, even in hyperglycaemic conditions. Remarkably, Tregs can secrete vascular endothelial growth factor (VEGF), particularly under hypoxic conditions, thereby inducing the proliferation of endothelial cells. Treg-mediated secretion of IL-10 has also been shown to induce post ischemic angiogenesis. Furthermore, Tregs are involved in heart tissue repair after myocardial infarction by stimulating the polarisation of macrophages towards an M2-like phenotype (12).

1.4 Sjögren’s syndrome

SS is a chronic rheumatic autoimmune disease characterised by the lymphocyte infiltration in the exocrine glands, primarily the salivary and lacrimal glands. Persistent focal infiltration causes glandular tissue destruction inducing dryness and sicca symptoms including oral (xerostomia) and ocular dryness (keratoconjunctivitis sicca) (149). Like in many other autoimmune diseases, a tremendous gender bias is observed in SS with the female:male ratio being 9:1, predominantly affecting post-menopausal women. Extraglandular manifestations are common and SS often co-occurs with other autoimmune diseases such as SLE and rheumatoid arthritis (150). The majority of patients with SS have autoantibodies against Sjögren’s syndrome antigen A or SSA/Ro and Sjögren's syndrome antigen B or SSB/La (151). These antibodies are of great diagnostic value and can even be detected several years before symptoms occur (152).

Due to the heterogeneity of SS and the delayed presentation of symptoms there are some discrepancies in the classification criteria making it difficult to determine the global incidence of the disease, but prevalence estimates range from 0.1-3% (149, 153-155). The complete aetiology and pathogenesis of SS remain elusive, however a genetic predisposition and hormonal, immunologic, and environmental factors have been established (156, 157). GWA studies in SS are sparse and statistically underpowered, but associations with SNPs in various genes have already been reported, for instance *HLA*, *IL2* and *CTLA4* (157). Interestingly, the association of *HLA* variants with SS is unique to a subgroup of patients positive for SSA/SSB autoantibodies (158).

Several studies have highlighted a relationship between IL-2R and SS. Firstly, elevated sIL-2R in serum and saliva was reported in patients with SS and considered a disease activity index (68, 159-164). In CD25 knock-out mice, a disruption of IL-2 signalling resulted in age-dependent Sjögren's syndrome-like autoimmune lacrimal-keratoconjunctivitis, further pointing towards a breakage in immune tolerance (165). Contrarily, in humans with SS an increased frequency of $CD4^{+}CD25^{++}$ T cells with suppressive activity was reported claiming an unimpaired Treg function in SS (166). However, in this study the definition criteria of Tregs were lacking phenotypic characteristics, such as CD127 negativity or FOXP3 positivity, and suppressive function was only tested in 4 individuals. Therefore, further studies are necessary to elucidate the role of IL-2R, Tregs and immune tolerance in SS.

2. Aims

The general aims of this thesis were to study the role of immune factors, specifically sIL-2R, in the development of diabetic complications. Further, we aimed to investigate IL-2/IL-2R signalling in immune cells from patients with Sjögren's syndrome.

The specific aims include:

I. Analyse human cytokines (30-plex) and immune cell populations (26-plex) in long-term type 1 diabetes patients with and without vascular complications in the Scandinavian cohorts PROLONG and DIALONG.

II. Investigate plasma sIL-2R and SNPs in *IL2RA* and *PTPN2* in different diabetes subtypes (T1D, T2D, LADA) with and without vascular complications in the Ukrainian cohort DOLCE.

III. Study the potential connection between sIL-2R and IL-2/IL-2R signalling efficacy in regulatory T cells in a Norwegian Sjögren's syndrome cohort.

3. Methodological considerations

The present work was based on data from different cohorts stemming from various countries, which provided a sound foundation to draw conclusions. However, each clinical study and analysis approach had its limitations requiring careful considerations. Materials and methods used for these studies are described in the respective papers and manuscripts, while assays and analyses are briefly discussed below.

3.1 Clinical cohorts

All cohorts included in the three papers targeted different aims, which were adapted to the performed immunologic analysis in question. This resulted in certain limitations regarding sample and clinical data availability in the respective cohorts.

In the first paper we worked with two cohorts, PROLONG and DIALONG, which are both collections of patients with long-term T1D. In both cohorts, non-progressors (NP) were defined as patients with a T1D duration of over 30 years without having progressed to vascular complications. Progressors (P) were defined as those who have developed severe vascular complications. Definitions of micro- and macrovascular complications were similar in both cohorts and adjusted to the clinical data available in each cohort. The regional ethical committees approved the studies (PROLONG: Denmark H-2-2013-073, Sweden 777/2009, Norway 2019/1324; DIALONG: Norway 2014/851) and all participants provided written informed consent.

The PROLONG study had cross-sectional design and was initially launched to identify protective factors against complications in long-term T1D individuals. Patients were recruited between 2011 and 2015 from seven departments of endocrinology/diabetes outpatient clinics in Sweden (Scania University Hospital in Malmö; Karolinska University Hospital, Danderyd Hospital and South Hospital in Stockholm; Sunderby Hospital in Luleå, University Hospital of Umeå; Sahlgrenska University Hospital in Gothenburg) and Denmark (Steno Diabetes Center in Gentofte). Detailed information on recruitment and classification criteria have been previously described (135). The PROLONG set of patients represents a strong set with which to analyse the clinical

properties of NPs. However, due to sampling at different collaborating institutions, underlying variabilities and accessibility of samples between hospitals must be considered. For instance, the majority of RPs were recruited at the Karolinska University Hospital and circulating immune cells were only collected from patients recruited at the Steno Diabetes Center in Gentofte.

The DIALONG study is a cross-sectional, controlled, retrospective Norwegian cohort based in Oslo, which also included a control group consisting of non-diabetic spouses or friends with a similar lifestyle. The cohort aims to investigate joint stiffness in older patients with T1D and recruitment protocols and study results have been previously reported (133, 167, 168). We collaborated with the DIALONG study to examine whether the same results can be attained in two independent but similar cohorts. The same classification criteria were applied to the DIALONG cohort after appropriately adjusting complication definitions to the available cohort data. Nevertheless, certain clinical differences were noticeable between the two cohorts regarding T1D duration. Namely, NPs and Ps were similar in DIALONG regarding clinical variables as age, HbA1c and diabetes duration, whereas those variables showed significant differences in PROLONG.

In paper II, we worked with the Ukrainian hospital- and primary health care-based study DOLCE (The Diagnostic Optimization and Treatment of Diabetes and its Complications in the Chernihiv Region). DOLCE enrolled over 6000 participants including patients with different types of diabetes and healthy first- or second-degree relatives. A more detailed description on the study design has been reported elsewhere (169). The hospital data in DOLCE only distinguished between T1D and T2D based on physician diagnosis, so we carefully re-categorised the patients including available clinical and biochemical data. Patients with documented T1D were grouped into classical and late-onset T1D based on their age of onset with a cut-off at 30 years. Documented T2D and seropositivity for GAD antibodies defined patients with LADA, who all had a diabetes onset at above 30 years of age. The remaining autoantibody-negative patients with documented T2D were grouped by their treatment status into insulin-treated T2D and T2D without insulin treatment. Each of these diabetes

subgroups included patients with and without diabetic complications. Subsequently, we selected individuals of similar age and sex between the diabetes subgroups and between patients with and without complications within each subgroup. This strict sample selection left us with 667 individuals for further analysis. The regional ethical committees approved the study (Ukraine: Dnr17/2011-09-14; Norway: 2019/1324) and all participants provided written informed consent. Working with various diabetes subtypes within one study as we did in DOLCE has many benefits, however it also leads to clinical differences due to the nature of each disease and requires careful consideration during statistical analysis. For instance, the age at diagnosis and C-peptide levels are lower in patients with T1D than those with T2D, as T1D is typically diagnosed during adolescence and patients are insulin-dependent due to the autoimmune destruction of pancreatic β cells. During statistical analysis we only adjusted for age and sex, but not for clinical variables such as age at diagnosis, diabetes duration, HbA1c or C-peptide, as they not necessarily categorise as covariates but disease-defining criteria instead.

For paper III, we included female individuals participating in the Sjögren's syndrome biobank at the Broegelmann Research Laboratory and recruited healthy women of similar age. All patients fulfilled the 2016 American College of Rheumatology/European League Against Rheumatism classification criteria for SS (170). This biobank provides comprehensive clinical information on each patient allowing for a rigid sample selection. Clinical characterisation includes oral, ocular and ultrasound examinations, broad autoantibody screenings, extensive treatment data and information on a range of other medical conditions (Diseases affecting the circulatory, digestive, endocrine, cutaneous, lymphatic, muscular, nervous, renal, respiratory and skeletal system; haematological diseases; oral/ocular complications; cancers; other autoimmune diseases). For this study we carefully selected female patients with SS by sample availability (plasma, PBMC, saliva) and presence of comorbidities. The patients were grouped by their salivary flow rate, where 1.0-3.0 ml/min was categorised as normal/high and ≤0.7 ml/min as low/pathologic salivary flow. Due to the strict sample selection, we observed few limitations regarding the clinical cohort. The

healthy volunteers were collected separate from the biobank, therefore clinical characterisation is scarce compared with the patients. Hence, we cannot exclude the possibility that some of our healthy controls might suffer from asymptomatic diseases. Due to the limited treatment available for patients with SS, many of our study participants are untreated allowing us to study the full immunological picture of SS without variations introduced by medication.

3.2 Patient samples

For cytokine analysis, we used EDTA plasma from overnight fasted patients stored at -80°C in all three papers. In each study the samples were carefully thawed on ice and aliquoted into plates in a randomised fashion prior to analysis. Multiple freeze-thaw cycles of plasma samples can affect the proteins to be analysed, which is why it is important that all samples are processed similarly to allow for robust comparisons (171). Storage time and anticoagulant can also affect the stability of cytokines (172, 173), but we did not observe an association between storage time and sIL-2R concentration in our studies.

Peripheral blood mononuclear cells (PBMC) were used for cell surface marker analysis in paper I and phospho-flow analysis in paper III. In paper I, PBMCs were isolated from peripheral blood collected in cell preparation tubes (CPT) by density gradient centrifugation and subsequently cryopreserved in human AB serum containing 10% dimethyl sulfoxide (DMSO) at -80°C. In paper III, PBMCs were also collected from peripheral blood by density gradient centrifugation using Lymphoprep™ and subsequently cryopreserved in 50% X-vivo 20, 42.5% Profreeze™-CDM™ NAO chemically defined freeze medium and 7.5% DMSO at -150°C. By using CPT, blood can be drawn directly into a vacutainer containing a high-density medium. Thereby careful and time-consuming layering of blood by trained laboratory staff can be spared, which is of advantage particularly in busy clinical environments. Studies have shown that CPT and Lymphoprep are comparable methods for PBMC isolation for immunological studies (174), however opposing results have been reported (175). Immune cells are sensitive to freezing conditions, which is why the use of a

cryoprotectant such as DMSO is important to minimise cell damage (176). In order to maintain viability of PBMCs, they need to be stored below -132°C, which is considered to be the temperature where all biological activity stops (177-179). However, it has been reported that viable cell recovery was not affected in PBMCs cryopreserved at -70°C/-80°C for 1.5 years (180). PBMC storage in PROLONG may not have been according to the best practice, but enough cells were recovered to perform a sound cell surface marker analysis. In paper I and III, cells were thawed rapidly at 37°C to avoid osmotic stresses and ice recrystallisation, and ensured an enhanced cell viability and recovery (181).

3.3 Cytokine analysis

In order to determine the plasma concentration of various cytokines and growth factors, we applied Luminex and enzyme-linked immunosorbent assay (ELISA). Both have advantages and disadvantages, which will briefly be discussed in this chapter.

3.3.1 Luminex technology

Luminex is a bead-based technique to quantify the presence of specific analytes in a sample. It enables simultaneous detection of multiple proteins in a single well, which saves sample, time and reagents. In this assay each magnetic bead has a different dye concentration which allows for its identification at the time of analysis. Additionally, each bead has a single target it can bind with determined by a capture molecule attached to the bead. Thereby, a range of analytes can be determined simultaneously in a well by combining different beads with their unique colour and target signature. Beads and samples are added to each well. If the target is present in a sample, it will bind to the capture molecule. The target is then bound to a reporter molecule, in our case streptavidin coupled to PE. This reporter molecule makes it possible to identify the presence of the target. In the Luminex machine the beads are analysed with flow cytometry, where each bead gets send through a fluidics system and is eventually hit by a laser beam. The diffraction of fluorescent light is captured by the detector and converted into digital data. The Luminex can quantify the signal from the reporter

molecule and associate it with the bead type using its unique colour signature. Thereby one can determine how much of each target was present in the analysed sample (182).

