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DEVELOPMENT OF IMMUNOMODULATORY RNA THERAPEUTICS FOR IMMUNE DISORDERS

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Cover illustration: It illustrates a T cell surrounded by cytokines and ASOs. The image was self-drawn and the T cell was inspired by artistic renderings of immune cells by Sandra Black.

Development of Immunomodulatory RNA Therapeutics for Immune Disorders
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Vaishnavi Srinivasan Iyer

The thesis will be defended in public at CMM Lecture Hall, Karolinska University Hospital, Solna, Sweden, on Friday the 26th of August 2022 at 9.00 am.

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To My Family. Without you, none of this would be possible.

~ The sky is no longer the limit when you’re reaching for the stars~

POPULAR SCIENCE SUMMARY OF THE THESIS

The immune system is important for survival; it helps to defend your body against attacks from harmful foreign organisms such as bacteria or viruses. However, sometime the cells that comprise the immune system make mistakes in identifying their target. When they identify a harmless foreign object as a pathogen it can lead to allergies. When they mis-identify certain harmless proteins produced by cells of our own body as harmful it can lead to autoimmune disease. In many autoimmune diseases, the cells of the adaptive immune system cause severe inflammation in various tissues causing pain and damage to these tissues. There are drugs that combat some of the symptoms, but they can only target certain proteins that are "druggable" and have the right structure for these small molecule drugs to bind to, and this limits the proteins we can target to modulate cells. However, there are newer classes of drugs that can potentially target any protein and thus modulate any cell type but haven't been used to treat immune diseases yet. Thus, the aim of this thesis project was to investigate if these newer drugs was a viable way to reduce the harmful over-activation of T cells in the autoimmune context.

The first step to drug development is to figure out what drug to use and what the particular target of the drug will be. In my project, I used an antisense oligonucleotide (ASO): a chain of 16 nucleotides, made of building blocks similar to that of our genome. When a cell wants to express a gene, the cell copies the gene in the form of RNA to be translated to a protein. The ASO binds to this RNA, causing degradation of the RNA and consequently reduced amount of this protein.

We validated a gene target by knocking out genes using a technique called CRISPR where we induce mutations in a gene to render it non-functional. A CRISPR mediated knock out of a gene will result in no protein at all, mimicking a situation where the ASO is 100% efficient and manages to take away all expression of the target protein. Using this technique, we validated in **paper I** that when LCP2 is knocked out, T cells were unable to get activated, and concluded that it would be a good target to develop ASOs against.

In **paper II**, we designed, synthesized and tested several ASO sequences against LCP2, identified the most efficient sequence. We used this sequence in artificial cells in the lab as well as cells taken from human donors and found that it indeed worked to reduce some of the activation signals in T cells.

The next steps were to study the pathogenic T cells further. It is thought that there are particular T cells that are specific to particular proteins that contribute to the disease, so **paper III** and **paper IV** study particular specificities of T cells that were found in patients with rheumatoid arthritis.

ABSTRACT

Autoimmune diseases such as Rheumatoid Arthritis (RA) are complex and involve many different cell types. Of these cell types, T cells are thought to be key drivers of the inflammation. The current treatment options available are restricted to small molecule drugs and Biologics, typically exerting its therapeutic activity by interfering with exact protein structures. Notably, only a fraction of the proteins in the body are predicted to be druggable by such drug modalities, greatly reducing the potential drug targets.

Antisense Oligonucleotides (ASOs) are synthetic oligonucleotides that are designed to manipulate expression of their target genes at the post-transcriptional level. As ASOs have the potential to target any gene, thus greatly expanding the universe of potential drug targets, it represents a viable therapeutic approach to modulate aberrant pathways in diseases like RA.

In **paper I**, we present a present a novel way of using CRISPR-mediated gene knock out to validate the role of target genes in particular cellular pathways of interest. We describe the RCC (rapid CRISPR competitive) assay, where we exploit the heterogeneity of the CRISPR-Cas9 mediated genome editing together with sanger sequencing to study the role of the target gene based on both the enrichment or depletion of mutated sequences but also the type of mutation (frameshift or silent mutations). In particular, LCP2 was validated as good target to reduce the activation potential of T cells. Thus in **paper II**, ASOs targeting LCP2 were designed and evaluated for their knockdown potential and their subsequent ability to reduce T cell activation. We show that these ASOs can modulate T cell behavior in human cell lines and human primary cells, validating the potential of ASOs as a therapeutic approach for immune diseases.
There has been a lot of research into the phenotype of T cells found in RA patients and how they differ from healthy controls. One of the hypotheses for the progression of the disease is that T cells reacting to specific peptides are thought to contribute to the disease. Thus, **paper III** and **paper IV** focus on studying TCR specificities to gain a better understanding of the particular T cells that could be the culprits. In particular, these papers focus on development of methods to identify interesting TCR sequences from patients and re-expression of these TCRs *in vitro* and *in vivo* to study them in detail with methods to recapitulate patient T cells *in vitro*.

Though focused currently on limited gene targets, this paves the way to answer the overarching questions forming the basis of my PhD: what is a good drug target, and how can technological developments transform the field of drug development?

LIST OF SCIENTIFIC PAPERS

I. Shen Y, Jiang L#, **Iyer VS**,**#**, Raposo B, Dubnovitsky A, Buddul SV, Kasza Z, Wermeling F.

A rapid CRISPR competitive assay for *in vitro* and *in vivo* discovery of potential drug targets affecting the hematopoietic system.
Comput Struct Biotechnol J. 2021 Sep 20; 19:5360-5370. doi: 10.1016/j.csbj.2021.09.020. PMID: 34745454
shared authorship

II. **Iyer VS**, Boddul SV, Johnsson AK, Raposo B, Sharma RK, Shen Y, Kasza Z, Lim KW, Chemin K, Nilsson G, Malmström V, Phan AT, Wermeling F.

Modulating T-cell activation with antisense oligonucleotides targeting lymphocyte cytosolic protein 2.
J Autoimmun. 2022 Jun 30, 131:102857. doi: 10.1016/j.jaut.2022.102857. PMID: 35780036

III. Boddul SV, Sharma RK, Dubnovitsky A, Raposo B, Gerstner C, Shen Y, **Iyer VS**, Kasza Z, Kwok WW, Winkler AR, Klareskog L, Malmström V, Bettini M, Wermeling F.
In vitro and ex vivo functional characterization of human HLA-DRB1∗04 restricted T cell receptors.
J Transl Autoimmun. 2021 Mar 3; 4:100087.

IV. **Iyer VS,** Sharma RK, Boddul SV, Malmström V, Wermeling F.
Citrullinated peptide-reactive T-cell receptors in patients with rheumatoid arthritis.
Manuscript

OTHER PUBLICATIONS

Not included in the thesis

I. Jiang L, Ingelshed K, Shen Y, Boddul SV, **Iyer VS**, Kasza Z, Sedimbi S, Lane DP, Wermeling F.
CRISPR/Cas9-Induced DNA Damage Enriches for Mutations in a p53-Linked Interactome: Implications for CRISPR-Based Therapies.
Cancer Res. 2022 Jan 1;82(1):36-45. doi: 10.1158/0008-5472.CAN-21-1692. PMID: 34750099

II. Panda SK, Wigerblad G, Jiang L, Jiménez-Andrade Y, **Iyer VS**, Shen Y, Boddul SV, Guerreiro-Cacais AO, Raposo B, Kasza Z, Wermeling F.
IL-4 controls activated neutrophil FcγR2b expression and migration into inflamed joints.
Proc Natl Acad Sci U S A. 2020 Feb 11;117(6):3103-3113. doi: 10.1073/pnas.1914186117. Epub 2020 Jan 24. PMID: 31980518 Free PMC article.

Reviews

III. **Iyer VS**, Jiang L, Shen Y, Boddul SV, Panda SK, Kasza Z, Schmierer B, Wermeling F.
Designing custom CRISPR libraries for hypothesis-driven drug target discovery.
Comput Struct Biotechnol J. 2020 Aug 18;18:2237-2246. doi: 10.1016/j.csbj.2020.08.009. eCollection 2020.PMID: 32952937

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LIST OF ABBREVIATIONS

ACPA	Anti-cirtullinated protein antibody
APC	Antigen presenting cell
ASO	Antisense Oligonucleotide
BCR	B cell receptor
Cas	CRISPR associated protein
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DC	Dendritic cell
DMARDs	Disease modifying anti-rheumatic drugs
DNA	Deoxyribonucleic acid
GADS	GRB2-related adaptor protein 2
GFP	Green fluorescent protein
GRB2	Growth factor receptor bound protein 2
GWAS	Genome-wide association studies
HA	Influenza hemagglutinin
HLA	Human leukocyte antigen
HLMC	Primary human lung mast cells
Ig	Immunoglobulin
IL2Rα	IL-2 receptor alpha (a.k.a CD25)
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine based activation motifs.
KD	Knock down
KO	Knock out
LATat	Linker for activation of T cells
LCP2	Lymphocyte cytosolic protein 2 (a.k.a SLP-76)
LNA	Locked nucleic acid
mAb	Monoclonal antibody
MHC	Major histocompatability complex
MMP	Matrix metalloproteinases

NCK	non-catalytic region of tyrosine kinase adaptor protein 1
NFκB	Nuclear factor-kappa B
NK cell	Natural killer cell
NSAIDs	Non-steroidal anti-inflammatory drugs
NTC	Non-targeting control
PAD4	Peptidyl arginine deaminase 4 (encoded by *PADI4* gene)
PBMC	Peripheral blood mononuclear cells
PS	Phosphorothioate
RA	Rheumatoid Arthritis
RCC	Rapid CRISPR competitive
RF	Rheumatoid factor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein (complex)
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SLP-76	Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76-kDa (a.k.a LCP2)
SNP	Single nucleotide polymorphisms
TCR	T cell receptor
TRAC	T cell receptor alpha subunit constant region
Treg	Regulatory T cell
VAV	Vav guanine exchange factor
WT	Wild type
ZAP-70	Zeta chain associated protein kinase of 70 kDa

1 INTRODUCTION

The immune system is essential to protect the body from pathogens. When an infection occurs, the cells of the immune system work together to recognize the infectious agent, eliminate it and the infected cells. In most cases, the body also manages to retain an immunological memory so that the next time the same or similar infectious agent attacks the body, the immune system can mount a faster and more specific response.

The immune system also has the capacity for self-regulation, dealing with modulation of immune responses by cells of the body. However, sometimes this self-regulation is imperfect and can result in a misidentification of self-antigens as harmful. When the body mounts a response against these harmless antigens, it can lead to chronic inflammation which can further develop into immune disorders.

