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# Redox Regulation of T Cells in Autoimmunity

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Cover illustration: Fine-tuning of cell responses via ROS by Julia Herrele

# Redox Regulation of T cells in Autoimmunity

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Eva & George Klein Hall, Biomedicum, KI, Stockholm 24th September, 2021

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*Journey before destination*

— Brandon Sanderson, Oathbringer

# POPULAR SCIENCE SUMMARY OF THE THESIS

Our immune system has over the ages evolved to not just protect us from outside threats, but also from ourselves. It is a fine-tuned machinery of astounding complexity that has learned to distinguish “good” from “bad” and to tolerate our own body’s organs, cells, and proteins, while ferociously attacking intruders. Troubles arise when this decision-making process goes awry, leading to a state of “autoimmunity” where an attack is mistakenly mounted toward harmless self-proteins. This is the basic tenet behind autoimmune diseases such as type 1 diabetes, rheumatoid arthritis, or multiple sclerosis. The list of such diseases is long. Their incidence is steadily rising in the western world and they can, in general, not be cured.

It is therefore of importance to study the underlying mechanisms of such conditions to be able to better address them clinically and this thesis is an attempt to do just that. Here we focus on rheumatoid arthritis (RA), an autoimmune disease affecting 0.5-1% of the population that results in the destruction of joints and bones. As is common for autoimmune diseases, RA is dependent on an individual’s genetic background as well as their environment. To address our questions we use mice, as their immune systems are fairly similar to ours and as they can, under the right conditions, develop a human-like arthritis.

Two of the studies presented here identify genes that regulate arthritis severity: Study III identified a potential new target for therapeutic interference while Study IV highlights the role of vitamin D in the immune system.

The focus of this work falls under the umbrella term “redox regulation” which encompasses the regulation of cellular processes by derivatives of oxygen. The related term “antioxidant” has made it into mainstream parlance, particularly in the world of dietary supplements, where reactive oxygen species (ROS) are portrayed as a foe that must be eliminated. However, our view on ROS has shifted tremendously over the past decades and they are now regarded as essential signaling molecules regulating a wide array of processes. In Studies I and II we show that ROS-mediated signaling effects impact inflammatory processes and arthritis development via regulation of two proteins: PTPN22 and LAT. This work adds to our understanding of important cellular pathways and highlights the importance of redox regulation.

# ABSTRACT

Autoimmune disorders affect a significant part of the population and therefore present a serious health and economic burden. One of the most common autoimmune diseases is rheumatoid arthritis (RA), affecting 0.5-1% of the population which is mediated by both genetic and environmental risk factors. A common thread throughout this thesis is the impact of various proteins on T cell signaling and how this affects autoimmune inflammation in rodents.

Studies I and II investigate the role of redox regulation on two major players in TCR signaling: PTPN22 and LAT. We targeted known redox-sensitive cysteine residues in these proteins and could thereby investigate their importance *in vivo*. Study I shows that PTPN22 function can be regulated by its non-catalytic cysteine 129 (C129) residue by forming a disulfide bond which protects the active site from irreversible oxidation; impaired redox regulation leads to enhanced T cell and inflammatory responses. In a similar vein, we found in Study II that cysteines 120 and 172 mediate redox regulation of LAT by affecting its phosphorylation and localization. Redox insensitivity of the LAT protein worsens T-cell dependent inflammation.

In the last two studies we have used a forward genetics approach to identify genetic determinants of RA susceptibility: In Study III we were the first to identify that loss of *Sh3gl1* leads to protection from autoimmunity due to alterations in the T cell signaling pathway, thereby providing an attractive new therapeutic target. Study IV shows that polymorphisms regulating vitamin D receptor expression affect T cell activation and T cell mediated inflammation.

Collectively, our results show the importance of physiological redox effects and expand the knowledge on RA genetics.