In paper I, we used a magnetic human premix Luminex assay to multiplex plasma proteins in PROLONG and DIALONG. First, DIALONG plasma samples were profiled for 30 different cytokines and growth factors using the Invitrogen™ Human Cytokine 30-Plex kit (Thermo Fisher Scientific). Subsequently we analysed PROLONG plasma samples with a custom-designed 4-Plex ProCartaPlex™ Human Cytokine Panel (Thermo Fisher Scientific). Both kits provided similar laboratory protocols and data was acquired on the same machine. Additionally, they were purchased from the same firm, however the daughter company was different. Unfortunately, we observed kit-to-kit variation in the quantified absolute values, where the sIL-2R concentrations were on average approximately 10-fold higher in the ProCartaPlex kit compared with the Invitrogen kit. Therefore, we could not use the absolute concentrations for this study and had to work with log-transformed data to compare the two studies. After contacting the mother company for help with troubleshooting we were informed that absolute values from different multiplex kits provided by different daughter companies often differ significantly. Hence, the major drawback of Luminex technology is, that inter-assay variations can occur, particularly when using different providers.

3.3.2 ELISA

In paper II and III, we used enzyme-linked immunosorbent assay (ELISA) to determine the sIL-2R concentration in EDTA plasma. ELISA is a biochemical technique to detect and quantify the presence of a specific compound, such as a cytokine, hormone or antibody. A major benefit of ELISA is that it is a very quick yet highly sensitive method to quantify a large number of samples in parallel at low costs.

In this study we used a so-called sandwich ELISA. For this type the wells of a polystyrene plate are coated with antibodies specific to an epitope of the antigen of interest, sIL-2R in our case. The wells are then filled with standards provided by the manufacturer or plasma of the patients. Only antigens specific to the antibody will bind

to the wells and a washing step removes all unbound compounds. Next, an antibody targeting another epitope on the antigen of interest is added. When it binds, a so-called sandwich is formed with the antigen of interest in-between the two antibody types. This secondary antibody is covalently conjugated to an enzyme, most commonly horseradish peroxidase (HRP). All wells are washed again to remove any unbound antibody. Finally, a solution of a colorimetric substrate is added, which generates visible colour when it interacts with the HRP enzyme. The development of colour can be quantified with an electronic plate reader, also known as a spectrophotometer. In our experiments we measured the absorbance at a 450 nm wavelength.

Whereas ELISA can only quantify one analyte at a time, trustworthy absolute values can be achieved, which are consistent between assays and cohorts (183).

3.4 Flow cytometry

Multicolour flow cytometry is a powerful tool allowing for the detailed characterisation of cell populations at a single-cell level. It involves a laser-based technology where cells are passed through a fluidic system where sheath fluid focuses the cells into a precise stream. One by one each cell is hit by a laser beam leading to the scattering of light. The forward scatter (FSC) is proportional to the size of the cell, whereas the side scatter (SSC) indicates the cell granularity or internal complexity. Further laser beams of specific wave lengths can excite fluorochromes typically conjugated to monoclonal antibodies targeted to bind cell proteins. All light signals generated as particles are converted to voltages by photodetectors, most commonly by photomultiplier tubes. The electrical current is converted into a digital signal allowing for data analysis on a computer. Flow cytometry data is typically presented as a dot plot, where each dot represents a single cell allowing for gating and analysis of the desired cell populations. Tagging of multiple biomarkers with different fluorochromes allows for a vast number of combinations of these parameters driving flow cytometry's potential in the phenotypic characterisation of cell populations further. Nevertheless, the physical properties of fluorochromes limit the number of markers that can be analysed simultaneously. Each fluorochrome has a wide emission spectrum and spectral overlap

may be an issue when multiple fluorochromes are applied together. Colour compensation is a tool that can mathematically correct for spectral overlap by calculating the spill-over from each fluorochrome into other detector channels (184). In paper I, we applied multi-colour flow cytometry to immunophenotype PBMCs from PROLONG patients using two panels including 14 and 16 parameters, respectively, allowing us to discriminate between a range of different cell populations. Analysis of multi-dimensional flow cytometry data can be biased by the person analysing. The use of algorithms reducing the dimension of the data to a two-dimensional map, such as t-distributed stochastic neighbour embedding (tSNE), can be helpful in defining a reliable gating tree and in data visualisation (185, 186).

Phospho-specific flow cytometry, also known as phospho-flow cytometry, is a specific application of flow cytometry enabling the study of phosphorylation states of intracellular proteins. It is a powerful tool providing information on the signalling response of cells to various stimuli. Phospho-flow cytometry has the potential to detect abnormal signalling in PBMCs from patients, which have been successfully used in the prediction of disease outcomes and treatments responses, particularly in haematological cancers (187). In paper III, we applied phospho-flow cytometry to investigate the IL-2 signalling response within Tregs from healthy individuals and patients with Sjögren's syndrome by determining the level of phosphorylation of STAT5. In our study Tregs were defined by positivity for CD3, CD4 and CD25 and negativity for CD127, but we did not include Foxp3 in our panel due to technical reasons. Nevertheless, it was previously shown that our gating approach is sufficient to define Foxp3-positive Tregs with suppressive activity (188).

3.5 Genetic analysis

In paper I and II, we used the available genotype data from the different cohorts to calculate potential associations between SNPs in the *IL2RA* and *PTPN2* gene regions and plasma levels of sIL-2R or clinical phenotypes. These calculations were performed by my skilled and experienced colleague Olena Fedotkina, who is a mathematician in training.

With the completion of the Human Genome Project, a multitude of genetic variations or polymorphisms have been found with SNPs being the most abundant type of these variations (189). SNPs are single base pair positions in the genome with an abundance of more than 1% in the human population (190). The majority of SNPs occur in non-coding regions of the genome making their interpretation often difficult. SNPs in regulatory regions can affect transcription of genes, whereas exonic SNPs can alter the protein structure and function (191). GWA studies focus on SNP associations with certain traits and have in the past decades uncovered a range of associations for many complex human diseases, such as diabetes and various autoimmune diseases. When samples of individuals are genotyped, only a subset of SNPs is directly analysed, thus tools are necessary to predict the remaining SNPs. Imputation describes the process of predicting the genotypes that are not directly assayed and uses whole genome sequencing data from the respective populations as reference. Certain SNPs are more likely to be inherited together measured by their linkage disequilibrium (LD) to each other. Using the matrix of LD values for every single SNP in the genome enables us to mathematically impute genotypes. The imputation score describes the quality of the imputation and the higher the score, the more reliable the prediction (192). In our studies we only included SNPs with an imputation score above 0.4 and an abundance (minor allele frequency, MAF) higher than 5%. Furthermore, we pruned our list of associated SNPs by excluding the ones in high LD to each other (R^2>0.8) to determine independent associations.

3.6 Statistical analysis

In all three papers we used the non-parametric Mann-Whitney U test in the comparison between different groups. This allowed us to accurately calculate significant differences without assuming a Gaussian distribution. Multiple linear regression was applied to adjust for covariates. Adjustment for covariates is important to identify differences or associations independent of confounding factors. Nevertheless, adjustment models must be handled with care particularly with small data sets as new values are being predicted. Prediction models become more reliable with a growing

sample size, as the reference group the prediction is based on is larger. Furthermore, multiple linear regression assumes a Gaussian distribution and compares means whereas the non-parametric Mann-Whitney U test compares medians (193). Differences were considered statistically significant when $p<0.05$. The studies were of an exploratory nature and hence no correction was made for multiple comparisons. All statistical calculations and the production of associated graphs were coded in R Studio.

4. Summary of results

Paper I: Increased plasma soluble interleukin-2 receptor alpha levels in patients with long-term type 1 diabetes with vascular complications associated with *IL2RA* and *PTPN2* gene polymorphisms

In this study, we investigated whether immune-related factors contribute to the development of vascular complications in patients with long-term T1D in two independent cross-sectional cohorts based in Scandinavian countries (Sweden, Denmark, Norway), PROLONG and DIALONG. In these cohorts we identified non-progressors, which are rare patients with long-term T1D who remarkably remained free from vascular complications despite a diabetes duration of more than 30 years. These patients were compared with progressors, who rapidly progressed to vascular complications.

We studied a panel of inflammatory markers in plasma of patients and found that elevated levels of sIL-2R positively associated not only with T1D itself, but also with a severe complication phenotype, particularly PDR. Furthermore, plasma sIL-2R significantly associated with SNPs in the *IL2RA* and *PTPN2* gene regions suggesting a potential causal relationship. The biological role of sIL-2R is not yet elucidated, but an imbalance in Tregs and effector T cells has been suggested. To investigate this further, we used multicolour flow cytometry and studied a broad panel of circulating immune cells in a subset of PROLONG patients. Interestingly, we did not observe a difference in CD25 cell surface expression or in the frequency of Tregs between the two patient groups. We did however find a shift from naïve to effector T cells in patients with vascular complications compared with those without. This shift could potentially be driven by an impaired suppressive function of Tregs, which may be reflected by an increased shedding of sIL-2R. Contrarily, the increased plasma levels of sIL-2R may also originate from the increased frequency of activated effector T cells which often express CD25.

Altogether our data indicates that *IL2RA* and *PTPN2* polymorphisms may not only increase the risk of T1D, but in addition the development of diabetic complications possibly by influencing sIL-2R and affecting T cell responsiveness. Thus, elevated

plasma sIL-2R could potentially act as a biomarker in monitoring diabetic complications in people with T1D enabling an earlier treatment and an improved patient care.

Paper II: Soluble IL-2 receptor alpha as a biomarker of diabetes severity and vascular complications

In the second paper, we studied whether the observed association between sIL-2R and diabetic complications is exclusive to T1D or if similar outcomes can be detected in other diabetes subtypes as well. The Ukrainian DOLCE cohort comprises healthy controls, individuals with classical and late-onset T1D, LADA, and T2D with and without insulin treatment, where each diabetes subgroup included patients with and without vascular complications. Plasma analysis revealed that not only classical T1D is associated with higher sIL-2R, but also late-onset T1D and insulin-treated T2D. Furthermore, we observed that, overall, all diabetes subgroups with vascular complications demonstrated significantly elevated plasma sIL-2R compared with those without. This data supports a role of immune-related factors in T2D and its associated complications, potentially even the involvement of immune tolerance due to the central role of IL-2R in maintaining a balanced immune response. Considering that LADA is largely considered an autoimmune disease, it was somewhat surprising that plasma levels of sIL-2R in patients with LADA compared to healthy controls in our dataset. However, these observations further support the proposition that LADA does not categorise as a classical autoimmune diabetes, but a mix of T1D and T2D instead.

Moreover, we identified SNPs in the *IL2RA* and *PTPN2* genes to be associated with sIL-2R and a vascular complication phenotype in individuals with T2D. It remains unknown whether higher plasma sIL-2R levels are a cause or a consequence of diabetes complications. We identified several *IL2RA* SNPs to be associated vascular complications independent of sIL-2R, suggesting that a certain genetic predisposition towards an impaired IL-2/IL-2RA signalling could lead to a more severe diabetes phenotype with vascular complications. Furthermore, certain *IL2RA* SNPs were associated with insulin-treated T2D compared with T2D without insulin treatment.

Combined, these data may indicate a relationship between sIL-2R, *IL2RA* polymorphisms and diabetes complications in various subtypes of diabetes. We believe that high plasma sIL-2R could potentially act as an indicator for diabetes severity including the development of vascular complications and, in the case of T2D, insulin treatment. Thus, the clinical use of sIL-2R could assist in early intensive management of diabetic complications and improve patient care.

Paper III: Impaired activation of STAT5 upon IL-2 stimulation in Tregs and elevated sIL-2R in Sjögren's syndrome

In our third study, we aimed to investigate the relationship between sIL-2R and IL-2/IL-2R signalling in Tregs in more detail. Increased sIL-2R has been associated with a range of different autoimmune diseases apart from T1D, one of them being SS. As we did not have the necessary material available in the cohorts included in paper I and II, we decided to start investigating this relationship in SS because all required samples were readily available to us through the Norwegian Sjögren's syndrome cohort. In this cohort we could carefully select female patients with SS who did not suffer from any additional autoimmune disease which is associated with high sIL-2R. Subsequently, plasma and PBMC samples were collected from healthy women of similar age.

As previously reported, we detected significantly elevated sIL-2R in patients with SS compared with healthy controls. Additionally, we observed a positive association between increased sIL-2R and severity of SS reflected by a pathologically low salivary flow. Interestingly, patients positive for anti-SSA and/or anti-SSB had the highest levels of sIL-2R. Together these results indicate an association of high sIL-2R and SS severity, similarly to what we have already observed in T1D and T2D.

In a subset of the study participants, we stimulated the isolated PBMCs with IL-2 and measured phosphorylation of STAT5 downstream of the IL-2/IL-2R signalling cascade. We identified a significant decrease in the frequency of $pSTAT5^+$ Tregs upon IL-2 stimulation in patients with SS compared with healthy controls. This decrease was specific to Tregs and was not observed in conventional T cells. Furthermore, there was no difference in the general frequency of Tregs between patients with SS and healthy controls. IL-2 induced STAT5 activation is crucial for the suppressive function of

Tregs. Therefore, the association of SS with reduced pSTAT5^{+} Tregs upon IL-2 stimulation likely indicates an impaired Treg function. Interestingly, we found that patients with SS had a higher frequency of pSTAT5^{+} Tregs at baseline, which positively correlated with sIL-2R and associated with seropositivity. Constitutively activated STAT5 could negatively affect signalling once the stimulus is actually present and may thereby drive the pathogenesis or activity of the disease. Despite detecting a significant association of SS with sIL-2R and a reduced frequency of pSTAT5^{+} Tregs upon IL-2 stimulation, the two variables did not significantly correlate. Further studies are necessary to fully elucidate the role of sIL-2R and Treg function in autoimmune diseases.