Many of these immune disorders are currently being treated by several classes of drugs. Among these are broadly anti-inflammatory corticosteroids, DMARDs (disease modifying anti-rheumatic drugs) such as methotrexate, and NSAIDs (non-steroidal anti-inflammatory drugs) which dampen the immune system in general. There are also more specific drugs that have a defined target; these include biologics that target certain receptors on cell surfaces or certain cytokines. While these have been shown to be effective for some patients, more can be done for those who don't respond or develop tolerance to these drugs.

There are many proteins involved in the signaling pathways in these inflammatory cells which could be modulated to reduce disease burden. Traditional small molecule drugs bind to certain 'pockets' in their target protein. However, not all proteins have a structure that enables the binding of these small molecule drugs, i.e. they are not traditionally "druggable". Thus, there is a mismatch in the availability of potential drug targets and the drugs that currently exist to target them. One solution to this is to expand to other classes of drugs such RNA therapeutics.

For a gene to be expressed, the gene is transcribed to an RNA sequence which is then translated to a protein sequence which folds into the final protein structure to be functional. RNA therapeutics encompass several different types of drugs including siRNA (small interfering RNA), shRNA (small-hairpin RNA) and ASOs (antisense oligonucleotides). Each of the different types of RNA therapeutics use different cellular enzymes to modulate gene expression at the RNA level, one step below the protein level. This enables the targeting of any protein in any pathway without limitations on the structure of the protein. Thus RNA therapeutics provides the possibility for any protein to be a potential drug target.

This thesis explores the field of drug discovery by first, validation of drug targets to modulate T cell activity and subsequently the development and validation of ASOs developed for these targets. Through my PhD work and hopefully continuing in the future I want to study the overarching questions of what a good drug target is and how we can use this information to transform the field of drug discovery.

2 LITERATURE REVIEW

Immune disorders are a vast collection of diseases afflicting the immune system. Rheumatoid Arthritis (RA) is one such autoimmune disease. There are treatments, but there is no cure yet. In order to understand the disorder and the potential therapeutic modalities that can be applied, it is first important to understand the immune system itself, and the delicate balance of the interactions of its many different components.

2.1 INTRODUCTION TO THE IMMUNE SYSTEM

The immune system is designed to protect the body from any internal and external threats. It comprises several organs including the spleen, bone marrow, thymus, lymph nodes, mucosal lymphoid tissue and the lymphatic vessels [1]. It is an intricate web of many different cells and tissues, interacting in specific ways to identify and neutralise any threat to the body, e.g. that of a pathogen. Many components work together to ensure homeostasis; when any one of these components are dysregulated, it can cause imbalance in the system and lead to many immune disorders.

The immune system is often divided into the innate immune system and the adaptive immune system [2]. The innate and adaptive responses complement each other; the innate immune system is responsible for the immediate response upon encountering an infectious organism, while the adaptive immune response responds much later, taking up to a few days to develop. The adaptive immune system drives a more targeted removal of pathogens and retains memory of the pathogen – to protect against future infections.

The immune system has 4 main tasks [3]:

1. Immunological recognition to detect the presence of an infectious pathogen.
2. Immune effector functions, e.g. the complement system, antibodies, and the cytotoxic activities of lymphocytes for pathogen elimination.
3. Immune regulation: self-regulation of effector cells. Dysregulation can lead to allergies and autoimmune diseases.
4. Immunological memory: to protect the body against recurring attacks by the same pathogen.

2.2 THE INNATE IMMUNE SYSTEM

The innate immune system forms the bulk of the various different immune cell types and is the first to respond to an infection. Its role is to eliminate as much of the threat as possible through generic pathogen trapping and killing.

There are two main types of committed progenitors in the bone marrow responsible for differentiating into most of the cells of the immune system: common myeloid precursors (CMPs) and common lymphoid precursors (CLPs) [4]. CMPs are precursors to the cells of the innate immune system and CLPs give rise to both cells of the adaptive immune system (B

and T cells) as well as the innate system i.e., innate lymphoid cells (ILCs) [5]. The myeloid progenitors form the granulocyte progenitor and erythrocyte/megakaryocyte progenitor. The granulocyte progenitor gives rise to neutrophils, eosinophils, basophils, mast cells and monocytes/macrophages. Myeloid progenitors can also give rise to immature dendritic cells (DCs), but DCs can also be derived from the CLPs.

Phagocytic neutrophils are the most in number and are very important in protection against infections. They phagocytose a variety of microorganisms and efficiently degrade them using degradative enzymes from their cytoplasmic granules [5]. The main role of macrophages and neutrophils are that of phagocytic pathogen clearance while the role of dendritic cells (DCs) are that of antigen presentation and priming of T cells. Mature DCs prime T cells by presenting the antigen and providing additional co-stimulatory signals. Thus DCs are also known as antigen presenting cells (APCs). However, macrophages also act as APCs while sampling the environment and B cells perform the role of an APC by activating T cells in tissues for example. Together, DCs, Macrophages and B cells are all important APCs with overlapping functions and distributions in the body [6].

The erythrocyte progenitor differentiates into red blood cells and platelets. Red blood cells carry oxygen throughout the body in their haem groups and platelets are responsible for blood clotting in the event of damage [1].

Innate lymphoid cells (ILCs) are an important group of cells that have features of both the innate and adaptive immune system [7]. ILCs are derived from a lymphoid precursor and produce cytokines similar to T cells but do not express antigen receptors the way B and T cells do. There are three major subgroups of ILCs, and natural killer (NK) cells are most similar to ILC1s and are thus now considered group I ILCs [8]. The NK cells is responsible for killing abnormal cells such as cancer cells or other virus-infected cells and are thought to be important for managing viral infections before the adaptive immune response is initiated.

2.3 THE ADAPTIVE IMMUNE SYSTEM

Cells that are specific for an antigen, namely B and T cells, comprise the adaptive immune system. The common lymphoid progenitor in the bone marrow gives rise to 4 main types of cells: T cell, B cells and ILCs and immature dendritic cells (DCs), of which the latter two are important to connect innate and adaptive immune responses. ILCs produce necessary activating cytokines to guide e.g. T cell responses [7] and DCs present peptides to T cells to direct antigen specific immune responses.

Many infections can be handled by the innate immune response. However, B and T cells also play a central role in eliminating acute infections, along with recurring infections. The adaptive response can specifically target a particular pathogen and provide lasting immunological memory as well as specificity in pathogen recognition and elimination during acute infections. The importance of B and T cells is shown in primary immune deficiencies (PID), a group of immune disorders with mutations or deficiencies in the innate or adaptive immune system that reduces the ability to clear infections from the body [9].

Lymphocytes traverse the body through the lymphatic system made up of lymphoid tissues and organs. Lymphoid organs are broadly classified into central (primary) or peripheral (secondary) organs. Lymphocytes are generated in primary lymphoid organs which are the bone marrow and the thymus. Peripheral lymphoid organs are comprised of the lymph nodes, spleen and mucosal lymphoid tissues; this is where mature naïve lymphocytes are stored and where adaptive immune responses can be initiated.

B and T cells have specialised receptors on their cell surface that recognise specific antigens. B cells have a B cell receptor (BCR) and T cells have their T cell receptor (TCR). A naïve B cell gets activated by a combination of antigen recognition as well as other stimuli from helper T cells. These activated B cells can then proliferate and differentiate into antibody secreting plasma cells or undergo class switching to IgG (or oter Igs) expressing B cells, some of which will go on to become memory cells. B cells can express different types of immunoglobulins (Ig), also known as antibodies, and they play a role in neutralising the target antigen. CD4+ T cells are important for the B cell activation and class switching [5]. T cells are responsible for recognising a peptide-MHC (major histocompatability complex) (discussed below), presented by antigen presenting cells (APCs) and mounting a response against it if recognised. Binding of the cognate antigen to the TCR of a T cell promotes activation and proliferation of the T cell.

2.4 T CELLS, T CELL RECEPTOR MEDIATED SIGNALLING AND THE ROLE OF LCP2

T cells develop from CLPs in the bone marrow differentiating into immature T cells. The immature T cells travel to the thymus for "education", where they develop into mature T cells, each with its own unique T cell receptor (TCR) sequences [10]. Each TCR sequence is formed from recombination of the V, D and J regions of the TCR alpha and beta loci in the genome [11]. An extensive TCR diversity is achieved by the VDJ recombination and the combinations of TCR-α and β variable chains. Theoretically, there can be 10^{15}-10^{20} potential TCR combinations, however a large portion get rejected during the "education" phase in the thymus. There are an estimated 10^{10} different TCRs in the body, with different specificities. Many of these TCRs may recognise the same or similar antigen, and similarly, the same MHC can present different peptides. Nevertheless, the polymorphism in the TCR and MHC loci provides a vast repertoire of T cells expressing different TCRs that can recognise any potential antigen the body may encounter.

T cells signal through the TCR. Antigen presenting cells (APCs) present a peptide in complex with the major histocompatibility complex (MHC) on the cell surface. The peptide-MHC complex interacts with the TCR and is stabilised by either CD4 or CD8 depending on the cell and MHC class (**figure 1**). In humans, MHC is encoded by the human leukocyte antigen (HLA) genes. There are two types of MHC – class I and class II. MHC class I mainly interacts with CD8+ T cells, and in humans have 3 main alleles: HLA-A, HLA-B and HLA-C (with HLA-E, F,G being minor alleles). MHC class II is encoded by the following alleles: HLA-DR, HLA-DQ, and HLA-DP are the major alleles (HLA-DM, HLA-DOA, HLA-DOB

are the minor alleles) [12], and presents to CD4+ T cells. This peptide-MHC complex interacts with the TCR complex and triggers the downstream signalling cascade. The TCR consists of a dimer of a TCRα chain and a TCRβ chain in complex with CD3 molecules. CD3 is necessary for proper TCR function. Together, CD3γ, CD3δ, CD3ε and CD3ζ complex with the TCR α/β dimer and chain to form the TCR complex (**figure 1**).