Collectively, a reduced Treg function due to an impaired IL-2/IL-2R signalling capacity observed in SS could encourage lymphocytic infiltration into salivary glands. Thereby oral dryness is induced which associated with plasma sIL-2R. Therefore, sIL-2R could potentially act as an indicator for severe SS advancing earlier diagnosis and treatment.

5. Discussion

To investigate the role of immune-related proteins in diabetic complications, we performed a screening of a range of cytokines in patients of long-term T1D with and without vascular complications. We found a significant association between the presence of complications and elevated sIL-2R, which we also detected in other subtypes of diabetes in our second study. Furthermore, we identified an association between sIL-2R and certain SNPs in *IL2RA* and *PTPN2* in both T1D and T2D. In our third study, which focussed on SS, we discovered that patients with a more severe phenotype had the highest levels of plasma sIL-2R. In addition, we observed an impaired IL-2/IL-2R signalling in Tregs from patients with SS.

5.1 What is the functional relevance of sIL-2R?

To this day the functional relevance of sIL-2R remains unclear, but several theories have been postulated. In our studies we did not only replicate the findings that plasma sIL-2R is increased in patients with the autoimmune diseases T1D and SS, but also identified an association with disease severity reflected by the presence of severe vascular complications and pathologic sicca symptoms, respectively. Furthermore, elevated sIL-2R also associated with insulin-treated T2D and with diabetic complications in general. As all of the mentioned disease phenotypes are accompanied by pro-inflammatory immune responses, our findings may lead to the conclusion that higher levels of sIL-2R simply reflect an ongoing inflammation. Indeed, increased sIL-2R has been found in patients with infections, inflammation and autoimmune diseases. Nevertheless, it would be contradicting for effector T cells to shed sIL-2R in order to boost an inflammatory response, as the IL-2R turns into an intermediate-affinity receptor once IL-2Rα is cleaved. Therefore, shedding of IL-2Rα may act as a negative feedback mechanism to contain the immune activation, which could lead to the generation of a harmless by-product, i.e. the sIL-2R (45). However, after its cleavage the sIL-2R retains the ability to bind IL-2 with low affinity suggesting that sIL-2R has a functional role instead of representing an innocuous by-product (65). By binding IL-2 in the microenvironment, sIL-2R could reduce the levels of IL-2 favouring immune

tolerance over immunity and supporting a functional role of sIL-2R in a negative feedback loop intended to restore an immune balance. Tregs constitutively and stably express CD25 and can therefore respond to low IL-2 levels better than effector T cells (45). This principle is employed by low-dose IL-2 treatment, which has shown promising effects in various autoimmune diseases, most prominently T1D (194-197). Regular injections of IL-2 at low doses are safe and selectively stimulates Tregs without stimulating effector T cells (88, 89, 198). Stratification of patients receiving this treatment in ongoing trials by their levels of sIL-2R would be of interest to investigate whether there is an association between sIL-2R and treatment response. Tracking plasma levels of sIL-2R over the course of a successful low-dose IL-2 treatment may assist in further unravelling the exact function of sIL-2R.

The association between sIL-2R and SNPs in *IL2RA* and *PTPN2* strengthens the premise that sIL-2R does have a function. In both PROLONG and our SS study we found elevated sIL-2R to be associated with disease severity, while the expression of CD25 and the frequency of Tregs was similar between groups. If *IL2RA* polymorphisms influenced sIL-2R via the expression of IL-2Rα, the surface expression of CD25 would also be affected. It is highly interesting that the well-known *IL2RA* SNP rs2104286 is not only associated with protection from T1D (85, 199), but also with reduced levels of sIL-2R (199). This association further supports our proposition that elevated sIL-2R could be clinically of use for monitoring the prognosis of a patient suffering from an autoimmune disease. Furthermore, both *IL2RA* and *PTPN2* encode proteins central in the IL-2/IL-2R signalling pathway which is essential in the maintenance of immune tolerance. Therefore, the genetic association with sIL-2R suggests that sIL-2R could be indicative of a breakdown of tolerance and impaired Treg function.

5.2 Treg dysfunction in autoimmune diseases

In paper III, we speculated that the impaired IL-2/IL-2R signalling in Tregs is likely to negatively affect their suppressive activity, thereby allowing effector T cells to infiltrate salivary glands. Shedding of sIL-2R from Tregs could be a mechanism in

which the cells try to compensate for the signalling defects. It is tempting to speculate that the same mechanism is at place in many diseases associated with sIL-2R, particularly in autoimmune diseases such as T1D.

The strong link between Tregs and autoimmunity becomes very clear when studying the phenotype of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), which is caused by loss-of-function mutations in the *FOXP3* gene (200). Over 80% of individuals with IPEX develop T1D before the age of 2 years, indicating a key role of Tregs in maintaining islet-specific tolerance (201). Additionally, many of the identified T1D susceptibility loci are likely to alter Treg function, for instance *IL2RA*, *IL2*, *PTPN2*, *CTLA4* and *IL10*. Indeed, functional defects in Tregs from patients with T1D have been reported independently by several research groups (202-207). Although there is a large body of evidence for an impaired Treg function in T1D, it remains poorly understood how much of it can be attributed to IL-2/IL-2R signalling. Yang et al. elegantly demonstrated that Tregs from patients with long-standing T1D and low IL-2/IL-2R signalling displayed a reduced suppressor function (208). Several studies observed that particularly patients with certain polymorphisms in *IL2RA* and *PTPN2* displayed a reduced Treg function (208, 209). These associations could even be detected in the absence of disease (74, 210).

Due to the mounting evidence on *IL2RA* and *PTPN2* genotype associations with Treg function and sIL-2R, we believe that sIL-2R has the potential to act as a clinical biomarker in various ways. In children at increased risk of developing T1D, elevating plasma sIL-2R levels may be indicative of a reducing Treg function prior to disease onset. These children may particularly benefit from Treg-strengthening therapies, which have the potential to delay and even prevent disease onset. These therapies do not only include low-dose IL-2 treatment, but also the use of adoptive Treg transfer (211, 212).

Most studies investigating Tregs in T1D focus on recent-onset patients in order to investigate the origin of the autoimmune disease and the observed Treg dysfunction may indeed initially drive disease development. However, a persistent Treg

dysfunction combined with a prolonged diabetes duration could have adverse effects on different organs. If pathogenic T cells are not held in check by regulatory mechanisms, the ongoing inflammation could spread to the kidneys, eyes or the cardiovascular system triggering the development of diabetic complications. However, studies focussing on the relationship between Tregs and diabetic complications are scarce. In a T2D mouse model, Treg depletion led to an enhanced insulin resistance and signs of diabetic nephropathy, as shown by increased albuminuria and glomerular hyperfiltration. In the same study, adoptive transfer of Tregs improved insulin sensitivity and kidney function, highlighting for the first time a critical role of Tregs in the pathogenesis of T2D and diabetic nephropathy (111). The interplay between pro-inflammatory T-helper 17 (Th17) cells and Tregs plays a role in the development of autoimmune diseases (213, 214) and has been extensively studied in different autoimmune conditions affecting the eyes, including SS (215-217). Similar mechanisms could be at place in the development of diabetic retinopathy as elevated levels of IL-17 have been detected in vitreous fluid in patients with PDR (218-222). In line with this, a Th17/Treg imbalance has also been reported to contribute to the systemic inflammation associated with obesity and T2D (223). The association between insulin resistance and a chronic, low-grade adipose tissue inflammation is well established and predominantly highlights the infiltration of VAT by pro-inflammatory macrophages. However, mouse studies identified a unique VAT-resident Treg population which is implicated in the inflammatory state of adipose tissue and insulin resistance in mice (224). Interestingly, the master regulator of adipocyte differentiation peroxisome proliferator-activated receptor γ (PPAR-γ) was shown to be crucial for the accumulation, phenotype and function of these VAT Tregs. Remarkably, PPAR-γ expression by VAT Tregs was necessary to restore insulin sensitivity in obese mice treated with PPAR-γ agonists (225).

Altogether, there is increasing evidence underscoring a functional role of Tregs in the pathogenesis of T2D and diabetic complications. Nevertheless, there is still a lack of biomarkers that could indicate a more severe diabetes phenotype, which is at least partially attributed to an impaired Treg function. Our data indicated an association of elevated sIL-2R and diabetes complications in three independent cohorts. Due to the

significance of IL-2R for Treg function, we believe that sIL-2R could assist in the earlier detection and treatment of patients with diabetes. As we did not have the required samples available to investigate Treg function in our diabetes cohorts, we can only speculate whether the associations between sIL-2R and Treg function, which we observed in SS, exist in diabetes as well. Particularly longitudinal studies, such as TEDDY, provide enormous potential and allow the study of Treg function in individuals with diabetes over time and could thereby shed light on new biomarkers.

In paper III, we found that patients with SS had a higher frequency of $pSTAT5^+$ Tregs at baseline, which associated with disease severity and seropositivity. Similar results have been reported previously in SS and other autoimmune diseases (226, 227). Potentially, the switch from not phosphorylated to phosphorylated STAT5 is of importance in IL-2/IL-2R signalling. Constitutively activate STAT5 could possibly inhibit Tregs from reaching the optimal level of phosphorylation once IL-2 binds to its receptor and may thereby contribute to a Treg dysfunction.

Another topic worthy of discussion is the degree of heterogeneity of Treg subpopulations in the circulation and tissues, which leads to inconsistencies between published studies. Tregs present at the site of tissue damage could display a different phenotype compared to circulating peripheral Tregs, especially in organ-specific autoimmune diseases. Unfortunately, human tissue samples are often difficult - if not impossible - to obtain due to the intrusive nature of sample collection or the vital necessity of the organ of interest. The study of human pancreatic tissue relies on post-mortem samples and programmes such as the JDRF-sponsored network for pancreatic organ donors with diabetes (nPOD) will help gain deeper insight into tissue-specific immune dysregulations associated with type 1 diabetes (201). A drawback of our Tregs analyses in paper I and III is that our gating strategy was limited to CD3, CD4, CD25 and CD127 and could thereby not distinguish between tTregs and pTregs. Therefore, we cannot exclude the possibility that the impaired IL-2/IL-2R signalling we observed in circulating Tregs from patients with SS only occurs in one of the Treg subtypes. The transcription factor Helius was shown to be selectively expressed by tTregs and the

inclusion of antibodies targeting Helius in future studies may help in the discrimination between tTregs and pTregs (228).

Overall, Treg dysfunction is a well-established phenotype in autoimmune diseases, but it is debatable whether this is a cause or effect of disease. Studies examining Treg function in recent-onset T1D and in individuals carrying a high-risk haplotype for T1D in the absence of disease indicated that Treg defects pre-date clinical disease, thus supporting a causative role for Treg dysfunction (201, 204, 229). It is highly interesting that autoimmune diseases share many features and often co-occur in one individual, yet we do not know what determines which autoimmune disease develops after a breakage of tolerance. Environmental factors, such as viral infections or tissue injuries, and genetic predispositions probably play a role in this process (5).

5.3 Inflammatory proteins in diabetic complications

We initiated our study of immune factors associated with vascular complications with an untargeted approach screening 30 different inflammatory proteins in a small subset of DIALONG participants. Unfortunately, the detection rate of many cytokines and chemokines analysed was considerably low and statistically did not allow for reliable comparisons. Therefore, we could neither confirm nor confound previous findings reporting an association between diabetic complications and prominent cytokines such as IL-6 and tumour necrosis factor α (TNF-α) (230). The reason for the low detection rate is unclear to us as we did not identify any technical issues regarding quality of the samples, the kit or the Luminex machine itself during analysis.

Nevertheless, we did detect elevated levels of MCP-1 and sIL-2R in patients with CVD compared with patients without complications. Both proteins are prominently involved in the development of vascular complications (231-233), particularly MCP-1 which recruits pro-inflammatory M1 macrophages thereby driving the formation of plaques further (234). In PROLONG, only limited information was available on CVD, therefore we were not able to investigate our findings in a larger cohort. Despite the rather small sample size in DIALONG, patient characterisation for CVD was extremely

detailed enabling reliable data analysis and the identification of asymptomatic coronary artery disease in patients with type 1 diabetes (167).

Considering the complex interplay between all immune cell types, it is highly likely that several immune-related mechanisms are simultaneously at place during the development of diabetic complications (143). Nevertheless, we focused on sIL-2R as it provided the strongest signal, which we could replicate in three independent diabetes cohorts. Our novel contribution to the research field of diabetic complications was the finding that elevated sIL-2R is clearly associated with the presence of vascular complications in T1D and other diabetes subtypes such as T2D. Remarkably, we observed phenotype associations between SNPs in the *IL2RA* and *PTPN2* gene regions and the presence of diabetes complications in patients with T2D, supporting the assumption that T2D pathogenesis encompassed autoimmune aspects. Nevertheless, the interpretation of these results should be handled with care as the observed associations were rather weak and in a small sample size, warranting the need for larger T2D cohorts to replicate these findings.

5.4 Metabolic features of Sjögren's syndrome

Naturally T1D and SS share plenty commonalities due to the autoimmune background in both diseases, but SS and other diabetes subtypes might at first glance seem like two entirely different diseases with distinctive symptoms. However, one should not ignore the central role of metabolism in health and disease, and metabolic mechanisms are likely contributing to the pathogenesis of SS. Indeed, a high frequency of metabolic syndrome has been reported in female patients with SS. Those patients were presented with signs of obesity, a higher frequency of hypertension and diabetes, and increased cholesterol, LDL, triglycerides and leptin. Furthermore, patients with SS had an abnormal adipocytokine profile with high serum levels of adiponectin and resistin. The same study also found an association of metabolic syndrome with elevated serum IL-1β, further supporting a role of inflammation in the pathogenesis of metabolic syndrome and insulin resistance. Interestingly, patients with SS and metabolic syndrome had exacerbated insulin resistance reflected by enhanced levels of insulin

and HOMA-IR (235). SS frequently occurs in post-menopausal women (22) and menopause has also been associated with metabolic syndrome (236). However, the study performed by Augusto et al. balanced the patient groups by their menopause and hormonal replacement therapy status, suggesting an menopause-independent association of SS and metabolic syndrome (235). The presence of metabolic syndrome increases the risk of developing CVD and T2D (80). In fact, a high prevalence of subclinical atherosclerosis (237-239) and cardiovascular events (240, 241) has been observed in patients with SS. Notably, an increased risk of developing T2D has also been reported in SS and many other autoimmune diseases (242). Additionally, a study performed in a T1D mouse model reported decreased salivary flow rate, increased salivary gland inflammation combined with an increase in IL-17-producing cells in peripheral blood, spleen and salivary glands. They suggested that metabolic abnormalities promote the development of SS by increasing the frequency of IL-17-producing cells and their infiltration into the salivary glands (243). A detailed metabolomics study performed in urine and plasma samples from patients with SS found disturbed levels of two lysophosphatidylcholines (244). Lysophosphatidylcholines have been implicated in the pathogenesis of obesity and T2D as well (245), suggesting that an altered phospholipid metabolism may be related to the increased risk of diabetes in patients with SS (244). Altogether, the increasing evidence on metabolic alterations in SS clearly demonstrates that SS and diabetes are more alike than initially assumed.

6. Concluding remarks

The work presented in this thesis highlights the association of circulating sIL-2R with disease severity in different autoimmune and metabolic diseases including T1D, T2D and SS. Together, paper I and II demonstrated that sIL-2R may be a clinically useful indicator assisting in earlier diagnosis and treatment of diabetic complications thereby improving patient care. Moreover, the results from paper III revealed a negative correlation of sIL-2R with salivary flow and described an impaired IL-2/IL-2R signalling in patients with SS.

The findings in paper I demonstrated that genetic polymorphisms in *IL2RA* and *PTPN2* are not only affecting T1D susceptibility, but in addition the development of vascular complications by influencing plasma levels of sIL-2R and potentially lowering T cell responsiveness. We conclude, that sIL-2R may serve as a biomarker in monitoring the risk for developing vascular complications in patients with T1D.

The results of paper II illustrated that *IL2RA* and *PTPN2* gene variants associated with the development of vascular complications in T2D. Furthermore, the association of sIL-2R with diabetic complications in several diabetes subtypes suggests that sIL-2R could potentially act as a general indicator for diabetes severity. This notion was further supported by the elevated plasma levels of sIL-2R in insulin-dependent patients with T2D. Altogether, these results signify a role of IL-2/IL-2R signalling in T2D as well as T1D.

The data of paper III indicated a reduced immunosuppressive function of Tregs due to the impaired IL-2/IL-2R signalling observed in SS. This could mediate a more aggressive lymphocytic infiltration into salivary glands inducing sicca symptoms such as oral dryness, which associated with plasma sIL-2R. In conclusion, sIL-2R could act as a biomarker for SS severity assisting with earlier diagnosis and treatment.

7. Future perspectives

While we studied both sIL-2R and *IL2RA* genotype in patients with T1D with and without vascular complications, we only had sufficient amounts of PBMCs available to characterise the phenotype of different immune cell populations in the circulation. Thus, it would be of great interest to investigate the function of circulating Tregs in terms of their ability to suppress effector T cells as well as study their IL-2/IL-2R signalling capacity. Further, it would be interesting to more deeply characterise the surface expression repertoire and metabolic phenotype of these cells and study different Treg subsets. Ideally, these analyses would be performed in a longitudinal cohort to determine at what timepoint disturbances in immune tolerance can be observed and whether certain immune markers predict the development of diabetic complications early on.

Furthermore, we would like to pursue the study of tolerance-related pathways in people with T2D and explore the metabolic phenotype and function of Tregs in visceral adipose tissue to identify the role of immune tolerance in the progression to insulin resistance.

In paper I and II we found a relevant association of sIL-2R with vascular complications in different subtypes of diabetes, however the sample sizes were too limited to allow for a reliable statistical analysis after stratifying the patients by the type of vascular complication. Therefore, we would like to validate our findings in much larger diabetes cohorts where we could group patients with vascular complications using a more stringent approach. Furthermore, a larger independent cohort would allow us to perform mendelian randomisation in order to investigate whether a causal relationship between sIL-2R, *IL2RA* SNPs and vascular complications can be identified.

In our third study we investigated plasma sIL-2R and Treg function in SS. However, the genetic data was not available. In the future we plan to genotype patients with SS to investigate associations of *IL2RA* and *PTPN2* SNPs with sIL-2R and Treg function. Furthermore, it is of great interest to study additional cytokines linked to SS, particularly IL-17A, to investigate a relationship between those and Treg function or

sIL-2R. Due to the potential involvement of metabolic alterations in the pathogenesis of SS, it would be highly interesting to investigate this in more detail. The Sjögren's syndrome biobank at the Broegelmann Research Laboratory follows patients with SS and longitudinal sample collection continues to expand. In several participants samples from multiple timepoints can already be investigated. A detailed screening of a panel of metabolites and hormones in these samples would allow us to study metabolic changes over time to further unveil the role of metabolism in SS. Combined with genotype data, this will become an extremely powerful and unique dataset in a few years' time.

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Increased Plasma Soluble Interleukin-2 Receptor Alpha Levels in Patients With Long-Term Type 1 Diabetes With Vascular Complications Associated With *IL2RA* and *PTPN2* Gene Polymorphisms

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Type 1 diabetes (T1D) is largely considered an autoimmune disease leading to the destruction of insulin-producing pancreatic β cells. Further, patients with T1D have 3–4-fold increased risk of developing micro- and macrovascular complications. However, the contribution of immune-related factors contributing to these diabetes complications are poorly understood. Individuals with long-term T1D who do not progress to vascular complications offer a great potential to evaluate end-organ protection. The aim of the present study was to investigate the association of inflammatory protein levels with vascular complications (retinopathy, nephropathy, cardiovascular disease) in individuals with long-term T1D compared to individuals who rapidly progressed to complications. We studied a panel of inflammatory markers in plasma of patients with long-term T1D with (n = 81 and 26) and without (n = 313 and 25) vascular complications from two cross-sectional Scandinavian cohorts (PROLONG and DIALONG) using Luminex technology. A subset of

PROLONG individuals (n = 61) was screened for circulating immune cells using multicolor flow cytometry. We found that elevated plasma levels of soluble interleukin-2 receptor alpha (sIL-2R) were positively associated with the complication phenotype. Risk carriers of polymorphisms in the *IL2RA* and *PTPN2* gene region had elevated plasma levels of sIL-2R. In addition, cell surface marker analysis revealed a shift from naïve to effector T cells in T1D individuals with vascular complications as compared to those without. In contrast, no difference between the groups was observed either in IL-2R cell surface expression or in regulatory T cell population size. In conclusion, our data indicates that *IL2RA* and *PTPN2* gene variants might increase the risk of developing vascular complications in people with T1D, by affecting sIL-2R plasma levels and potentially lowering T cell responsiveness. Thus, elevated sIL-2R plasma levels may serve as a biomarker in monitoring the risk for developing diabetic complications and thereby improve patient care.

Keywords: cardiovascular disease, Cluster of Differentiation 25 (CD25), diabetes complications, nephropathy, regulatory T cells, retinopathy, sIL-2R

INTRODUCTION

Type 1 diabetes (T1D) is characterised by T cell mediated autoimmune destruction of insulin-producing pancreatic β cells (1, 2), causing T1D individuals to become insulin-dependent throughout their life (3). Most of the patients with T1D develop macrovascular complications such as cardiovascular disease (CVD), and microvascular complications, including proliferative diabetic retinopathy (PDR), chronic kidney disease (CKD), and peripheral neuropathy (PN) (4). The rising prevalence of T1D and its associated long-term vascular complications clearly confer a humanistic (5) and socio-economic burden (6). Vascular complications in T1D individuals are a common cause of mortality related to end-organ damage as compared to the non-diabetic population (4, 7). Remarkably, few patients with T1D do not progress to these vascular complications despite long disease duration and chronic hyperglycaemia, and therefore exert great potential to evaluate end-organ protection (8).

Although T1D individuals with complications show considerable derangement in immunological processes like having elevated concentrations of C-reactive protein (CRP), a marker of inflammation, proinflammatory cytokines interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) as compared to individuals without complications (9), the extent of contribution of immunological factors to the development of vascular complications in patients with T1D is poorly understood.

Over the past decades, both genetic (10, 11) and immunological (12, 13) studies revealed IL-2 receptor (IL-2R) and its downstream signalling pathways as central players in the pathogenesis of T1D (14). Upon binding of IL-2 to its receptor IL-2R, a cascade of signalling events is initiated. These events are negatively regulated by the ubiquitously expressed phosphatase tyrosine-protein phosphatase non-receptor type 2 (PTPN2) (14, 15). IL-2 signalling is critical for the function of regulatory T cells (Tregs), a type of suppressive immune cell, which plays an indispensable role in maintaining immune homeostasis (16) and prevention of autoimmune diseases (17, 18). In addition, elevated levels of soluble IL-2R alpha (sIL-2R; alternative: IL-2RA, CD25) have been reported to be an important factor in the development of diabetic retinopathy in non-insulin-dependent diabetes patients (19) and coronary artery calcification in T1D patients (20). However, a limitation of both studies was that the patient group without complications had a considerably shorter diabetes duration compared to the patient group with the respective complications. Therefore, some of the patients in the complications-free group could have later progressed to vascular complications. Furthermore, Colombo et al. (2019) reported that elevated levels of sIL-2R were associated with a decline in estimated glomerular filtration rate (eGFR) in T1D patients (21).

The aim of the present study was to evaluate plasma levels of inflammatory proteins including but not limited to sIL-2R in long-term T1D individuals with and without vascular complications. Additionally, a subset of patients was screened for circulating immune cells to investigate cell populations associated with developing vascular complications in T1D individuals. Finally, plasma sIL-2R levels were correlated to genetic risk variants in *IL2RA* and *PTPN2*.

METHODS

Study Design

This immunological investigation forms a part of the PROtective genetic and non-genetic factors in diabetic complications and LONGevity (PROLONG) study, which was launched to identify protective factors against complications in long-term T1D individuals. Patients were recruited from seven departments of endocrinology/diabetes outpatient clinics in Sweden and at the Steno Diabetes Center in Denmark. As an extended collaborative effort, we included T1D individuals from a Norwegian cohort, DIALONG. The DIALONG study also included non-diabetic spouses or friends as a control group.

We classified T1D patients into two groups comparing their diabetes complications status. Non-progressors (NP) were

defined as patients with a T1D duration of over 30 years without having progressed to any of the specified complications. Patients who progressed to complications within 25 years of T1D diagnosis were defined as rapid progressors (RP). We defined late progressors (LP) as T1D patients that did progress to complications >25 years post diagnosis. For this study RPs and LPs were pooled into one group referred to as progressors (P).

In PROLONG, PDR was defined by the presence of proliferative retinopathy in at least one eye, confirmed laser treatment (panretinal photocoagulation) or blindness. For CKD we used the following inclusion criteria: presence of nighttime albuminuria over 200 µg/min, macroalbuminuria over 300 mg/g or a documented diabetic kidney disease diagnosis. None of the PROLONG participants were treated with SGLT2 inhibitors. Documented events including non-fatal myocardial infarction, stroke (haemorrhagic or ischemic), balloon angioplasty, or coronary artery bypass were defined as CVD. There was limited information on CVD, as the original focus of PROLONG was on microvascular complications.

In DIALONG (22) macro- and microvascular complications were defined using similar criteria as in PROLONG. PDR was defined by the presence of proliferative retinopathy or blindness. Laser treatment was not used as criteria here as it was not exclusively applied to proliferative retinopathy in this study. CKD was adjusted to include patients with persistent microalbuminuria or proteinuria. None of the DIALONG participants were treated with SGLT2 inhibitors. CVD was defined by stroke or myocardial infarction events, coronary artery bypass or percutaneous coronary intervention (PCI), diagnosed peripheral vascular disease or heart failure.

The regional ethical committees approved the studies (PROLONG: Denmark H-2-2013-073, Sweden 777/2009, Norway 2019/1324; DIALONG: Norway 2014/851) and all participants provided written informed consent.

Blood Sampling

Collected EDTA plasma was aliquoted and stored at -80°C for ~6 years (PROLONG) and ~3 years (DIALONG).