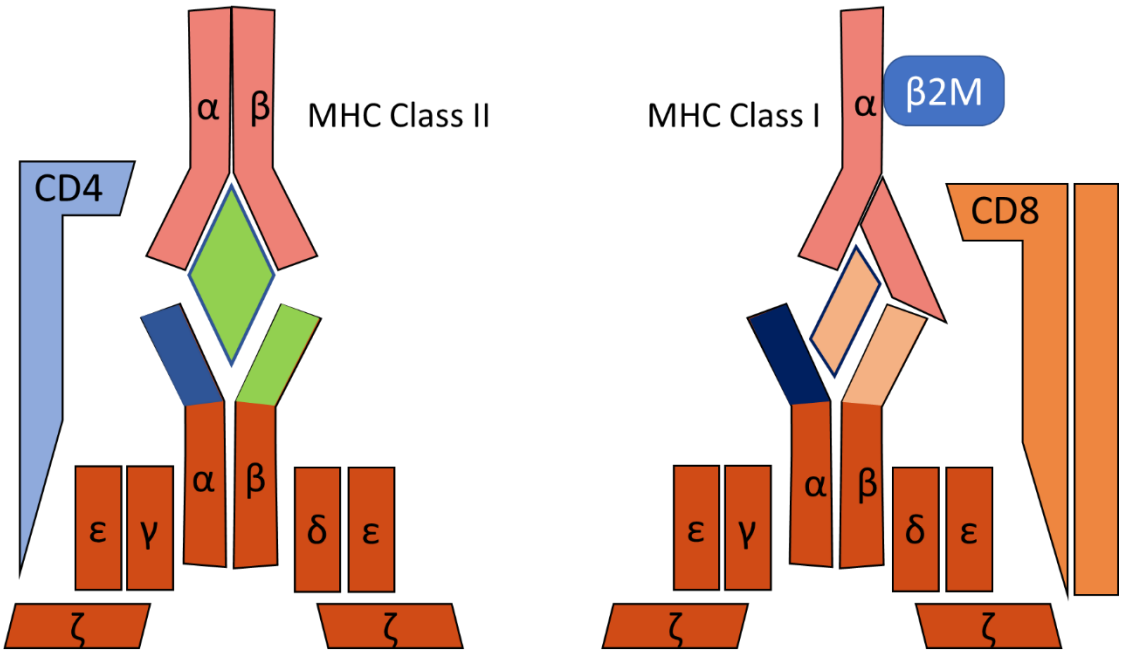

Figure 1: TCR-MHC interaction. The variable domains of the MHC and TCR αβ chains are specific for different peptides. CD4+ T cells interact with MHC class II and CD8 positive T cells interact with MHC class I.

Naïve T cells require two signals from an APC to be activated: the first signal links the CD3/TCR complex to the peptide-MHC complex on the APC, the second signal binds CD28 on the T cell to CD80/86 on the APC [13]. Upon receiving both signals, a signal cascade will occur resulting in T cell activation and proliferation in a process known as priming. After the initial priming, CD4+ T cells will still prefer both signals for strong activation, while CD8+ T cells only require antigen presentation via MHC I for activation. A natural feedback mechanism also exists to stop proliferation after it is no longer required: CTLA-4 is upregulated on the T cell surface and it binds to CD80/86, blocking the second signal and preventing the re-activation of the cell [14]. CD4+ T cells interact with APCs which are one of the major classes of cells that express MHC class II. MHC class II expression is to a large extent limited to APCs such as DCs, macrophages and B cells [15], and in thymic epithelial cells [16]. In contrast, CD8+ T cells interact with many other cell types, patrolling the body for foreign proteins presented by MHC class I, which is broadly expressed by cells around the body. Once the initial priming has occurred, the activated state is maintained by cytokine release e.g. IL2 and upregulation of the respective receptors e.g. IL2Rα.

The TCR signalling cascade aims to achieve three important goals linked to T cell activation: cytoskeletal restructuring, Ca^{2+} signalling and cytokine/activation marker production. Upon

TCR-MHC binding, the CD3-ζ-chain phosphorylates Zap-70 [17]. Zap-70 further phosphorylates the LAT complex, among which is the important signalling protein lymphocyte cytosolic protein 2 (LCP2), also known as SLP-76. LCP2, together with ITK, phosphorylates and activates PLCγ1 [18, 19]. This signalling leads to the following downstream effects: PIP2 is converted to DAG and IP3 [20]. IP3 leads to the opening of calcium channels and the influx of calcium from both intra and extracellular sources activates calcineurin mediated NFAT activation [21], and subsequent IL-2 (cytokine) transcription. DAG interacts with PKCθ, leading to the activation of NFκB [22]. Phosphorylated LCP2 interacts with VAV and NCK, which along with DAG mediated Ras pathway activation, leads to the activation of other transcription factors such as AP-1[23] which, for example, transcribe genes involved in cytoskeletal restructuring and CD69 as an early activation marker [24] (**figure 2**).

When looking at T cell activation in my studies, I look for the following measurable variables: Cytokines such as IL-2, activation markers such as CD69 and CD25 and transcription factor mediated activation (cloned with GFP markers) such as NFAT and NFκB.

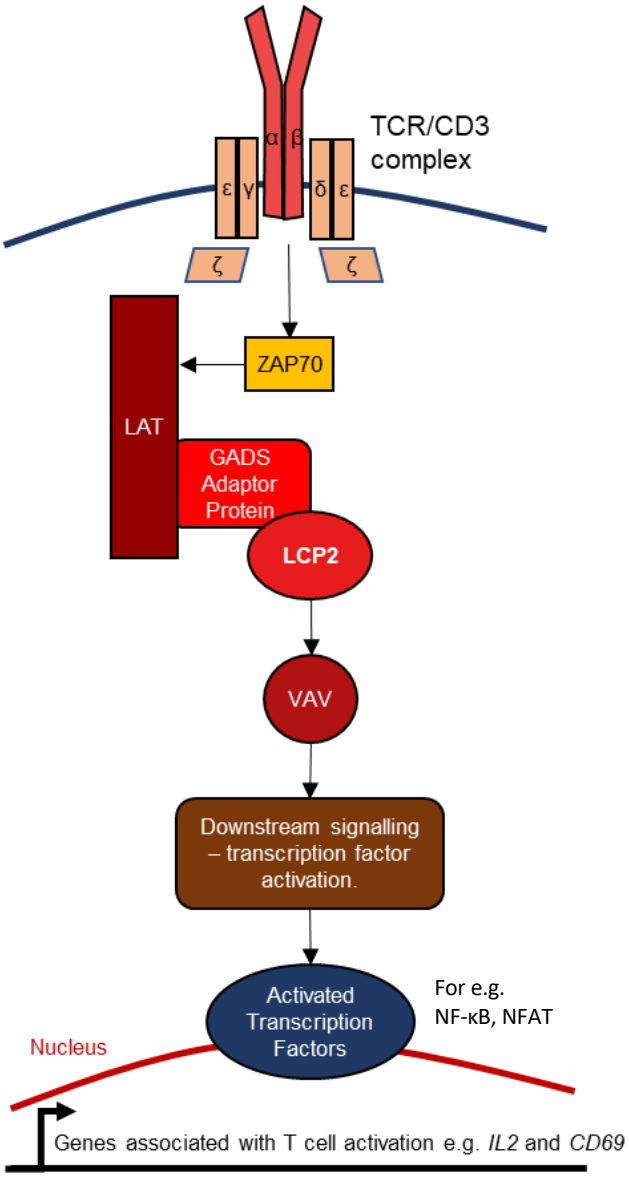

Figure 2: The TCR signalling pathway. Signal transduction in a T cell after TCR cross-linking. Figure adapted from Iyer et. al, 2022 [25].

2.4.1 Lymphocyte cytosolic protein 2 (LCP2)

LCP2 is also known as SLP-76 (Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76-kDa) is a 533 amino acid long adaptor protein. In itself, LCP2 does not catalyze reactions; it has several binding pockets into which various other molecules dock to form larger signaling complexes. It has integral functions in cells with surface receptors containing intracellular ITAMs or Fc regions. LCP2 can be split into 3 main domains: the C-terminal domain (a.k.a the SH2 domain), the central proline rich SH3 binding domain and the N-terminal acidic SAM (sterile α motif) SH2 binding domain [26]. The C-terminal binds various proteins including but not limited to, ADAP (a.k.a FYB) [27, 28], and phosphorylated CD6 [29]. The central domain has binding sites for the SH3 domains of GRB2 and GADS, other adaptor proteins involved in TCR signaling that form LAT complex. Phosphorylated VAV1 and NCK1 bind to the phosphorylated tyrosine residues in the N-terminal SH2 binding domain [30] (**figure 3**). LCP2 is an integral part of the TCR signal transduction pathway and thus modulation of LCP2 should lead to modulation of T cell activity. This is supported by studies showing that *Lcp2* knock-out (KO) mice have shown a lack of mature T cell development and lack of proper positive and negative selection in the thymus [31, 32]. It has also been shown to be essential for mast cell and platelet function [23, 33]. While *LCP2* KO in the human genome is not the solution to over-activated cells, knocking down LCP2 in order to reduce T cell activation may be a viable solution in, for example, inflamed joints of RA patients.

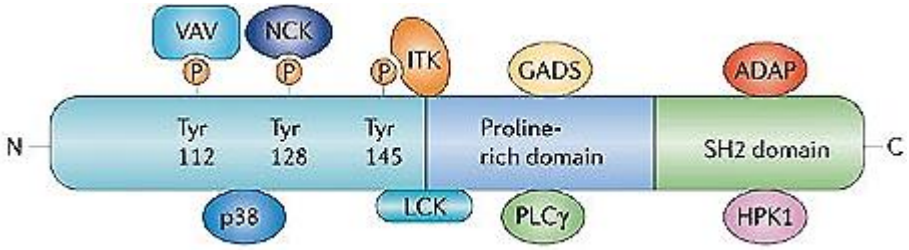

Figure 3. Structure of LCP2. The structure of LCP2 a.k.a SLP-76, its domains and binding partners are shown. Figure adapted from Koretzky et. al [34].

2.5 INTRODUCTION TO RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an autoimmune disease that is characterised by chronic inflammation of the synovial joints hand and feet [35, 36]. The disease affects 0.5-1% of the global population, disproportionately affecting women, and currently has no cure [37, 38]. Patients are commonly divided into seropositive and seronegative RA based on the presence of autoantibodies (anti-citrullinated protein antibodies (ACPA), and rheumatoid factor (RF)). [39, 40]. Chronic synovial inflammation will start to damage the cartilage around the joints, resulting in worsening joint pain and possibly reducing mobility in affected joints. Disease progression beyond this stage will lead to bone erosion as the inflammation has destroyed the cartilage, potentially deforming the bone structure of the affected joints. Late-stage RA is characterised by severe joint pain, loss of mobility and even loss of muscle strength and a loss of quality of life for the patients.