For peripheral blood mononuclear cells (PBMCs) isolation, peripheral blood from patients with T1D (PROLONG) with and without complications was collected in CPT tubes (BD Vacutainer) at the Steno Diabetes Center, Denmark. PBMCs were subsequently isolated by density gradient centrifugation and cryopreserved in human AB serum containing 10% DMSO at -80°C for ~5 years.

Cytokine Analysis

In DIALONG, plasma cytokines were measured in 79 individuals using an Invitrogen™ Human Cytokine 30-Plex kit (LHC6003M, Thermo Fisher Scientific) according to the manufacturer's instructions. The following adjustments were made to the protocol: (a) one additional standard was included in the serial dilution, making the standard range from 1:3 to 1:2,187; (b) undiluted plasma samples that underwent one freeze-thaw cycle were measured. The following biomarkers were detected in >90% of samples (CCL11, IFN-α, IL-12, sIL-2R, CXCL10, CCL2, CCL3, CCL4 and CCL5), whereas others were only detected in 40–75% (FGF basic, G-CSF, HGF, IL-13, IL-1RA, CXCL9, VEGF) and <17% of patients (EGF, IFN-γ, IL-10, IL-15, IL-17A, IL-1β, IL-2, IL-4, IL-6, TNF-α). GM-CSF, IL-5, IL-7, and IL-8 were not detected in any samples.

In PROLONG, we used a custom-designed ProCartaPlex™ Human Cytokine Panel (sIL-2R, CCL2, CCL11, IFN-α; Thermo Fisher Scientific) according to manufacturer's instructions to measure plasma cytokines. The following adjustments were made to the protocol: (a) one additional standard was included in the serial dilution, making the standard range from 1:4 to 1:32,768 (in pg/ml: sIL-2R 10.06–82,425; CCL2 0.45–3,650; CCL11 0.07–550; IFN-α 0.08–625); (b) undiluted plasma samples that underwent one freeze-thaw cycle were measured.

Data was acquired on a Luminex® 100™, counting 3,000 (DIALONG) and 600 (PROLONG) beads per well. The five-parameter logistic algorithm [weighted by 1/y, (V2.4)] and raw median fluorescence intensity values were used for the creation of standard curves.

Genetic Analysis

The DNA samples in the PROLONG study were genotyped using InfiniumCoreExome-24v1-1 array. Standard quality control steps for GWAS were performed. Imputation was performed using Michigan Imputation Server (https://imputationserver.sph.umich.edu/index.html) using Haplotype Reference Consortium Release 1.1 (HRC.r1-1, GRCh37) as a reference panel. Variants with minor allele frequency (MAF) <5% were excluded before imputation. In the present study, we extracted the region for the *IL2RA* (Chromosome 10: 6,052,652-6,104,288, build GRch37) and *PTPN2* gene (Chromosome 18: 12,785,477-12,929,642, build GRch37) for analysis. Only variants with imputation quality R^2 >0.4 and with MAF >5% were included in the analysis.

Principal component analysis (PCA) was performed on pruned, directly genotyped SNPs using 1,000 Genomes' reference panel version 5A. Population outliers were examined based on visual inspection of PC1 and PC2, and outliers were excluded from the further analysis. Only individuals with complete data on sIL-2R, sex, complication group, HbA1c and age at visit were included. In total, there were 330 individuals analyzed. We used linear regression adjusted for sex, age, and complication group to identify associations between genetic variants and log-transformed plasma levels of sIL-2R.

Antibodies Used for Flow Cytometry

We designed two flow cytometry panels using multiple fluorochrome-conjugated antibodies. Panel 1 includes 14 markers (14 colors), which can discriminate the main mononuclear immune cell types (B cells, T cells, NK cells, monocytes, dendritic cells), and endothelial progenitor cells. Panel 2 includes 16 markers (15 colors) and focuses specifically on different types of T cells. Pacific orange dye (250 ng/ml; Life Technologies) was used as a live/dead marker in both panels. The monoclonal antibodies used in the two panels during the flow cytometry protocol are listed in **Supplementary Table 1**.

Fluorescent Staining for Flow Cytometry

Before staining, cryopreserved PBMCs were rapidly thawed using a water bath set to 37°C and washed once in cold PBS (without calcium and magnesium, Lonza) containing 5% AB serum and Benzonase® Nuclease (1:10,000; Merck Millipore) by centrifugation at 450 x g for 5 min at 4°C. The PBMCs were then resuspended in cold PBS and stained with pacific orange (250 ng/ml; Life Technologies) for 20 min on ice in the dark. Following live/dead staining, cells were washed once, taken up in cold FACS-buffer (PBS containing 0.5% BSA) and incubated with 2 µl Fc receptor block (Miltenyi Biotec) per 1 x 10^6 cells for 10 min on ice. Cells were then subdivided into two parts and incubated for 30 min on ice in the dark with the respective antibody staining panel. The samples were subsequently washed once and re-suspended in FACS-buffer prior to analysis at the flow cytometer.

Flow Cytometry Analysis

Samples were acquired on a LSRI Fortessa flow cytometer (BD Biosciences) with BD FACSDiva™ Software (BD Biosciences) at the Bergen Flow Cytometry Core Facility, University of Bergen, Norway. The flow cytometer was equipped with 407, 488, 561, and 635 nm lasers, and emission filters for PerCP-Cy5.5 (Long Pass (LP): 685, Band Pass (BP): 695/40), Alexa Fluor 488 (LP: 505, BP: 530/30), PE-Cy7 (LP: 750, BP: 780/60), PE (LP: -, BP: 582/15), APC (LP: -, BP: 670/14), Pacific blue (LP: -, BP: 450/50), Pacific orange (LP: 570, BP: 585/42), and BV786 (LP: 750, BP: 780/60). The cytometer was routinely calibrated with BD cytometer setup and tracking beads (BD Biosciences). An average of 138,635 (panel 1) and 122,287 (panel 2) events were recorded in the intact single cell gate and mean percentage of live cells was 98 and 96% for panels 1 and 2, respectively. Flow cytometry data was analyzed in FlowJo™ 10 (Tree Star). Compensation beads (eBioscience) stained with the respective antibody were used as controls to calculate the compensation matrix. The representative gating strategies for both panels are shown in **Supplementary Figures 1** and **2** and were validated with the unsupervised gating method using the tSNE algorithm (**Supplementary Figures 3B, C**). For accurate gating, fluorescence minus one (FMO) controls were run regularly for both panels.

Statistical Analysis

sIL-2R was log2 transformed prior to analysis. Values above the ordinary range were removed by biological consideration and literature review. The Mann-Whitney U test was used in the comparison between the complication groups in the analyses of plasma cytokines. To evaluate the association between two variables we used the Pearson correlation formula. In flow cytometry analysis, multiple linear regression was applied and adjusted for the age and sex covariates. Differences were considered statistically significant when $p < 0.05$. The study was of exploratory nature and hence no correction was made for multiple comparisons. Comparisons between patient groups, correlations and the production of associated graphs were done using R Studio (Version 1.1.456). Figures were arranged in Adobe Illustrator CS6.

RESULTS

Elevated sIL-2R in T1D Individuals

Baseline characteristics of DIALONG study participants are given in **Table 1**. There were 26 T1D individuals with vascular complications (progressor, P), of whom 10 had CKD, 11 had CVD, and all apart from one had PDR. As the matching groups we included 25 T1D individuals without any vascular complications (non-progressors, NP) and 28 healthy controls. In brief, progressors had significantly higher BMI and slightly elevated HbA1c. The groups were balanced regarding age, sex, and eGFR.

There was a significant increase of sIL-2R ($p = 0.0011$) in T1D as compared to healthy individuals (**Figure 1A**). The increase was gradual in relation to vascular complication status, being highest in the progressor group (Control vs. NP: $p = 0.014$; Control vs. P: $p = 0.0021$; NP vs. P: $p = 0.47$) (**Supplementary Figure 4A**). None of the other analyzed cytokines showed significant differences between the T1D groups in relation to their complication status (**Supplementary Figure 5A**). An overview over the detection rate for each investigated cytokine is provided in **Supplementary Figure 5B**.

To investigate complication status further, we stratified progressors into those with CKD, CVD, or PDR. These analyses revealed that progressors with CVD had significantly elevated sIL-2R plasma levels ($p = 0.029$) as compared to NPs (**Supplementary Figure 4B**). Plasma sIL-2R was slightly increased in progressors with CKD ($p = 0.19$) as compared to NPs (**Supplementary Figure 4C**), and sIL-2R correlated negatively with eGFR (T1D: $R = -0.42$, $p = 0.0037$) (**Supplementary Figure 4D**). Adjusting for eGFR did not change the observed result with CKD ($p = 0.35$). Plasma sIL-2R was elevated in progressors with PDR compared to NPs ($p = 0.36$) (**Supplementary Figure 4E**). Monocyte chemotactic protein-1 (CCL2, alternative: MCP-1) plasma levels were significantly higher in progressors with CKD ($p = 0.021$) and CVD ($p = 0.013$) compared to NPs (**Supplementary Figures 4F, G**). CCL2 was slightly elevated in progressors with PDR compared to NPs ($p = 0.09$) (**Supplementary Figure 4H**).

Elevated sIL-2R in T1D Individuals with Vascular Complications

In order to confirm our cytokine findings in DIALONG in a larger and independent cohort, we measured 4 nominally associated cytokines (sIL-2R, CCL2, CCL11, IFN-α) in plasma from PROLONG patients with T1D with and without complications (n = 394). Clinical characteristics for this cohort are summarized in **Table 2**. We included 81 patients with T1D with vascular complications (progressors, P), of whom 40 had PDR, 58 had CKD, and on 2 we had information on CVD. Those individuals were compared to 313 T1D individuals without any vascular complications (non-progressors, NP). Progressors were significantly younger in age, displayed significantly higher BMI and HbA1c and lower eGFR and diabetes duration compared to NPs. The groups were balanced regarding sex.

TABLE 1 | Clinical characteristics of the DIALONG study participants.

Cohort	Healthy control	NP	Progressors	p-value
N	28	25	26	
Age (years)	62.2 ± 6.3	63.1 ± 6.5	62.2 ± 6.5	ns
BMI (kg/m^3)	26.6 ± 4.2[a]	25.1 ± 3.3	27.3 ± 3.9	3.66×10^{-2}
Diabetes duration (years)	NA	50.5 ± 3.4	51.3 ± 5.1	ns
Age at diagnosis (years)	NA	12.6 ± 5.6	10.8 ± 6.5	ns
Sex (% female)	57%	48%	54%	ns
HbA1c (%)	5.5 ± 0.2	7.3 ± 0.8	7.6 ± 0.8	ns
GAD AA positive (%)	7%	29%[b]	32%[c]	ns
IA-2 AA positive (%)	4%	8%[b]	16%[c]	ns
Insulin AA positive (%)	0%	71%[b]	68%[c]	ns
ZnT8 AA positive (%)	0%	4%[b]	8%[c]	ns
AA positive (%)	7%	75%[b]	80%[c]	ns
eGFR (ml/min/1.73 m^3)	83.4 ± 16.4	85.4 ± 15.1	78.5 ± 26.1	ns
C-peptide (nmol/L)	718.3 ± 225.3	undetectable	undetectable	ns
CRP (mg/L)	1.8 ± 2.3[d]	3.3 ± 6.1[e]	3.0 ± 3.3[e]	ns
Statins	6 (21%)	9 (36%)	17 (65%)	3.87×10^{-2}
Beta-blocker	1 (4%)	2 (8%)	10 (38%)	1.16×10^{-2}
ACEi/ARB	6 (21%)	7 (28%)	19 (73%)	1.49×10^{-3}
Antiplatelet agent	6 (21%)	1 (4%)	14 (54%)	1.16×10^{-4}
Loop diuretics	0 (0%)	1 (4%)	7 (27%)	2.69×10^{-2}
PDR/CKD/CVD (n)	NA	NA	25/10/11	

Values for continuous variables are presented as mean ± SD. P-values were calculated between NPs and progressors by Mann–Whitney U test. NP, non-progressor; BMI, body mass index; HbA1c, haemoglobin A1c; GAD, glutamic acid decarboxylase; AA, autoantibody; IA2, islet cell antigen-2; ZnT8, zink transporter 8; eGFR, estimated glomerular filtration rate; C-peptide, connecting peptide; CRP, C-reactive protein; ACEi, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; PDR, proliferative diabetic retinopathy; CKD, chronic kidney disease; CVD, cardiovascular disease.

[a]n = 27, [b]n = 24; [c]n = 25, [d]n = 21, [e]n = 20.

Three cytokines were detected in 100% of samples (sIL-2R, CCL2, CCL11), while IFN-α was only detected <16% of samples (**Supplementary Figure 6B**). We observed a significant increase of sIL-2R (p = 0.0064) in progressors compared to NPs (**Figure 1B**). This observed difference was even more pronounced (p = 0.00084) when comparing NPs with progressors with PDR (**Figure 1C**). Additionally, sIL-2R was slightly increased in patients with CKD (p = 0.077) compared to NPs (**Supplementary Figure 6C**), and sIL-2R correlated negatively with eGFR (R = -0.12, p = 0.025) (**Supplementary Figure 6D**). Adjusting for eGFR using linear regression, resulted in a significant increase in sIL-2R between progressors with CKD and NPs (p = 0.041). Comparisons for CVD could not be made due to the small sample size of progressors with information on CVD (n = 2). As observed in the DIALONG cohort, there was no significant difference between the complication groups in CCL2 (p = 0.46), CCL11 (p = 0.25), and IFN- α (p = 0.40) (**Supplementary Figure 6A**). In PROLONG, CCL2 was not elevated in progressors with CKD (p = 0.39) (**Supplementary Figure 6E**). Progressors with PDR and NPs showed similar levels of CCL2 (p = 0.97) (**Supplementary Figure 6F**).