RA develops as an interplay between genetic and environmental factors [41]. The genetic factors refer to certain HLA-DRB1*04 alleles (like DRB1 *04:01 and 04:04) that are associated with increased risk of developing RA [42, 43]. HLA-DRB1*04 alleles, formerly known as DR4, (along with the MHC α chain) mediates peptide presentation to CD4+ T cells. Aside from the strong genetic link to HLA-DRB1, there are several genetic, epigenetic and environmental factors which could affect the dysregulation in the central tolerance and peripheral tolerance processes, increasing susceptibility to RA. The central tolerance processes that should halt the development of some of these auto-reactive T cells in the thymus do not function as they should, perhaps due to lack of presentation of these self-antigens or due to mutations in genes that affect signalling and thus don't engage the processes that would otherwise induce clonal deletion/diversion of these auto-reactive T cells in the thymus. Similarly, mutations in signalling genes among other factors in these auto-reactive cells fail to engage anergic/apoptotic mechanisms in the periphery [10]. Some of these other genetic factors include single nucleotide polymorphisms (SNPs) in genes such as *PTPN22, IL23R, TRAF1, CTLA4, IRF5, STAT4, CCR6, PADI4* have been linked to increased RA susceptibility [44, 45]. Moreover, epigenetic modifications that demethylate promoters of inflammatory cytokines such as IL-6 [46] or dysregulation in the methylation patterns of MMPs (matrix metalloproteinases) in synovial fibroblasts can lead to greater joint destruction [47]. RA progression has also been linked to the overexpression of microRNAs such as miRNA-155 [48, 49]. Additionally, environmental factors such as smoking [50] or other lifestyle factors also affect disease development and severity of the symptoms. It has been shown that smoking in combination with HLA-DR alleles may induce reactions against citrullinated proteins [51]. As can be seen from the evidences above, there are many factors that contribute to the development and progression of RA.

PADI4 encodes an enzyme known as PAD4 (peptidyl arginine demainase type 4) which modifies arginine to citrulline posttranslationally. These citrullinated proteins could be the targets to which anti-citrullinated protein antibodies (ACPAs) develop and bind [52]. Citrullinated antigens have been found in many RA patients and the presence of ACPAs generally denotes a more severe disease.

While the exact mechanism is still unknown, several studies have elucidated many of the players in the disease progression [53]. Although T cells are thought to play a central role in RA, other immune cells such as B cells, macrophages and APCs have been found to infiltrate the synovium in patients. B cells in particular have been studied for their production of ACPAs, which are found in the serum of approximately 60% of RA patients and are linked to disease manifestation [54, 55].

2.5.1 T cells in RA and RA disease activity

The fact that HLA-DRB1*04 alleles is the strongest genetic link to developing RA suggests a central role for T cells in driving RA. In addition, other loci linked to RA such as CTLA4 or IL23R (mentioned above) are linked to T cell signalling and proliferation, further strengthening the centrality of T cells in RA. This is further corroborated by another biologic therapeutic for RA – Abatacept [56, 57]. Abatacept is a fusion of an antibody Fc domain and the extracellular domain of CTLA-4, a protein on the T cells surface that binds to CD80/86 on the APC with higher affinity than CD28-CD80 bindng [13]. Upregulation of CTLA-4 is a

natural mechanism to block the 2nd signal once proliferative signals are no longer required [58]. Thus Abatacept's mode of action has been shown to be the blockade of the second signal and suppression of T cell activation, indicating that over-active T cells contribute to disease progression. Animal models of RA-like disease also highlight the importance of T cells in the disease, such as the K/BxN model of autoimmune arthritis [59].

It is hypothesized that CD4+ T cells infiltrate the synovium of joints [60] and through a combination of activation signals and cytokine release in the synovium leading to the initial inflammation that later develops into RA [61]. In a homeostatic environment, T cells with self-reactive TCRs are not supposed to have an effector function. RA causing T cells have escaped this regulation, through a combination of epigenetic and environmental factors.

It is well known that ACPAs are found in many RA patients. It has been hypothesized that the HLA-shared epitopes (HLA-SE) present these citrullinated antigens to T cells and these T cells help B cells recognise these citrullinated antigens [62] and induce class switching to produce different isotypes of ACPAs. T cells are important in B cell activation and aiding in affinity maturation to produce high affinity antibodies, further corroborating the role of T cells in RA. These autoreactive B cells can then further re-activate T cells which can lead to an inflammatory cycle [63, 64].

In combination with the presence of autoantibodies such as ACPAs, recent advances in sequencing technology have enabled the discovery and characterisation of T cells with TCR sequences against citrullinated proteins from RA patients. These TCR reactivities include citrullinated vimentin [65], tenascin C [66], fibrinogen, CILP (Cartilage-intermediate layer proteins) [67] among others. The molecular characterisation of these TCRs has been more thorough in recent years with studies of these TCR epitopes and model cell systems to study the interaction between the TCR and their target antigen-MHC complex more in depth [68, 69].

All this evidence corroborates that RA is a product of immune dysregulation and can potentially be treated by rectifying this dysregulation of T cells. Especially due to the presence of ACPAs as a hallmark of RA, and the role of T cells in B cell maturation, isotype switching and antibody production, T cells are an especially attractive target. In conclusion, RA is a complex disease with many facets and would benefit from a novel therapeutic approach targeting T cell signalling with RNA therapeutics.

2.6 CURRENT TREATMENTS FOR RA

The current treatments for RA all fall into the category of disease modifying anti-rheumatic drugs (DMARDS). DMARDS are broadly classified anti-inflammatory drugs that are immunomodulators and applied across many immune disorders. The two main sub-classes of available drugs are conventional synthetic DMARDs and biologic DMARDs [70].

Conventional DMARDs include small molecule drugs such as methotrexate, leflunomide and hydroxychloroquine among others [71]. Biologic DMARDs target more specific pathways involved including cytokine blockers (e.g. Enbrel, blocking TNF-α) [72], or antibody-based

drugs, cell depleting/blocking antibodies (e.g. Rituximab, depleting B cells, and Abatacept, suppressing T cell activation) [73, 74]. Methotrexate is the most common treatment prescribed to patients when they first present at the clinic and is shown to be effective at reducing inflammatory symptoms for a majority of patients [75]. The advantage of conventional DMARDs like methotrexate lies in its effectiveness [76] and its low cost [71]. When conventional DMARDS are no longer effective, or not tolerated due to the side-effects, more expensive and specific biologic DMARDs such as TNFα blockade or Abatacept are prescribed, which has also shown to be effective at improving the patients' prognosis [77].

While conventional DMARDs have been effective to varying degrees in limiting disease progression in patients, the field is moving towards more targeted therapies with more specific targets to exert their anti-inflammatory effects. In addition to conventional synthetic DMARDs such as methotrexate, leflunomide etc., targeted synthetic DMARDs were developed. This group includes JAK inhibitors like Tofacitinib [78]. These, given in combination with conventional DMARDs have proven effective in some patients. However, they cannot achieve long-term remission for patients, and thus new therapies are required [79]. While targeted DMARDs is a good approach, a limited number of intracellular proteins are considered druggable using traditional drug modalities. It is estimated that only around 15% of all the protein coding genes are druggable by small molecule drugs due to the structure of the protein and the requirement of 'binding pockets' for these small molecule drugs, though more work is being done to identify new druggable proteins [80, 81]. There are many genetic factors and intracellular proteins that have been identified to be linked to RA and to the activity of immune cells [64]. These genes may cause immune cell dysregulation and as a result play a part in disease progression. In order to access these genes and proteins and widen the scope of targets for RA treatments, alternative therapeutic modalities such as antisense oligonucleotides (ASOs) should be considered. ASOs target the mRNA of a gene, and thus open up the pool of targetable genes, from the previous group restricted to "druggable" proteins and antibodies.

More aggressive approaches to autoimmune diseases (as well as lymphoid cancers) have tried to completely deplete T and B cells in an attempt to "remove" the pathogenic cell type. One such example is Alemtuzumab, (CAMPATH-1H), also known as anti-CD52 mAb (monoclonal antibody), a lymphocyte depleting antibody. Other T cell depleting antibodies previously considered as a potential treatment for RA (and subsequently abandoned) include anti-CD7, anti-CD5, and anti-CD4 [82]. There were many challenges with the T cell depletion therapies including the need for prolonged antibody treatment, especially for the anti-CD4 mAbs. Complete depletion of all CD4+ T cells seemed to be necessary to reduce disease burden as even a few remaining cells could maintain disease progression [82]. Follow up studies with these patients did reveal a slower regeneration of CD4+ T cells after depletion but not a significant improvement of the clinical parameters [83, 84]. These therapeutic options did not show any clinically relevant action, they provided insight into the role of CD4+ T cells in the RA. A few of the patients in the CAMPATH-1H trial had side effects linked to immune system activation (fever, nausea, vomiting, etc.) and some infections. It also showed only a moderate short-term effect and showed toxicity in some of the RA trial participants [85]. More recently in 2018, FDA warned of other more severe side effects such as blood vessel wall tears and spontaneous intracranial haemorrhage when used for MS

(multiple sclerosis) patients. Anti-CD25 mediated depletion of Tregs in the CIA (collagen induced arthritis) in mice lead to an increased severity of disease, as expected [86]. This goes to show that in RA as well as perhaps other autoimmune diseases where T cells play a central role, the solution lies in the ability to modulate T cells back to a properly regulated state as depletion causes a whole host of problems while still running the risk of disease progression. Armed with this knowledge, we aimed to develop a therapeutic strategy that focuses on immunomodulation rather than immunosuppression.

2.7 ANTISENSE OLIGONUCLEOTIDES (ASO)

RNA therapeutics is an emerging therapeutic modality due to its versatility and potential to target any gene [87]. This group includes Antisense Oligonucleotides (ASOs), short interfering RNAs (siRNA) and short-hairpin RNAs (shRNAs). In particular, antisense oligonucleotides (ASO) are emerging players as therapeutics for many diseases [88]. While all these therapeutic modalities target RNA, they employ different mechanisms of action. ASOs can employ either steric hindrance or RNAse H mediated RNA degradation. siRNAs recruit the RISC complex for RNA cleavage and are sometimes formed by the cleavage of shRNA by the DICER complex [89]. In addition, siRNAs and shRNAs are typically produced as double-stranded compared to the single stranded ASOs. ASOs are short stretches of modified oligonucleotides ~ 12-30 nucleotides long, that are complementary in sequence to its target RNA [90]. ASOs bind to the complementary RNA of its target gene consequently recruiting RNAse-H [91]. RNAse-H is an enzyme specialising in the cleavage of RNA polynucleotide stretches in an RNA-DNA duplex, leaving the DNA intact. Therefore, ASO mediated knockdown involves RNAse-H mediated cleavage of mRNA sequence in the mRNA-ASO duplex, while leaving the ASO (DNA-based) sequence intact [92]. The cleavage of the mRNA will render it non-functional, and unable to be translated, and thus results in knockdown of expression of the target gene.

ASOs are commonly chemically modified at two locations: the sugar-phosphate backbone and the 2’OH group in the sugar ring. The state-of-the-art ASO design involves a phosphorothioate (PS) backbone which replaces one of the oxygens to a sulphur in the backbone. The full PS backbone protects the ASO from nucleases, without hampering activity [93]. The sugar ring is modified to a locked nucleic acid (LNA) configuration, connecting the 2’O to the 4’C with a methylene bridge in the ribose ring (**figure 4**). This increases stability by making it resistant to nucleases and increases affinity for the mRNA strand. However, RNAse H cannot cleave sites binding to LNA modified nucleotides and requires unmodified DNA nucleotides for binding. Thus, the state-of-the-art approach creates a ‘gapmer’, 16-nucleotide long ASO with 3 LNA-modified nucleotides flanking the 10 middle unmodified nucleotides, allowing the cleavage of the target RNA at the middle of the binding sequence [91].

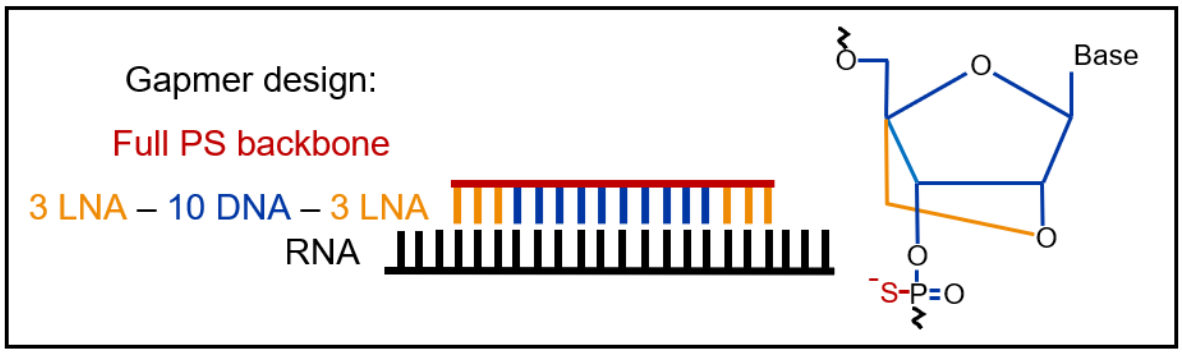

Figure 4: Structure of the ASO. The figure shows the chemical modifications to the backbone and the flanking nucleotides in the 16 nucleotide Gapmer. Adapted from Iyer.et al, 2022 [94].

The advantage of RNA therapeutics, including ASOs over small molecule inhibitors is that the former targets the RNA of a specific gene in order to modulate expression, and thus is not limited in the genes it can target. Small molecule inhibitors can only target the "druggable genome", i.e. genes that encode proteins that have a ligand binding pocket that modulates its activity[95]. The druggable genome is predicted to be only 10% of the total protein coding genes in the body. Thus small molecule drugs limit the number of disease-causing genes that can be targeted for treatment. This problem can be easily solved with the use of ASOs, which can modulate the expression of any gene through interaction with the RNA transcribed from the target gene[96]. In addition, the intervention by ASOs early in the gene expression process at the post-transcriptional level, allows for more efficient control of gene and subsequent protein expression compared to small molecule drugs that target fully formed proteins.

Unfortunately, there are still some factors that make it difficult for ASOs to become a widely used therapeutic modality. Firstly, while ASOs have been shown to be effective at knockdown of target gene expression, delivery of these ASOs into the target cells can be a challenge[97]. Secondly, libraries of small molecules have already been established, and are fairly standardised. The same level of development for ASOs and RNA therapeutics is yet to be achieved, which could deter researchers and companies from considering ASOs over small molecules. As small molecules have been extensively studied over the last few decades, the understanding of how to design and develop small molecules as therapeutics as well as accompanying technology has improved greatly. Thus small molecules are easier to develop as therapeutics, with the current infrastructure of traditional pharmaceutical companies [92]. However, with the recent roll-out of the COVID mRNA vaccines and the largescale test of the safety and efficacy of the delivery method, the role for ASO in the advent of personal medicine is likely to greatly increase. Additionally, novel programmable delivery vehicles for ASO/siRNA have been shown to be effective and herald a new age for RNA therapeutics [98, 99].

While ASOs have yet to be approved to treat RA in humans, they have shown great promise in animal models of autoimmune arthritis. In both rat and mouse models of RA, Anti-miRs, i.e. RNA-ASOs specifically designed to target microRNA (miRNA), have been reported to decrease excessive inflammation via miRNA inhibition [100-103]. Anti-miRs have been

shown to treat inflammation in mice with CIA (collagen induced arthritis), a mouse model of RA [49] and ASOs against NFκB were shown to reduce paw inflammation in rats with adjuvant-induced arthritis [104]. Furthermore, ASOs have good safety and low toxicity profiles and are safe to be administered as a drug [105]. These studies provide support for the use of RNA therapeutics for rheumatoid arthritis.

2.8 CRISPR

CRISPR/Cas9 is a widely used genome editing tool derived from bacteria [106]. The most used CRISPR-Cas system combines a guide RNA (gRNA) and the Cas9 endonuclease [107]. The gRNA is designed to be complementary in sequence to the target region that is to be modified. The Cas9 protein, which, with the help of gRNA recognises and binds to the target DNA, cuts the DNA next to the protospacer-adjacent motif (PAM) sequence [108]. This creates a double-stranded break, which is sometimes faultily repaired by the cell. This can result in a loss-of-function mutation or "knock-out" (KO) of the target gene (**figure 5**). The subsequent cellular phenotype of the KO cell shows the function of the targeted gene. This technique is useful for studying the function of a gene as it removes the production of the native protein. CRISPR as a technique is also useful in the field of drug target discovery. A KO of a potential target gene can validate both the function of the gene as well as show the phenotype of the cell in response to the absence of this gene. This can serve as validation for the use of therapeutics that will manipulate or knockdown the target gene expression, for example, an ASO. Thus, CRISPR provides a positive control for a 100% effective knockdown of target gene expression and serves as an example of the expected phenotype if an ASO was developed against that target [109]. Targets for ASO-mediated knockdown can be selected through a hypothesis driven CRISPR screen targeting genes in the pathway of interest. The screen can identify which genes would affect the pathway, and how they would affect the phenotype of interest and therefore identify which genes would be good drug targets. The screen can also rank the targets in order of important to the pathway and identify certain redundancies.

Therefore, CRISPR is useful to validate the function of a gene and thereby identify potential drug target. CRISPR also serves as a positive control to assess the effect of knockdown based therapeutics on pathways of interest.

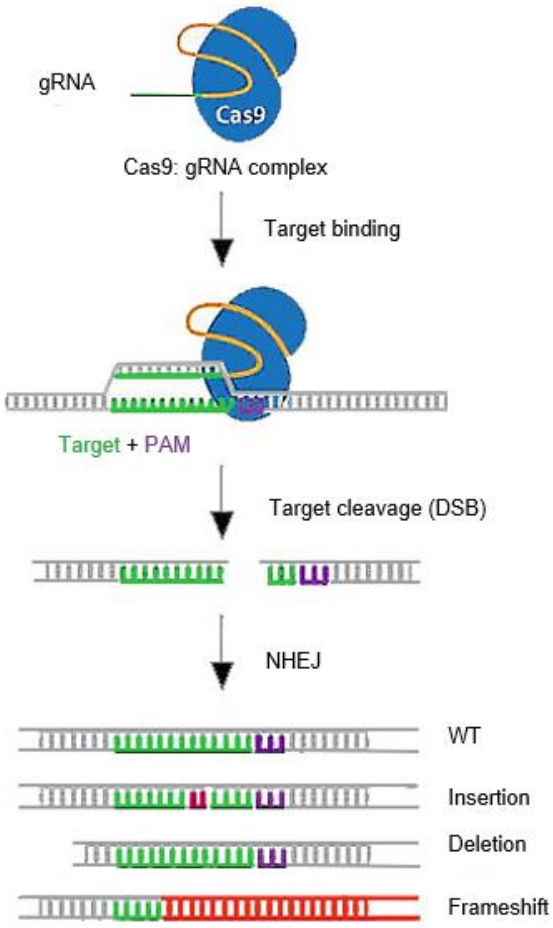

Figure 5. CRISPR-Cas mediated gene KO. The figure depicts the process of CRISPR-Cas mediated genome editing. Figure adapted from addgene.

3 RESEARCH AIMS

The main aims of my thesis project have been to (*i*) validate drug targets for ASOs targeting T cells, (*ii*) design and evaluate the ASOs for efficacy and potency and (*iii*) to study the characteristics of T cells and TCRs in a disease context.

Specific Aims

Paper I: To develop and evaluate the Rapid CRISPR competitive assay to quickly and efficiently analyze genes affecting various phenotypes of cells in the hematopoietic system.

Paper II: To design and evaluate the efficacy of ASOs targeting LCP2 in knockdown potential and subsequent effect on the activation of T cells and mast cells.

Paper III: To generate artificial T cells mimicking immune responses through antigen-specific stimulation of cell lines expressing exogenous TCRs.

Paper IV: To study and validate the peptide specific responses for patient-derived TCRs reactive against citrullinated peptides.

MATERIALS AND METHODS

All materials and methods used to generate the data for this thesis are described at length in **papers I-IV.** The specific methods used and the paper they were used in are summarized below in **Table 1**. However, some of the frequently used methods will be expanded upon in this section.

Table 1. Materials and methods used to generate data for this thesis.