Association of sIL-2R Levels With IL2RA and PTPN2 Gene Variants

To identify associations between genetic variants in *IL2RA* and plasma levels of sIL-2R, we used linear regression adjusting for sex, age, and group. Plasma levels of sIL-2R were significantly associated with 68 SNPs in the *IL2RA* gene (**Table 3**), with rs12722489 showing the statistically strongest association ($p = 5{,}19 \times 10^{-7}$). Two of our identified SNPs are located in exon 8, namely rs12722606 and rs12722605. The majority of the associated SNPs (n = 51) are located in the large intron 1 area of *IL2RA*.

Furthermore, sIL-2R levels were significantly associated with 53 intronic SNPs in the *PTPN2* gene region (**Table 4**). The variant rs12971201 showed the strongest association with plasma sIL-2R ($p = 1.09 \times 10^{-3}$). When we conditioned this analysis for the T1D-associated SNP rs2104286 in *IL2RA*, we could still identify 42 *PTPN2* variants to be independently associated with sIL-2R (**Table 4**). Here rs592390 had the strongest association with plasma sIL-2R ($p = 2.16 \times 10^{-3}$).

Cell-Surface Marker Analysis on PBMC of T1D Individuals

Our flow cytometry panels enabled us to investigate a range of different cell populations, which are summarized in **Supplementary Figure 3A**. The applied gating strategies are provided in **Supplementary Figure 1** and **2**. Baseline characteristics for this subset of PROLONG patients are provided in **Table 2**. In total, we performed flow cytometry analysis on 61 T1D samples, of which 17 were from progressors. The groups were balanced regarding age and sex.

We identified a significant decrease of $CD8^+$ naïve T cells ($CD3^+CD8^+CD45RA^+CCR7^+CCR5^-$) (p = 0.0046) and increase of $CD8^+$ effector T cells ($CD3^+CD8^+CD45RA^+CCR7^-$) (p = 0.070) in progressors compared to NPs (**Figures 2A, B**). Furthermore, progressors had significantly increased $CD4^+$ effector T cells ($CD3^+CD4^+CD45RA^+CCR7^-$) (p = 0.045) and decreased $CD4^+$ naïve T cells ($CD3^+CD4^+CD45RA^+CCR7^+CCR5^-$) (p = 0.14) compared to NPs (**Figures 2C, D**). To summarize, we observed

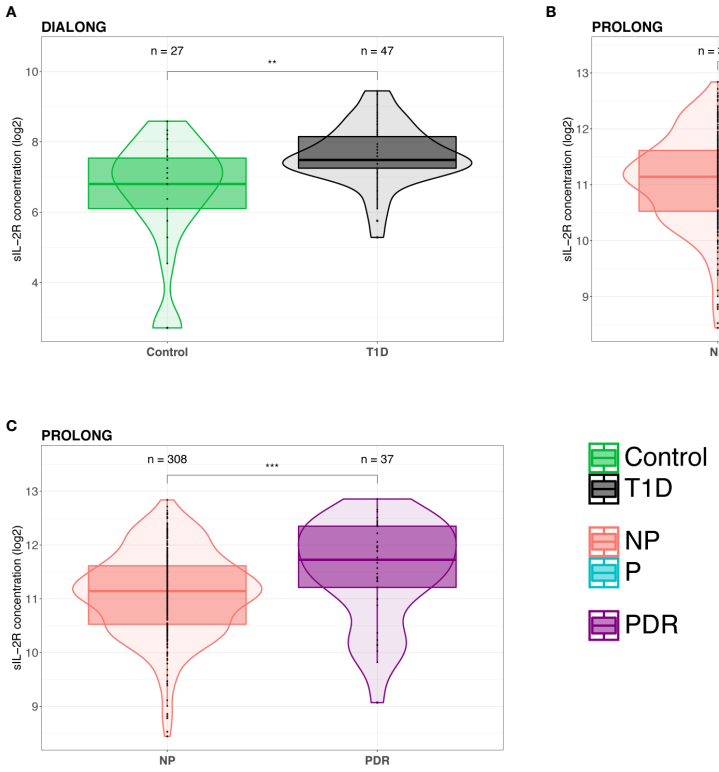

FIGURE 1 | Elevated plasma levels of sIL-2R in patients with type 1 diabetes (T1D). **(A)** Patients with T1D from the DIALONG cohort had significantly elevated sIL-2R plasma levels compared to healthy controls. **(B)** In PROLONG, sIL-2R plasma levels were significantly increased in T1D with vascular complications (progressors, P) compared to T1D patients without complications (non-progressors, NP). **(C)** PROLONG progressors with proliferative diabetic retinopathy (PDR) showed significantly higher sIL-2R plasma levels compared to progressors with other vascular complications. (The Mann-Whitney U test was used in the comparison between the different groups. **p < 0.01 and ***p < 0.001).

TABLE 2 | Clinical characteristics of the PROLONG participants.

Cohort	Cytokine assay			Flow cytometry		
	NP	Progressors	p-value	NP	Progressors	p-value
n	313	81		44	17	
Age (yrs.)	58.1 ± 10.6	44.6 ± 13.7[a]	1.08×10^{-13}	50.6 ± 7.2	50.8 ± 15.5	ns
BMI (kg/m^3)	24.8 ± 3.7[b]	26.4 ± 4.7	3.94×10^{-3}	25.3 ± 4.2	25.3 ± 4.7	ns
Diabetes duration (years)	40.6 ± 8.6	22.4 ± 8.3	$<2.2 \times 10^{-16}$	37.7 ± 5.1	29.9 ± 13.4	9.49×10^{-3}
Age at diagnosis (years)	17.5 ± 9.9	22.0 ± 14.4[a]	ns	12.9 ± 6.0	20.9 ± 13.2	4.63×10^{-2}
Sex (% female)	58%	53%	ns	59%	53%	ns
HbA1c (%)	7.6 ± 0.9	8.9 ± 1.5	3.33×10^{-14}	7.4 ± 0.9	8.5 ± 0.8	6.35×10^{-5}
GAD AA positive (%)	50%[c]	68%	8.15×10^{-3}	50%[d]	90%[e]	2.41×10^{-2}
eGFR (ml/min/1.73 m^3)	90.2 ± 15.5	97.4 ± 30.8[f]	3.66×10^{-3}	94.6 ± 16.6	93.3 ± 29.7	ns
C-peptide (nmol/L)	0.03 ± 0.07[g]	0.03 ± 0.05[h]	ns	0.01 ± 0.02[i]	0.02 ± 0.01[j]	3.37×10^{-2}
PDR/CKD/CVD (n)	NA	40/58/2		NA	10/12/0	

Values for continuous variables are presented as mean ± SD. P-values were calculated by Mann–Whitney U test. NP, non-progressor; BMI, body mass index; HbA1c, haemoglobin A1c; GAD, glutamic acid decarboxylase; AA, autoantibody; eGFR, estimated glomerular filtration rate; C-peptide, connecting peptide; PDR, proliferative diabetic retinopathy; CKD, chronic kidney disease; CVD, cardiovascular disease.

[a]n = 77; [b]n = 308; [c]n = 303; [d]n = 40; [e]n = 10; [f]n = 76; [g]n = 296; [h]n = 68; [i]n = 35; [j]n = 7.

TABLE 3 | Imputed *IL2RA* genotypes and their significant associations with sIL-2R in plasma.

Chr	SNP	bp*	Intron/Exon	A1	n	Beta (95% CI)	p-Value
10	rs12722489	6102012	Intron 1	T	330	–0.28 (–0.39, –0.17)	5.19×10^{-7}
10	rs12722517	6081040	Intron 1	C	330	–0.24 (–0.34, –0.14)	2.23×10^{-6}
10	rs9663421	6055604	Intron 7	T	330	0.25 (0.15, 0.35)	3.17×10^{-6}
10	rs12722553	6071284	Intron 1	A	330	–0.26 (–0.37, –0.15)	3.30×10^{-6}
10	rs12722551	6071379	Intron 1	T	330	–0.26 (–0.37, –0.15)	3.30×10^{-6}
10	rs791593	6083292	Intron 1	G	330	–0.23 (–0.33, –0.14)	4.22×10^{-6}
10	rs2104286	6099045	Intron 1	C	330	–0.24 (–0.33, –0.14)	4.63×10^{-6}
10	rs41294713	6080354	Intron 1	C	330	–0.25 (–0.36, –0.15)	5.23×10^{-6}
10	rs12722515	6081230	Intron 1	A	330	–0.25 (–0.36, –0.15)	5.23×10^{-6}
10	rs791590	6090322	Intron 1	T	330	–0.25 (–0.36, –0.15)	5.23×10^{-6}
10	rs2246031	6092210	Intron 1	T	330	–0.25 (–0.36, –0.15)	5.23×10^{-6}
10	rs7078614	6075831	Intron 1	T	330	–0.21 (–0.30, –0.12)	5.50×10^{-6}
10	rs7920946	6074634	Intron 1	C	330	–0.22 (–0.31, –0.13)	5.53×10^{-6}
10	rs4625363	6072504	Intron 1	G	330	–0.25 (–0.36, –0.15)	5.54×10^{-6}
10	rs12722527	6077328	Intron 1	T	330	–0.25 (–0.36, –0.15)	5.54×10^{-6}
10	rs12722523	6078390	Intron 1	A	330	–0.25 (–0.36, –0.15)	5.54×10^{-6}
10	rs12722561	6069893	Intron 1	T	330	–0.25 (–0.36, –0.15)	6.22×10^{-6}
10	rs12722559	6070273	Intron 1	A	330	–0.25 (–0.36, –0.15)	6.22×10^{-6}
10	rs12722606	6053133	Exon 8	A	330	0.25 (0.14, 0.36)	6.98×10^{-6}
10	rs11256335	6055222	Intron 7	A	330	0.25 (0.14, 0.36)	6.98×10^{-6}
10	rs12722605	6053163	Exon 8	A	330	0.29 (0.17, 0.42)	7.27×10^{-6}
10	rs12722497	6095928	Intron 1	A	330	0.35 (0.20, 0.50)	1.22×10^{-5}
10	rs11256464	6082558	Intron 1	T	330	0.32 (0.18, 0.47)	2.55×10^{-5}
10	rs11597237	6079017	Intron 1	T	330	–0.23 (–0.33, –0.12)	3.07×10^{-5}
10	rs11256416	6075359	Intron 1	T	330	–0.21 (–0.31, –0.11)	3.67×10^{-5}
10	rs7910961	6077796	Intron 1	T	330	0.20 (0.11, 0.30)	4.04×10^{-5}
10	rs4747837	6058735	Intron 7	G	330	–0.23 (–0.34, –0.12)	4.34×10^{-5}
10	rs7900385	6062748	Intron 4	A	330	–0.23 (–0.34, –0.12)	4.34×10^{-5}
10	rs12722588	6060433	Intron 6	T	330	–0.22 (–0.33, –0.11)	5.83×10^{-5}
10	rs12722587	6060630	Intron 6	T	330	–0.22 (–0.33, –0.11)	5.83×10^{-5}
10	rs7093069	6063319	Intron 4	T	330	–0.22 (–0.33, –0.11)	5.83×10^{-5}
10	rs11816044	6074082	Intron 1	A	330	0.20 (0.10, 0.30)	8.91×10^{-5}
10	rs7100984	6078539	Intron 1	A	330	0.20 (0.10, 0.30)	9.07×10^{-5}
10	rs12722574	6066462	Intron 2	A	330	–0.20 (–0.29, –0.10)	9.63×10^{-5}
10	rs4749894	6058323	Intron 7	G	330	0.22 (0.11, 0.33)	1.00×10^{-4}
10	rs4749924	6082396	Intron 1	C	330	0.20 (0.10, 0.29)	1.00×10^{-4}
10	rs6602398	6082953	Intron 1	T	330	0.20 (0.10, 0.29)	1.00×10^{-4}
10	rs7900744	6065611	Intron 3	G	330	–0.20 (–0.29, –0.10)	1.19×10^{-4}
10	rs791588	6089342	Intron 1	G	330	0.15 (0.06, 0.24)	7.71×10^{-4}
10	rs11256342	6057231	Intron 7	G	330	0.17 (.07, 0.26)	8.37×10^{-4}
10	rs12253981	6092346	Intron 1	G	330	0.16 (0.07, 0.25)	8.84×10^{-4}
10	rs2025345	6067688	Intron 2	G	330	0.16 (0.07, 0.26)	9.75×10^{-4}
10	rs12358961	6066195	Intron 3	A	330	0.16 (0.07, 0.26)	1.03×10^{-3}
10	rs1924138	6096158	Intron 1	A	330	0.16 (0.06, 0.25)	1.06×10^{-3}
10	rs11256497	6087794	Intron 1	A	330	0.15 (0.06, 0.25)	1.36×10^{-3}
10	rs10795752	6072354	Intron 1	T	330	0.15 (0.06, 0.25)	1.51×10^{-3}
10	rs2245675	6095577	Intron 1	A	330	0.16 (0.06, 0.25)	1.55×10^{-3}
10	rs2256852	6096923	Intron 1	A	330	0.16 (0.06, 0.25)	1.55×10^{-3}
10	rs791587	6088699	Intron 1	A	330	0.14 (0.05, 0.23)	1.62×10^{-3}
10	rs12251836	6091281	Intron 1	A	330	0.15 (0.06, 0.24)	1.65×10^{-3}
10	rs6602368	6062915	Intron 4	C	330	0.15 (0.06, 0.24)	1.67×10^{-3}
10	rs2476491	6095410	Intron 1	T	330	0.16 (0.06, 0.26)	1.74×10^{-3}
10	rs4749926	6085312	Intron 1	A	330	0.15 (0.05, 0.24)	2.60×10^{-3}
10	rs11256457	6080794	Intron 1	G	330	0.15 (0.05, 0.24)	2.72×10^{-3}
10	rs10905641	6072293	Intron 1	C	330	0.14 (0.05, 0.23)	3.41×10^{-3}
10	rs6602379	6073374	Intron 1	G	330	0.14 (0.05, 0.24)	3.51×10^{-3}
10	rs809356	6091148	Intron 1	C	330	0.13 (0.04, 0.22)	3.60×10^{-3}
10	rs2256774	6097165	Intron 1	C	330	0.14 (0.05, 0.23)	3.87×10^{-3}
10	rs1323657	6072427	Intron 1	A	330	0.13 (0.04, 0.23)	3.95×10^{-3}
10	rs7072398	6079846	Intron 1	A	330	0.13 (0.04. 0.22)	5.33×10^{-3}
10	rs10795763	6096199	Intron 1	G	330	0.13 (0.04. 0.22)	5.34×10^{-3}
10	rs7917726	6096600	Intron 1	G	330	0.13 (0.04. 0.22)	5.34×10^{-3}
10	rs706779	6098824	Intron 1	C	330	0.12 (0.03. 0.21)	7.97×10^{-3}