Method	Resource/material	Paper(s)
Isolation and culture of PBMCs	Buffy coats from healthy donors	I, II
Isolation of T cells/ TCR sequences	Synovial fluid and blood from RA patients	IV
RNA extraction, cDNA synthesis and qPCR	Cell lines, human primary material (from blood and lungs)	II
Mice	C57BL/6 CD45.1 (I) C57BL/6 Cas9+/GFP+ (I) Cas9+GFP+CD45.1+ (I) RAG-/-, I-Ag-7+/+, DRB1*04:01 on a NOD/C57BL/6 background (III) TCRb-/- I-Ag-7+/+, DRB1*04:01 on a NOD/C57BL/6 background (III)	I, III
Cell lines	Jurkat (II) Jurkat-NFκB-GFP (I, II)	I, II, III, IV

	C57BL/6 Ca9+GFP+ - ER-HoxB8 (a.k.a. HoxB8 cells) (I) HEK293T (I, II, III, IV) 58 -/- cells (58 alpha-beta-TCR negative cells) transduced with hCD4 and NFAT GFP (III, IV)	
Viral preparation and transduction	Retrovirus particles generated in HEK cells to transduce target cell lines.	I, III, IV
gRNA Design	Green listed software	I, II
CRISPR KO and genotyping by sanger sequencing and ICE (Inference of CRISPR Edits)	Cell lines, primary human cells, primary mouse cells	I, II
Flow cytometry and Fluorescence assisted cell sorting	Cell lines, primary human cells, primary mouse cells	I, II, III, IV
Protein detection by western blot, ELISA	Cultured cell lines and primary cells	II
ASO design, synthesis, and purification		II
Statistical analysis		I, II, III, IV

CRISPR mediated gene editing

CRISPR-Cas9 was an important tool to validate the functions of different genes and their importance to different pathways in this project and was used to KO genes in paper **I** and paper **II**. The first step was to design single guide RNAs (sgRNAs) to target the genes of interest. For all the genes targeted, gRNAs were designed using Green listed software and ordered from Sigma-Aldrich or Synthego as 2'-o-methyl and phosphorothioate modified versions to protect from degradation. The gRNAs were delivered by electroporation via the neon transfection system, for which more details can be found in the papers. CRISPR KO was done by either transfecting gRNA into a Cas9 expressing cells or transfecting the RNP (ribonucleoprotein) complex (gRNA+Cas9) into the target cell. To confirm the KO, genotyping was done by performing Sanger sequencing on PCR amplified genomic DNA. Primers were designed in house to amplify ~250bp flanking either side of the target PAM sequence and ordered from Thermofisher. The knockout percentage was confirmed by analyzing the sequencing results using the ICE (inference of CRISPR edits) software [Synthego].

Cell and animal models

The cell models used in the PhD thesis are of both human and mouse origin. The human material include the Jurkat (and its variants) and HEK cell lines as well as PBMCs from human blood and hLMCs (human lung mast cells) from human lung. The mouse material includes the 58-/- cell line (alpha beta TCR knockout) (**III, IV**) as well as the following mouse strains: TCRb-/- I-Ag-7+/+, DRB1*04:01 on a NOD/C57BL/6 background, RAG-/-, I-Ag-7+/+, DRB1*04:01 on a NOD/C57BL/6 background (**III**), C57BL/6 Cas9+/GFP+CD45.1 and C57BL/6 Cas9+/GFP+CD45.2 (**I**).

Some of these cell models were retrovirally modified to generate reporter cell lines. Retrovirus particles were generated by co-transfection of the transfer plasmid of interest, and the EcoPac gag-pol-env into HEK293T cells. The target cells were spin infected with these particles and selected for successful integration of the plasmid of interest. Jurkat cells were transduced with pSIRV-NF-κB-eGFP to generate the Jurkat NFκB-GFP cell line (**I, II**) and BM from C57BL/6 Ca9+GFP+ was transduced with ER-HoxB8 **(I**). 58-/- mouse cells were transduced first with pSIRV-NFAT-eGFP to generate the 58-/-NFAT.GFP cell line. This was further transduced with pMX hCD4 plasmid to generate the 58-/-NFAT.GFP.hCD4 cell line. Different TCR constructs were transduced into 58-/-NFAT.GFP.hCD4 cell line (**III, IV**).

Primary human material used in paper **II** and **IV** were isolated as follows. PBMCs (peripheral blood mononuclear cells) were isolated from human blood and buffy coats using Ficoll-paque plus density gradient. The PBMCs were frozen in vials of 10 million each and thawed for use. Mast cells were isolated from human lung biopsies using the optimized protocol from Ravindran et. al [110].

Functional assays

I used various functional assays to measure changes in gene and protein expression. To study changes in gene expression in paper **II**, I used the RT-qPCR (real-time quantitative PCR) technique. RNA was extracted from human cell lines as well as PBMC cultures, and human lung tissue using TRIzol and the Direct-zol MiniPrep Kit (Zymo research), reverse transcribed into cDNA using the high-capacity RNA-to-cDNA kit (Invitrogen). The cDNA was amplified with TaqMan gene expression assay (FAM-gene of interest and VIC-β-actin) in a CFX 384 Real-Time PCR machine (Bio-Rad), quantified using the ddCT method and normalized to β-actin as the housekeeping gene.

To measure protein levels I used three methods: flow cytometry for cell surface and intracellular proteins (**I-IV**), western blot for intracellular LCP2 (**I**) and ELISA for secreted IL-2 (**II and III**). Flow cytometry for intracellular proteins involved fixation with formaldehyde before staining with the appropriate antibodies whereas surface proteins were stained before fixation. The Sony SH800S was used for fluorescence assisted sorting. For flow cytometry, the following cytometers were used: BD Accuri, BD LSR Fortessa, BD FACS Verse and Cytek Aurora. All results were read as FCS files and analyzed using FlowJo version 10 (FlowJo, LLC). Western blot was performed on cell lysates from Jurkat cells. These lysates were separated on a gel and blotted on a PVDF membrane. The membrane was incubated with primary anti-rabbit-β-actin (housekeeping) and anti-rabbit-LCP2/SLP-76, washed then incubated with secondary rabbit-IgG HRP-linked antibody before incubation with the substrate and imaging on the Amersham Imager. ELISA was performed on supernatant from cultured and treated primary cells using the ELISA MAX IL-2 kit from Biolegend.

Cell stimulation assays contributed in large part to the ability to study cell functionality in this thesis. In papers **I-IV**, stimulation assays were performed on T cell lines such as Jurkat and 58-/- and their derivatives, primary T cells and hLMCs and splenocytes from mice. They were either stimulated with anti-CD3/28, used as soluble antibodies or antibodies coated on magnetic beads (**I-IV**), PMA/Ionomycin (**I, II**) or with their cognate peptide/HLA-monomers (**III, IV**). The effect of this stimulation was assessed by flow cytometry or IL-2 ELISA.

4 RESULTS AND DISCUSSION

4.1 DRUG TARGET DISCOVERY IN CELLS OF THE HEMATOPOIETIC SYSTEM USING THE RAPID CRISPR COMPETITIVE (RCC) ASSAY (PAPER I)

The CRISPR-Cas9 system can cause mutations in target genes with a gene specific gRNA. However, this system generates a heterogenous population with many different genotypes; some will be gene inactivating mutations resulting in a "knock-out" of the target gene, and some will retain the WT conformation. In Paper I, we exploit this heterogeneity to rapidly study the function of target genes in a studied phenotype. In short, if we want to study the role of the gene "A" in activation of T cells, we generate a heterogenous "A" KO population in T cells. Then we activate the population with T cell activating antibodies and sort the activated and non-activated populations. Using standard sanger sequencing and the ICE software (inference of CRISPR edits), we determine the percentage of KO and type of mutations (insertions/deletions/frameshift) in each sorted population. Based on the enrichment of the KO in either of the sorted populations, we can make conclusions about the function of the target gene in the studied phenotype. The general principle of the RCC is illustrated below in **figure 6**.

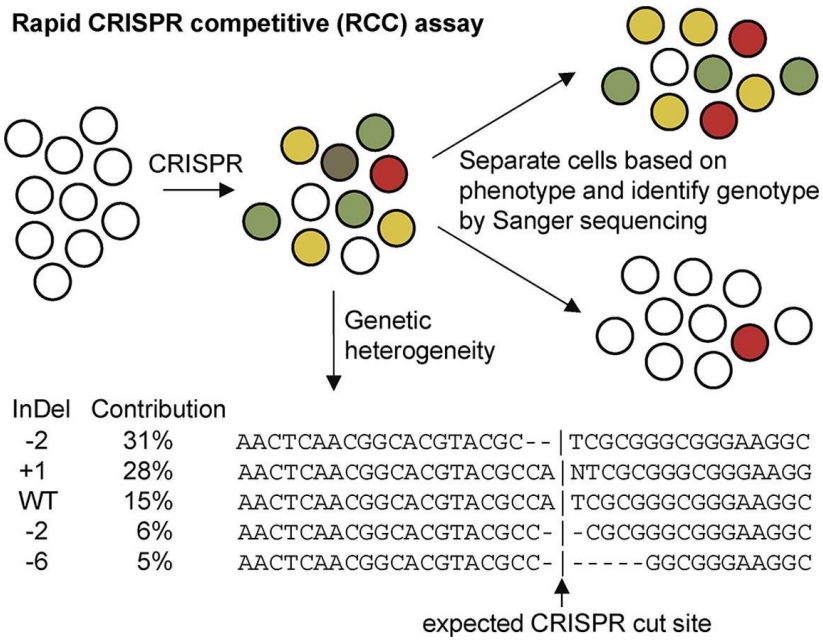

Figure 6. The principle of the RCC assay [111].

In order to ensure that primary cells can be efficiently modified by CRISPR and the KO can be accurately evaluated by sanger sequencing methods we first tested the ability of the CRISPR-Cas9 system to KO GFP in lineage negative (lin-) bone marrow (BM) cells that were constitutively expressing GFP (from Cas9+GFp+ C57BL/6 mice). A GFP specific gRNA was delivered to these BM cells, and the level of GFP KO was evaluated by flow cytometry. From this experiment, we verified the efficacy of the GFP targeting gRNA, the effect different gRNA concentrations had on KO efficiency as well as the best electroporation condition to have a high efficiency of KO but also a good cell viability using a combination of sanger sequencing and flow cytometry (**Paper I, Fig 1**).

After optimization of electroporation and gRNA treatment conditions, we applied the optimized protocol into mouse models to generate *Zap70* KO iCR (immune-CRISPR) mice, as proof of principle. To generate the *Zap70* iCR mice, *Zap70* was targeted by a gRNA in lin- BM cells from Cas9+ mice *in vitro* and the resultant genetically heterogenous *Zap70* cells were subsequently transferred to irradiated recipient mice. Zap70 has been shown to be important for T cell development but does not play such a role in B cells. As expected, the percentage of B cells in *Zap70* iCR and WT mice did not differ, but T cells were significantly depleted from the *Zap70* iCR mice compared to WT mice. In addition, we sorted B and T cells from the spleen of iCR and WT mice and sequenced for *Zap70* and found that B cells and total cells in the blood carried a very high percentage of *Zap70* KO alleles, showing that Zap70 did not really affect their function, but all T cells found in *Zap70* iCR mice had WT *Zap70*, confirming that Zap70 is indeed necessary for T cell development. To increase the iCR population, we developed a secondary transfer model where BM from a successful primary iCR mouse is injected into irradiated mice creating more iCR mice via a secondary transfer. This process is fairly efficient, reliable, cost effective and has great potential for rapidly studying gene function *in vivo* (**Paper I, Fig 2**).

We also showed that *in vitro* CRISPR KO of GFP in lin- BM cells did not affect the ability of the cells to differentiate, showing that the RCC assay could be used to study such behaviors *in vitro*. As an example, we differentiated GFP KO lin- BM cells into macrophages and DCs (dendritic cells). They differentiated into the correct phenotypes, shown by the upregulation of surface markers, without any significant reduction in GFP KO percentage. This showed that CRISPR modification does not alter normal physiological responses to cytokines or other stimuli. (**Paper I, Fig 3**).

I also wanted to apply the RCC method to human cells and used the assay to validate the importance of LCP2 for T cell activation. Literature has shown that LCP2 is important for signaling downstream of the TCR [32]. We electroporated LCP2 targeting RNP complexes (Cas9+gRNA) directly into Jurkat reporter cells. To confirm that LCP2 is important only for TCR-mediated activation, we activated the resultant genetically heterogenous *LCP2* population with either anti-CD3/28 to activate the TCR or PMA/Ionomycin, which activates T cells independent of TCR crosslinking, and sorted the activated or non-activated populations. We found that the *LCP2* KO cells were enriched in the non-activated population of only the anti-CD3/28 cells, not the PMA/Ionomycin stimulated cells showing that not only is LCP2 necessary for T cell activation but that it is specific to the TCR signaling cascade. (**Paper I, Fig 4).** We also showed that primary human T cells could be modified by the RCC assay to study similar pathways with a proof of principle KO of *TRAC* (T cell receptor alpha constant region) (**Paper I, Fig S3**). This set the stage for our next study to develop ASOs against genes affecting T cell activation.

Lastly, we assessed the potential of the RCC assay in studying genes in hematopoietic malignancies. As an example, we transduced an inducible HoxB8 (a proto-oncogene) construct into BM cells, and subsequently used the RCC assay to study the role of genes

influencing HoxB8. HoxB8 promotes unlimited proliferation, similar to acute leukemic cells and do not differentiate. In a heterogenous HoxB8 KO population, differentiated (lin+) cells showed 100% mutated HoxB8 while the undifferentiated (lin-) showed very low percentage of inactivating or frameshift mutations. To test the applicability of the RCC assay to study genes influencing *HoxB8, Meis1* and *Pbx1* were knocked out in the HoxB8 cells and the subsequent sequencing of lin+ cells showed that *Meis1* KO was able to induce differentiation, similar to *HoxB8* KO, but *Pbx1* KO did not show such an effect, gleaning an insight into their roles in the HoxB8 pathway (**Paper I, Fig 5**).

In conclusion, in this paper, we showed a rapid method that takes advantage of the heterogeneity in CRISPR-mediated gene mutations to study the function of different genes in both human and mouse cells in various disease contexts.

4.2 DEVELOPMENT OF ANTISENSE OLIGONUCLEOTIDES (PAPER II)

There are quite a few autoimmune diseases of which T cells are key drivers of disease pathogenesis. Of these, RA is known to have a T cell mediated disease phenotype, and therapeutic strategies that target T cell activity in one way or another. This includes drugs like abatacept which block T cell interaction with APCs or TNF blockers which blocks an important cytokine that influences T cell and other immune cell activity. However, these do not work for all patients and new therapeutics need to be developed to be used as single agents or in combination with existing strategies.

Antisense oligonucleotides are still under-utilised classes of therapeutics with a lot of potential. Especially in the context of RA and other autoimmune diseases where T cells are key drivers of the disease, but current therapies cannot exploit the range of intracellular targets to modulate its activity. The druggable genome is a concept estimating the number of proteins with a structure or binding pocket amenable for small molecule binding and subsequent alteration of protein function. It estimates that less than 15% of all protein coding genes in the human body are druggable with traditional small molecule drugs. There are other classes of existing drugs such as biologics or soluble receptors like abatacept, but these most commonly need to target extracellular proteins.

To truly show the potential of ASOs we wanted to identify intracellular undruggable targets amenable that would be important for T cell activation. We decided that to specifically modulate T cell activity, we should target proteins involved in TCR mediated signalling specifically. From paper I, we validated LCP2 as a good target to modulate T cell activation as the KO cells did not respond to activation signals but were able to survive indicating that LCP2 KO was not toxic. A genome wide CRISPR screen also prioritised LCP2 and VAV1 as good targets to modulate T cell activation and proliferation [112]. As we had also validated LCP2 on our own, we focused our ASO design on LCP2 and VAV1, a protein downstream of LCP2.

The ASOs can be designed to be complementary to any sequence. Since it has been shown that the 3'UTR has important functions in the regulation of translation and of mRNA stability [113] while also being partially specific to the gene itself, we designed our ASO sequences to be complementary to the 3'UTR of VAV1 and LCP2 mRNAs. The ASOs were designed with our own in-house approach that ensured sequence specificity and minimal off-targets.

We designed 18 sequences targeting LCP2 and 10 targeting VAV1 and did a small-scale screen to determine the best sequence for both genes. Our main selection criterion demanded that the knockdown was >80%, i.e. <20% of LCP2 or VAV1 mRNA remained when treated with 5uM of the ASO. For VAV1, none of the designed sequences met this criterion. However, 4 sequences targeting LCP2, namely LCP2_008, 009, 013, and 017 achieved this rate of knockdown. When probed further for consistency in a dose response as well as ability to modulate downstream activation, LCP2_009 performed the best and thus was the contender for further studies (**Paper II, Fig S3 and S4**).

To ensure that the ASOs are stable, have high affinity binding to the target mRNA and resistant to nucleases, they were all designed as 3-10-3 gapmers, with 10 unmodified nucleotides in the middle flanked by 3 LNA modified nucleotides on either end, with a fully sulphurised backbone (PS backbone). We ran initial experiments to determine the efficiency and potency of LCP2_009 in Jurkat cells. A dose dependent response of LCP2_009 determined its IC50 to be ~523nM. We also found that LCP2_009 mediated LCP2 knockdown was stable for at least 96 hours, and that the knockdown at mRNA and protein level correlated very well (**Paper II, Fig 1**). We also ensured that LCP2_009 was not toxic to the cells. Our current method involved a passive uptake of the ASO, an approach which is highly coveted because a delivery vehicle is unnecessary. However, we wondered if a delivery method would improve potency, and indeed found that when cells were electroporated with the ASO, the same concentration induced a much better knockdown (**Paper II, Fig S5**). We think this is a good future direction to continue such ASO development project, discovering the most efficient delivery methods, such as non-toxic engineered lipid nanoparticles and extracellular vesicles, some of which are already in development [98, 99].

Continuing the technical analysis of the ASOs, we tested them in Jurkat reporter cell lines, which express GFP when activated. We thus tested the ability to of LCP2_009 to reduce activation of Jurkat cells when activated by either anti-CD3/28 or PMA/Ionomycin. Similar to the RCC assay set up in paper I, when LCP2 was knocked down in Jurkat cells, the GFP level, corresponding to activation, was reduced in a dose dependent manner only when Jurkat cells were stimulated with anti-CD3/28. There was no difference in GFP level between ASO treated or control cells when stimulated with PMA/Ionomycin, proving that the LCP2_009 ASO is indeed specific to LCP2 and the TCR pathway. This corroborates the validation in paper I, as well as showing the specificity/lack of off-target activity of the ASO. To further confirm this phenotype, the same Jurkat cells were also analysed by flow cytometry and qPCR for 2 additional well-known markers of activation: CD69 and CD25 (IL2Rα). These

markers were also reduced in a dose dependent manner when treated with LCP2_009, with the mRNA levels and cell surface expression levels showing a nice correlation (**Paper II, Fig 2**). After systematically confirming that LCP2_009 does indeed knockdown LCP2 and this consequently decreases the activation potential in cell lines, we decided to expand to primary human material.

4.3 EVALUATION OF THE EFFICACY AND POTENCY OF LCP2_009 IN AFFECTING T CELL AND MAST CELL FUNCTION (PAPER II)

LCP2 has been shown to be important for different immune cell types, including T cells and mast cells. Thus we set out to determine if LCP2_009 mediated knockdown of LCP2 would alter the activation potential of T cells through their TCR and mast cells through FcεRI signaling.

In the pursuit of developing ASOs for eventual use in the clinics, we decided on an approach that would mimic the *in-vivo* context and proceeded with treatments in a mixed PBMC culture, with PBMCs isolated from healthy donors. First, we wanted to confirm if LCP2_009 was able to knockdown LCP2 in primary cells, and if the ASO was taken up in T cells without a delivery agent. We treated the PBMCs with LCP2_009 over the course of 72 hours and sorted the PBMCs based on CD3 expression into two populations (CD3+ and CD3-). We then performed qPCR on these populations to determine the level of LCP2 KD in these cells. Both the CD3+T and CD3-non-T cell populations showed LCP2 KD, implying that while LCP2_009 was indeed taken up by other cells in the culture, it was possible to KD LCP2 with this ASO in T cells (CD3+) in the competitive setting of a mixed PBMC culture. We also performed a smaller dose response test in mixed PBMCs, showing that LCP2_009 was indeed able to maintain a knockdown at 72 hours in PBMCs, albeit at lower KD percentages compared to Jurkat cells, which is to be expected given the different cell types and their natural differences in endocytosis. We also wanted to delve deeper into the mechanism of action of the ASO, and thus decided to perform phosphoflow on LCP2_009 treated cells. This technique involves the flow cytometric analysis of intracellular phosphorylated proteins. Previous literature shows LCP2 signals downstream to VAV1, resulting in phosphorylation of VAV1 (p-VAV1) [114]. Knockdown of LCP2 should therefore reduce p-VAV1. However, p-ZAP70, which is upstream of LCP2 should not be affected by the reduction in LCP2 following TCR crosslinking. As expected, when I performed this experiment, the results showed that there was no difference in p-ZAP70 levels between ASO treated and untreated groups, but there was a significant reduction in p-VAV1 levels in the LCP2_009 treated group following TCR crosslinking. This confirmed that the LCP2_009 mediated knockdown of LCP2 did indeed have a specific effect on proteins downstream of LCP2 in the TCR signaling pathway (**Paper II, Fig 3**).

We then tested the effect of the LCP2 KD on T cell activation in the mixed PBMC culture. We found that CD69 was downregulated in both CD4 and CD8 T cells in the ASO treated group compared to the control group upon stimulation with anti-CD3/28. IL-2 secretion into the supernatant of these stimulated cultures was also reduced in the ASO treated condition.

Interestingly, PD1, an inhibitory receptor, was upregulated in all of these ASO treated and TCR crosslinked samples, indicating a different mechanism which may be linked to inhibitory nature of PD1 or perhaps other mechanisms linked to NFκB activation. Taken together, this implies a decrease in activation potential in ASO treated samples, when LCP2_009 is knockdown before the TCR is crosslinked (**Paper II, Fig 4**).