(Continued)

TABLE 3 | Continued

Chr	SNP	bp*	Intron/Exon	A1	n	Beta (95% CI)	p-Value
10	rs10905656	6086093	Intron 1	A	330	0.11 (0.02. 0.20)	1.51×10^{-2}
10	rs3793713	6059704	Intron 7	G	330	0.11 (0.01. 0.20)	2.56×10^{-2}
10	rs4749920	6071453	Intron 1	C	330	0.11 (0.01. 0.21)	3.73×10^{-2}
10	rs4749921	6071654	Intron 1	C	330	0.11 (0.01. 0.21)	3.73×10^{-2}
10	rs4747845	6074441	Intron 1	A	330	0.11 (0.01. 0.21)	3.73×10^{-2}

SNP positions according to the Genome Reference Consortium Human Build 37 (GRch37).
Linear regression model: p-value = adjusted for age, sex and complication group. Chr, chromosome; SNP, single nucleotide polymorphism; bp, base pair; A1, minor allele; CI, confidence interval.

a shift from naïve to effector T cells ($CD4^+$ and $CD8^+$) in PBMCs from T1D patients with vascular complications compared to those without.

Since we observed significant differences in plasma sIL-2R in the two PROLONG groups, we also investigated cell surface expression of interleukin-2 receptor alpha (CD25) on PBMCs. Remarkably, we did not see differences between NPs and progressors in neither $CD25^+$ T cells (p = 0.84) nor Tregs ($CD3^+CD4^+CD25^{++}CD127^-$) (p = 0.27) (**Supplementary Figures 7A, B**).

DISCUSSION

The present study revealed that plasma sIL-2R levels are reproducibly elevated in individuals with long-term T1D with severe vascular complications as compared to those who remained free from to vascular complications despite more than 30 years of diabetes duration. Further, plasma levels of sIL-2R were associated with SNPs in the *IL2RA* and *PTPN2* gene regions, which might suggest underlying genetic determinants and possibly biological causal inference. Finally, our results are in agreement with published studies confirming an increase of circulating sIL-2R in patients with T1D when compared to healthy controls, which might further emphasize that immune factors contributing to diabetes pathogenesis might early on govern progression to vascular complications (12, 23).

The biological function of sIL-2R is not yet completely understood, but there is evidence that it reflects an imbalance in Treg and effector T cell (T_{EFF}) activity (14, 24). It has been suggested that there is a reduced immunosuppressive function of Tregs due to impaired IL-2 signalling in T1D (24–28), a defect which may subsequently lead to a more aggressive immune destruction of pancreatic β cells by T_{EFF} (12, 28). In addition, defects in the intracellular IL-2 pathways and a decreased regulatory function have recently been reported in patients with type 2 diabetes (T2D) (29). In many autoimmune diseases, such as multiple sclerosis (MS) (30), rheumatoid arthritis (31), primary Sjögren's syndrome (32), scleroderma (33), and inflammatory myopathies (34), but also in various cancers (31), sIL-2R has been proposed to be a biomarker for disease activity.

Patients with T1D develop the disease at an early age and a large proportion of them will progress to devastating vascular complications representing a major problem because the tools for monitoring when and how disease deteriorates are not available (4). Diabetes retinopathy is the most common vascular complication of diabetes (35) and the proliferative form of diabetic retinopathy (PDR) is the leading cause of vision loss in adults (36). Previous studies show that several inflammatory cytokines are involved in the pathogenesis and progression of PDR, including sIL-2R (19), however, not all of the results have been replicated. In the present study, both PROLONG and DIALONG progressors with PDR had higher plasma levels of sIL-2R compared to NPs, supporting the notion that sIL-2R could emerge as a contributing player not only in the pathogenesis of T1D but also in disease progression.

An additional evidence in the present study to biological importance of sIL-2R were our findings that 68 *IL2RA* SNPs are associated with sIL-2R plasma levels in PROLONG patients with T1D and correlated with the elevated sIL-2R levels observed in progressors accordingly. *IL2RA* rs12722489 showed the strongest association with sIL-2R plasma levels in patients with T1D and is located in the large intron 1. This particular SNP has been identified as a risk factor for MS in several studies (37–39). However, a secondary association was suggested due to the nearby location of rs2104286 (Linkage disequilibrium D' = 1, R^2 = 0.58), which is a well-recognized T1D risk factor (11, 37) and also significantly associated with plasma sIL-2R in our dataset. Interestingly, one of our identified SNPs, rs2256774, was associated with higher levels of Rubella antibodies (40), and Rubella viral infections have been associated with the onset of T1D (41, 42). Additionally, multiple *IL2RA* variants have been shown to correlate with sIL-2R levels in T1D (10) and MS (37) and *IL2RA* gene variants are associated with susceptibility of T1D (10, 11, 43). Further, functional studies support these results by suggesting that specific *IL2RA* variants cause defects in immune homeostasis due to impaired IL-2 signalling in Tregs (25, 27, 44).

Interestingly, seventeen of our identified SNPs are positioned within the first 15 kb of the first intron of the *IL2RA* gene, which has been described as a super-enhancer region due to a cluster of histone 3 lysine 27 acetylation (H3K27ac) elements in this area (45). Notably, many of the *IL2RA* SNPs related to autoimmune diseases fall into this region and affect transcription factor binding and enhancer activity (46). Several of our identified SNPs have been reported to be associated with DNA methylation at the *IL2RA* promoter locus, particularly rs6602398 and rs4749926 (47). Despite having detected a number of *IL2RA* SNPs to be associated with sIL-2R, it is challenging to conclude the direct effect of those mostly intronic SNPs on the expression of IL-2R itself and in-depth research is scarce. However, it has

TABLE 4 | Imputed *PTPN2* genotypes and their significant associations with sIL-2R in plasma.

Chr	SNP	bp*	Intron/Exon	A1	n	Beta (95% CI)	p-Value$_a$	p-Value$_b$
18	rs12971201	12830538	Intron 4	A	330	0.16 (0.07, 0.26)	1.09×10^{-3}	3.01×10^{-3}
18	rs2542162	12820900	Intron 5	T	330	0.16 (0.06, 0.26)	1.13×10^{-3}	2.91×10^{-3}
18	rs2847281	12821593	Intron 5	G	330	0.16 (0.06, 0.26)	1.13×10^{-3}	2.91×10^{-3}
18	rs2852151	12841176	Intron 2	A	330	0.16 (0.06, 0.26)	1.16×10^{-3}	3.38×10^{-3}
18	rs3826557	12843263	Intron 2	T	330	0.16 (0.06, 0.26)	1.16×10^{-3}	3.38×10^{-3}
18	rs674222	12848349	Intron 2	C	330	0.16 (0.06, 0.26)	1.16×10^{-3}	3.38×10^{-3}
18	rs2847273	12856908	Intron 2	C	330	0.16 (0.06, 0.26)	1.16×10^{-3}	3.38×10^{-3}
18	rs641085	12824930	Intron 5	T	330	0.15 (0.06, 0.24)	1.61×10^{-3}	2.22×10^{-3}
18	rs592390	12822314	Intron 5	C	330	0.15 (0.06, 0.24)	1.67×10^{-3}	2.16×10^{-3}
18	rs12957037	12829065	Intron 4	G	330	0.15 (0.06, 0.24)	1.67×10^{-3}	2.16×10^{-3}
18	rs588447	12832842	Intron 3	C	330	0.15 (0.06, 0.24)	1.69×10^{-3}	2.48×10^{-3}
18	rs8087237	12834359	Intron 3	A	330	0.15 (0.06, 0.24)	1.69×10^{-3}	2.48×10^{-3}
18	rs478582	12835976	Intron 3	C	330	0.15 (0.06, 0.24)	1.69×10^{-3}	2.48×10^{-3}
18	rs559406	12857002	Intron 2	G	330	–0.14 (–0.24, –0.05)	1.94×10^{-3}	2.44×10^{-3}
18	rs960550	12827697	Intron 4	T	330	0.15 (0.06, 0.25)	2.08×10^{-3}	6.02×10^{-3}
18	rs4797709	12882359	Intron 1	C	330	0.15 (0.05, 0.24)	2.43×10^{-3}	6.64×10^{-3}
18	rs2292759	12884343	upstream	A	330	0.15 (0.05, 0.24)	2.43×10^{-3}	8.09×10^{-3}
18	rs2542157	12787247	Intron 10	G	330	0.14 (0.04, 0.23)	5.57×10^{-3}	4.98×10^{-3}
18	rs2847291	12808713	Intron 8	A	330	0.14 (0.04, 0.24)	6.86×10^{-3}	1.43×10^{-2}
18	rs11663472	12810471	Intron 8	A	330	0.14 (0.04, 0.24)	6.86×10^{-3}	1.43×10^{-2}
18	rs2847286	12817815	Intron 6	G	330	0.14 (0.04, 0.24)	6.86×10^{-3}	1.43×10^{-2}
18	rs2847285	12818224	Intron 6	A	330	0.14 (0.04, 0.24)	6.86×10^{-3}	1.43×10^{-2}
18	rs45456495	12792228	Intron 10	T	330	0.13 (0.03, 0.24)	9.11×10^{-3}	1.77×10^{-2}
18	rs2542167	12795849	Intron 9	T	330	0.13 (0.03, 0.24)	9.11×10^{-3}	1.77×10^{-2}
18	rs2847298	12800120	Intron 9	G	330	0.13 (0.03, 0.24)	9.11×10^{-3}	1.77×10^{-2}
18	rs2542160	12789246	Intron 10	C	330	0.13 (0.03, 0.23)	1.06×10^{-2}	1.94×10^{-2}
18	rs2847299	12801337	Intron 9	A	330	0.14 (0.03, 0.24)	1.10×10^{-2}	3.06×10^{-2}
18	rs7227207	12819616	Intron 5	T	330	–0.13 (–0.23, –0.03)	1.15×10^{-2}	2.13×10^{-2}
18	rs72872125	12876915	Intron 1	T	330	0.19 (0.04, 0.34)	1.24×10^{-2}	1.72×10^{-2}
18	rs60474474	12792736	Intron 10	T	330	–0.14 (–0.25, –0.03)	1.68×10^{-2}	2.03×10^{-2}
18	rs45450798	12792940	Intron 10	G	330	–0.14 (–0.25, –0.03)	1.68×10^{-2}	2.03×10^{-2}
18	rs60751993	12795420	Intron 9	A	330	–0.14 (–0.25, –0.03)	1.68×10^{-2}	2.03×10^{-2}
18	rs60735058	12795470	Intron 9	A	330	–0.14 (–0.25, –0.03)	1.68×10^{-2}	2.03×10^{-2}
18	rs8096138	12808140	Intron 8	G	330	–0.14 (–0.25, –0.03)	1.68×10^{-2}	2.03×10^{-2}
18	rs1893217	12809340	Intron 8	G	330	–0.14 (–0.25, –0.03)	1.68×10^{-2}	2.03×10^{-2}
18	rs11663253	12789556	Intron 10	G	330	–0.13 (–0.25, –0.02)	1.84×10^{-2}	2.12×10^{-2}
18	rs10502416	12822702	Intron 5	T	330	–0.13 (–0.24, –0.02)	2.01×10^{-2}	2.14×10^{-2}
18	rs78637414	12826836	Intron 4	A	330	–0.13 (–0.24, –0.02)	2.01×10^{-2}	2.14×10^{-2}
18	rs62097820	12834649	Intron 3	T	330	–0.13 (–0.24, –0.02)	2.01×10^{-2}	2.14×10^{-2}
18	rs8096327	12887750	Upstream	G	330	–0.10 (–0.19, –0.01)	2.91×10^{-2}	3.71×10^{-2}
18	rs3737361	12831324	Intron 3	C	330	–0.11 (–0.22, –0.01)	3.07×10^{-2}	5.45×10^{-2}
18	rs16939910	12837993	Intron 2	A	330	–0.11 (–0.22, –0.01)	3.07×10^{-2}	5.45×10^{-2}
18	rs3786158	12843275	Intron 2	A	330	–0.11 (–0.22, –0.01)	3.07×10^{-2}	5.45×10^{-2}
18	rs11080605	12847329	Intron 2	C	330	–0.11 (–0.22, –0.01)	3.07×10^{-2}	5.45×10^{-2}
18	rs62097858	12862581	Intron 1	A	330	–0.11 (–0.22, –0.01)	3.07×10^{-2}	5.45×10^{-2}
18	rs8091720	12865186	Intron 1	T	330	–0.11 (–0.22, –0.01)	3.07×10^{-2}	5.45×10^{-2}
18	rs7244152	12854294	Intron 2	C	330	–0.11 (–0.22, –0.01)	3.23×10^{-2}	5.44×10^{-2}
18	rs11080606	12867969	Intron 1	C	330	–0.11 (–0.22, –0.01)	3.23×10^{-2}	5.44×10^{-2}
18	rs7242788	12820330	Intron 5	A	330	–0.11 (–0.22, –0.01)	3.30×10^{-2}	5.79×10^{-2}
18	rs12959799	12900695	Upstream	G	330	0.11 (0.01, 0.21)	4.01×10^{-2}	5.41×10^{-2}
18	rs80262450	12818922	Intron 6	A	330	–0.13 (–0.25, –0.01)	4.13×10^{-2}	3.45×10^{-2}
18	rs56946650	12916943	Upstream	T	330	–0.11 (–0.22, –0.00)	4.16×10^{-2}	6.45×10^{-2}
18	rs2847282	12819820	Intron 5	G	330	–0.09 (–0.19, –0.00)	4.79×10^{-2}	6.62×10^{-2}