So far with our *in-vitro* assays we tried to accurately capture the *in-vivo* setting as accurately as possible with mixed PBMC cultures to simulate the mixed cell environment of the inflamed joints. However, thus far, the ASO treatment was given before TCR crosslinking, whereas in a clinical setting it is likely to be used after inflammation had already begun. I wanted to take the study further and perform a pilot test to mimic the administration of the ASO after TCR crosslinking. In this pilot test, there was no significant different in the activation potential of T cells in ASO treated patients post stimulation (**Paper II, Fig S6**). However, this is not unexpected in an *in-vitro* setting, with a flood of activation signals upon CD3/28 addition to the culture. In the *in-vivo* setting it is more likely that cells do not all get activated at once but rather there is a constant flow of cells and activation signals. LCP2 is important for signaling downstream after TCR crosslinking, but T cells do not continuously depend on TCR mediated activation after the initial signal, instead relying on cytokine mediated signaling, by-passing LCP2. Thus while this pilot test provided some confirmation of the role of LCP2 early after TCR crosslinking, it perhaps did not provide a complete picture of the potential of LCP2_009 *in vivo*.

Finally, we explored the effect of ASO mediated LCP2 KD in primary human lung mast cells (HLMCs). When FcεRI is crosslinked by IgE, the signal is transduced through different signaling molecules including LCP2. Mast cells continuously produce granules containing histamine, among other molecules. These granules also express CD63 on the inside. Upon activation, these granules fuse to the surface membrane to release the contents, and CD63 is expressed on the surface. Thus degranulation can be measured by CD63 expression on the surface of mast cells as well as histamine release in the supernatant. LCP2_009 was able to knockdown LCP2 in HLMCs, and this led to a decrease in CD63% as well as a corresponding decrease in histamine following FcεRI crosslinking, implying decreased degranulation (**Paper II, Fig 5**). As mast cell degranulation plays an important role in allergic reactions, LCP2_009 could have potential use as an immunomodulatory agent in allergy.

The advantages of our ASO development and evaluation system include the systematic testing of the potency and efficacy of LCP2_009 in both cell lines and primary cells. The mixed PBMC culture simulates an *in-vivo* environment. Additionally, the number and variety of donors further mimics the scenario in clinics and allows the results of the study to be applied more widely. Our donors were both male and female, and had a wide age range from 20-65, giving an unbiased view of the general population. The large variability in the donors also provided a perspective into the challenges of drug development with regards to natural variation in human responses; while the trend of downregulation of activation signals with ASO treatment was similar for all the donors, the actual levels of these markers showed

variation. To study the mast cells, we got HLMCs to get a more clinical perspective of mast cell behavior (compared to cell lines), and our ASO showed potential to modulate their behavior in vitro.

4.4 DEVELOPMENT OF MODEL SYSTEMS TO CHARACTERIZE T-CELL RECEPTORS (TCR) (PAPER III & IV)

A lot of research has gone into studying the intricacies of RA as a disease, and to identify components that are unique to RA. These unique "signatures" can help to not only diagnose the disease but could also be potential therapeutic targets. Among these are unique TCR repertoires from RA patients, some of which are public clones, i.e. they overlap between patients. These are particularly interesting because the target of the TCRs could provide an insight into the peptides that might trigger an immune response. However, since these TCRs are unique and found in patient material they are not as easy to study. Thus papers III and IV are concerned with the development and validation of model systems to characterize patient TCRs and their reactivities.

It has been previously shown that HLA DRB1*04 is a risk allele for developing RA. Thus, we aimed to characterize HLA DRB1*04 restricted TCRs derived from human material. We first wanted to establish the system and so tested the model system with a HA (Influenza hemagglutinin) specific TCR and a GAD (glutamic acid decarboxylase) specific TCR. In order to have a reliable and robust expression of the TCRs on HEK cells, we found that the TCRs benefited from being co-transfected not only with CD3 but hCD4 (human CD4). In this transient transfection model, the TCR could be expressed in HEK cells, and peptide/HLA tetramer binding ability of these TCRs could be assessed by flow cytometry (**Paper III, Fig 1**). However, this method of assessing the binding specificities of the TCRs relied on transient TCR expression and we wanted to develop a more stable expression system that could easily be used to determine expression. Thus, we used lentiviral transduction methods to create stable cell lines with the mouse cell line 58. We used a version of this cell line called 58-/-, lacking expression of the endogenous TCR. We transduced the 58-/- cell line with hCD4 and the TCRs of interest, as well as a NFAT-GFP construct creating cell lines that stably expressed the TCRs that could bind to the cognate peptide and expressed GFP as an easy read out for TCR responsiveness to the peptide. The binding of the peptide was compared to anti-CD3/28 stimulation as a control and the NFAT-GFP read out was also corroborated by measurement of IL-2 secretion. It is important to note that the hCD4 only made a difference in peptide/HLA mediated stimulation as CD4 stabilizes the peptide-MHC-TCR complex, while CD4 is not necessary for anti-CD3/28 crosslinking (**Paper III, Fig 2**). We wanted to extend this further and ensure that our system was peptide and HLA specific. Thus we compared GAD and HA TCR cell line activation when the cognate peptide (or control peptide) was presented by DR4+ or DR4- APCs. We confirmed that only the cognate peptide presented by DR4+ APCs elicited an IL-2 or NFAT-GFP response, confirming the specificity of our system (**Paper III, Fig 3**). We also found that peptide/HLA monomeric protein (monomers) with the correct peptide/HLA combinations were also able to stimulate

the cell lines in a specific manner (**Paper III, Fig 4**). After confirming that our system was specific and reliable, we focused on extending the use of these TCR constructs to broader applications by ensuring that these TCRs could be used in mouse models. To this end, we generated retrogenic mice by transducing bone marrow from RAG.DR4 mice (Rag-/-DRB1*04:01tg) with the HA TCR construct and grafted these cells together with some TCRb-/-DR4+ bone marrow into TCRb-/-DR4 irradiated mice and collected splenocytes 8 weeks afterwards. These splenocytes were used for similar peptide response assay, with different variants of the HA protein. We found that the splenocytes only responded to the H3 subtype of the HA protein and not the H1 subtype, which is the HA subtype that the TCR recognizes, once again confirming the specificity of this method in studying TCR-peptide recognition (**Paper III, Fig 5**).

After developing the method, we extended it to TCRs clinically relevant for RA. It is well known that B and T cells recognize citrullinated antigens in ACPA+ RA and this is linked to the fact that ACPA+ RA generally has a more severe disease. Therefore we focused on studying TCRs against these citrullinated antigens. We first identified citrulline reactive TCRs from the blood of RA patients by expanding citrulline specific T cells by culturing with citrullinated peptides and subsequently sorting these cells based on binding to HLA-DRB1*04:01 tetramers loaded with citrullinated vimentin (cit-vim), citrullinated fibrinogen (cit-fib) or HA (control) (**Paper IV, Fig 1**). Different reactive clones were identified from the patients, and these TCRs were cloned into plasmids, which were further transduced into 58-/-NFAT.GFP.CD4 cells using a similar method to that used in paper III. The successfully transduced TCR positive cells were sorted and expanded, and the peptide reactivities of these TCRs were assessed using monomer stimulation compared to anti-CD3/28 stimulation as a control. The method showed success with the HA and cit-fib reactive TCRs, but not with the cit-vim reactive TCRs (**Paper IV, Fig 2**). In the case of the HA and cit-fib reactive TCRs, they also responded to their respective monomers in a dose dependent manner, showing that this method was successful to analyze these TCRs (**Paper IV, Fig S1**). However, there is still some optimization to be done in citrullinated TCR identification, as with the case of the cit-vim TCR. The non-response could be due to differential binding of the TCRs to the tetramers in the initial sorting phase leading to some TCRs that are more specific compared to others. Nonetheless, this TCR identification, re-expression and reactivation method to study TCRs can be successfully applied in multiple scenarios.

5 CONCLUSIONS

Drug development of any form is a complicated process involving many steps. One needs to decide on the most appropriate class of therapeutic as well as which disease to target. Other important steps to consider are target validation, drug screening, development and evaluation which can then be extended to clinical trials when a suitable drug candidate has been evaluated. In this PhD project, we show all these pre-clinical steps involved in the development of ASOs for immunomodulation, particularly to modulate T cells in the context of autoimmune diseases. We started with target validation, to identify and validate good targets to be modulated by ASOs for the purpose of decreasing T cell activation, as well as modulating other cells of the hematopoietic system, the main focus of **Paper I**. Once LCP2 was validated as a good target, we followed up with screening of the potential therapeutic molecules to select the most potent and efficient ASO, followed by evaluation of the effectiveness of the ASO in both cell lines and primary cells, shown in **Paper II**. Now that we had established a good therapeutic, we wanted to study the target cell type further. While it is widely agreed that CD4+T cells play an important role the etiology of RA, and that specific TCRs are expanded in RA patients compared to healthy controls, we still need better tools to study these TCRs. **Papers III and IV** deal with the study of the specific citrullinated-protein reactive TCRs and the *in-vitro* recapitulation of the responses of these TCR expressing T cells to their cognate antigens. We developed a methodology to easily study these TCRs *in vitro* in different contexts, in hopes that this will provide a better understanding of how these T cells drive the inflammation in RA and how we can more specifically target the therapeutics to these pathogenic T cells.

6 POINTS OF PERSPECTIVE

This thesis work has been a proof of concept for the development of immunomodulatory ASOs for potential use in autoimmune diseases, but it also provides an insight into the different steps in the process of drug development. Though focused on limited drug targets, I believe that the underlying questions I tried to answer through my PhD are guiding principles for the future of drug discovery: What a good drug target is and how can we use this information to transform the field of drug discovery?

With the advent of CRISPR mediated genetic modification techniques, gene manipulation has become increasingly accessible as well as flexible with different types of Cas proteins. Our RCC assay adds to the growing repertoire of CRISPR based assays to study gene function and to enable simpler hypothesis mediated drug target discovery. CRISPR can easily help us understand not only the role of a gene but identify redundancies, intricate interactions, and the feasibility of manipulating the gene without altering other essential cellular and tissue functions. These characteristics will help answer some of the questions surrounding the suitability of a gene to be a drug target for a pathway of interest.

The ability of an ASO to target any sequence in the human genome is one its greatest advantages and is one of the important criteria to bring it to the forefront of therapeutics to be used for personalized medicine. The advent of personalized medicine demands personalization with efficiency, and that is something I think the ASO can provide, setting it apart from traditional small-molecule-based therapeutics.

The implications of the works comprising my thesis on the future of drug discovery is hopefully one of increasing accessibility and ease of drug development on a smaller scale. I believe that basic research into the mechanisms of action of these RNA therapeutics will further enhance the future therapeutic options for diseases that currently do not have a cure.

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