**SNP positions according to the Genome Reference Consortium Human Build 37 (GRch37)*

Linear regression models: p-value$_a$ = adjusted for age, sex and complication group; p-value$_b$ = adjusted for age, sex, complication group and rs2104286. Chr, chromosome; SNP, single nucleotide polymorphism; bp, base pair; A1, minor allele; CI, confidence interval.

been reported that one of our identified SNPs, rs12251836, is associated with *IL2RA* expression on acutely triggered T_{EFF}, but not in Tregs (48), suggesting a likely biological causal inference of IL-2R gene locus in T1D.

Another exciting and supporting genetic observation included 53 *PTPN2* SNPs to be significantly associated with sIL-2R plasma levels, importantly 42 of which were associations independent of *IL2RA* variant rs2104286. Our most significant SNP in *PTPN2* is rs12971201, which has previously been associated with T1D, however a secondary association has been suggested due to rs1893217 (49). This particular variant is considered a risk variant for T1D and celiac disease (50, 51),

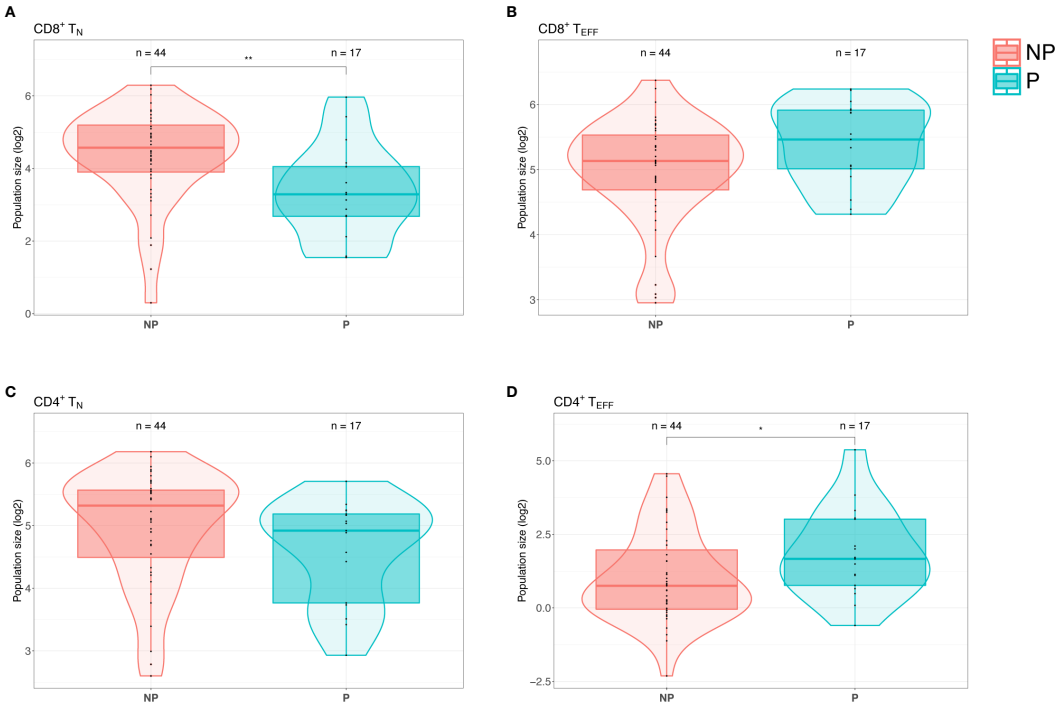

FIGURE 2 | Patients with type 1 diabetes (T1D) with complications display a shift from naïve T cells to effector T cells. **(A)** Flow cytometry screening of PBMCs from PROLONG patients revealed a significant decrease of $CD8^+$ naïve T (T_N) cells ($CD3^+CD4^+CD45RA^+CCR7^+CCR5^-$) in progressors (P) compared to non-progressors (NP). **(B)** Progressors displayed elevated $CD8^+$ effector T (T_{EFF}) cells ($CD3^+CD4^+CD45RA^+CCR7^-$) simultaneously (p = ns). **(C)** $CD4^+$ T_N cells were also declined in patients with T1D with complications as compared to NPs (p = ns). **(D)** In progressors $CD4^+$ T_{EFF} cells were significantly elevated compared to NPs. (For the comparison between the different groups, multiple linear regression was applied and adjusted for the age and sex covariates. *$p < 0.05$ and **$p < 0.01$).

and correlated with impaired IL-2 signalling in $CD4^+$ T cells as measured by decreased phosphorylation of STAT5 (pSTAT5) but also reduced PTPN2 expression (52). One of our identified SNPs, rs2847281, was shown to significantly associate with CRP levels along many other *PTPN2* variants (53). PTPN2 is a negative regulator in the IL-2 signalling cascade and several SNPs in the *PTPN2* gene region have been linked to different autoimmune diseases including T1D, rheumatoid arthritis and Crohn's disease (54, 55). Furthermore, different genetic variants in *PTPN2* were reported to be associated with diminished IL-2 responsiveness in naïve Tregs from patients with long-standing T1D (56). PTPN2 has been shown to modulate interferon gamma signal transduction in pancreatic β cells and regulate cytokine-induced apoptosis, which could potentially contribute to the pathogenesis of T1D (57).

As described above, elevated sIL-2R levels are likely to reflect an imbalance in Treg and T_{EFF} activity. PBMCs from PROLONG patients with T1D with vascular complications displayed a shift from naïve T cells (T_N) to T_{EFF}, however, we cannot distinguish whether this shift is the cause or the result of diabetic complications. The interpretation of these results was difficult due to the small and heterogeneous sample size in the progressors group. One can speculate that progressors have increased T_{EFF} due to impaired Treg suppression leading to a more destructive form of T1D, thereby more active course of the disease. This systemic long-term inflammation could subsequently drive the development of vascular complications by affecting tissues aside from the pancreas. Further studies testing suppression capacity of Tregs isolated from patients with T1D with and without complications are crucial to confirm this notion.

In PROLONG, we observed significantly higher levels of sIL-2R in progressors which associated with *IL2RA* SNPs, however the surface expression of IL-2R on circulating immune cells was similar between progressors and NPs. This may be confirmatory for the theory that it is not the number of cells expressing IL-2R making a difference, but the efficiency of IL-2 signalling within the cells themselves. Paradoxically, the IL-2/IL-2R signalling pathway is important in immunity and tolerance, which is further complicated by shedding of sIL-2R. How sIL-2R is involved in the pathogenesis of different diseases remains a

puzzle. The high-affinity receptor for IL-2 consists of 3 protein chains, namely IL-2Rα, IL-2Rβ, and IL-2Rγ. Upon proteolytic cleavage of the ectodomains of the membrane-bound IL-2Rα and release into the extracellular space, sIL-2R retains the ability to bind IL-2 with low affinity, which can lead to different outcomes. Firstly, sIL-2R may function as a decoy-receptor reducing the bioavailability of IL-2 and favor tolerance controlled by Tregs over immunity. Tregs constitutively express the high-affinity IL-2R, which enables them to out-compete conventional T cells with the intermediate-affinity receptor (IL-2Rβ and IL-2Rγ) when less IL-2 is available, thereby boosting immune tolerance (58). This difference in affinity is exploited in clinical trials in T1D where the administration of low-dose IL-2 has shown promising effects expanding and activating Tregs (59, 60). Alternatively, the binding of sIL-2R to IL-2 can enable *in trans* presentation of IL-2 to T-cells which only express the intermediate-affinity IL-2R. Overall, increased shedding of sIL-2R and it binding to IL-2 can have opposing effects depending on the cell type affected (58).

Stratified analysis of different vascular complications revealed increased sIL-2R and CCL2 levels in DIALONG patients with CVD in comparison to patients with T1D with other diabetic complications. Elevated sIL-2R has been described as a marker for coronary artery calcification progression in both individuals with and without T1D independent of traditional CVD risk factors (20). CCL2 plays a critical role in the development of atherosclerotic plaque formation by attracting monocytes to the vessel lumen where they will differentiate into macrophages and become foam cells by the uptake of low-density lipoprotein (61). Elevated plasma levels of CCL2 have also been associated with CVD (61–63). The observed increases in sIL-2R and CCL2 were based on a small sample set in DIALONG, however, the patient characterisation for CVD was performed thoroughly using computed tomography coronary angiography, which enabled the identification of asymptomatic coronary artery disease (64). Nevertheless, we were not able to investigate this finding in PROLONG due to the limited information on CVD.

Previously it was shown in the EURODIAB study that patients with T1D with complications have increased IL-6 and TNF-α as compared to individuals without complications (9). We could neither confirm nor confound this finding due to the low detection rate of IL-6 and TNF-α (< 20%) in our study, which statistically did not allow for reliable comparisons. In general, the detection rates in our cytokine screening were considerably low, where many of the investigated biomarkers were not detected at all. This could be due to technical differences and kit quality, however all kits used were validated for plasma usage by the respective providers, we followed manufacturer's instructions accordingly and did not experience technical issues during analysis.

Our future perspective is to unravel the role of IL-2R in the progression to diabetic complications in general, larger cohorts analyzing sIL-2R levels in other types of diabetes, such as T2D with no autoimmunity and latent autoimmune diabetes of adults, are of importance. To investigate the predictive power of sIL-2R levels in the development of diabetic complications, longitudinal studies in children and adolescents would be a great asset. Furthermore, it is of great interest to study the relationship between sIL-2R and IL-2 signalling efficacy and Treg function in patients with T1D.

In summary, we conclude that *IL2RA* and *PTPN2* gene variants may not only increase the risk of T1D, but in addition the development of diabetic complications possibly by influencing sIL-2R plasma levels and lowering T cell responsiveness. Thus, sIL-2R could potentially act as a biomarker for monitoring vascular complications in people with T1D thereby enabling early treatment and improving patient care.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of GDPR and ethical restrictions. Requests to access the datasets should be directed to valeriya.lyssenko@uib.no.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by PROLONG-Sweden: Regional Ethics Review Board, Department 1, Lund, Sweden, Dnr 777/2009; PROLONG-Denmark: Scientific-Ethical Committee for the Capital Region of Denmark, Hillerød, Denmark, Dnr H-2-2013-073; DIALONG: Regional Committees for Medical and Health Research Ethics (REC), South-East regional health authority, panel D, Norway, 2014/851; Data analysis of both studies at University of Bergen: REC, West regional health authority, Norway 2019/1324. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VL, RJ, and SA conceived the study, and MK and SA designed the immunological part of the study. TM, CL, HF, TN, S-BC, GJ, LG, ME, BE, KB, and PN contributed to the study design and data collection. VL is the PI of the PROLONG study. TB is the PI of the DIALONG study. MK conducted the flow cytometric and cytokine analysis and wrote the manuscript. MK, OF, EP, BB, and SA analyzed and processed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.575469/full#supplementary-material

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