# LIST OF SCIENTIFIC PAPERS

I. **Jaime James**, Yifei Chen, Clara M. Hernandez, Florian Forster, Markus Dagnell, Qing Cheng, Amir A. Saei, Hassan Gharibi, Gonzalo Fernandez Lahore, Annika Åstrand, Rajneesh Malhotra, Bernard Malissen, Roman A. Zubarev, Elias S.J. Arnér, Rikard Holmdahl. **Redox regulation of PTPN22 affects the severity of T cell dependent autoimmune inflammation.** Manuscript

II. **Jaime James***, Gonzalo Fernandez Lahore*, Clara M. Hernandez, Florian Forster, Bernard Malissen, Rikard Holmdahl. **Redox regulation of LAT enhances T-cell mediated inflammation.** *equal contribution Manuscript

III. Ulrika Norin, Carola Rintisch, Liesu Meng, Florian Forster, Diana Ekman, Jonatan Tuncel, Katrin Klocke, Johan Bäcklund, Min Yang, Michael Y Bonner, Gonzalo Fernandez Lahore, **Jaime James**, Klementy Shchetynsky, Maria Bergquist, Inger Gjertsson, Norbert Hubner, Liselotte Bäckdahl, Rikard Holmdahl (2021). **Endophilin A2 deficiency protects rodents from autoimmune arthritis by modulating T cell activation.** *Nature Communications*, 12(1):610

IV. Gonzalo Fernandez Lahore, Bruno Raposo, Marie Lagerquist, Claes Ohlsson, Pierre Sabatier, Bingze Xu, Mike Aoun, **Jaime James**, Xiaojie Cai, Roman A Zubarev, Kutty Selva Nandakumar, Rikard Holmdahl (2020). **Vitamin D3 receptor polymorphisms regulate T cells and T cell-dependent inflammatory diseases.** *Proceedings of the National Academy of Sciences*, 117(40):24986-24997.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACPA | Anti-citrullinated protein antibody |
| AD | Autoimmune Diseases |
| CII | Collagen type 2 |
| CAIA | Collagen antibody induced arthritis |
| CIA | Collagen-induced arthritis |
| DMARD | Disease-modifying anti-rheumatic drug |
| EAE | Experimental autoimmune encephalomyelitis |
| EBV | Epstein–Barr virus |
| HLA | Human leukocyte antigen |
| LAT | Linker for Activation of T cells |
| MHC | Major histocompatibility complex |
| MMP | Matrix metalloprotease |
| mTEC | medullary thymic epithelial cells |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| PAD | Peptidylarginine deiminase |
| PTP | Protein tyrosine phosphatase |
| PTPN22 | Protein tyrosine phosphatase, non-receptor type 22 |
| QTL | Quantitative trait locus |
| RA | Rheumatoid Arthritis |
| RF | Rheumatoid Factor |
| ROS | Reactive Oxygen Species |
| TCR | T cell receptor |
| Th | T-helper |
| Treg | Regulatory T cell |
| VDR | Vitamin D Receptor |

# 1 INTRODUCTION

## 1.1 AUTOIMMUNITY

A key function of our immune system is to discriminate self from non-self. Self-tolerance describes the unresponsiveness of the immune system towards substances or tissues that could otherwise evoke an immune response. It is mediated by two processes: central and peripheral tolerance. Central tolerance eliminates autoreactive B and T lymphocytes in the bone marrow and thymus, respectively. During T cell development many of the randomly arranged T cell receptors are unusable, as they cannot bind to self-MHC. Positive selection ensures that only T cell progenitors that can interact with MHC-peptide complexes receive survival signals and proceed in their development (MHC restriction). Negative selection then eliminates the majority of high-affinity autoreactive T cells through the processes of clonal deletion, and to a lesser extent through anergy and receptor editing. During this stage, tissue-restricted antigens are presented by medullary thymic epithelial cells (mTECs), tolerizing maturing thymocytes to peripheral organs [1][2]. As central tolerance is not perfect and self-reactive cells “leak” through, peripheral mechanisms exist to curb autoreactivity. Disruption of either of these mechanisms can trigger autoimmunity.

Many chronic, inflammatory diseases are of autoimmune nature, such as rheumatoid arthritis (RA), Hashimoto's thyroiditis, type 1 diabetes, or multiple sclerosis. Previous decades have seen a sharp increase in the incidence of autoimmune diseases in the West with a prevalence of around 5% and a female preponderance. They cause great individual suffering, represent an enormous socio-economic burden, and are among the leading causes of death [3]–[5].

## 1.2 RHEUMATOID ARTHRITIS

One of the most prevalent autoimmune diseases is rheumatoid arthritis (RA), affecting around 0.5-1% of Caucasian individuals [6]. RA is a chronic, complex, and heterogeneous disease with variable clinical presentation and pathogenic mechanisms. Roughly described, environmental factors trigger loss of tolerance in genetically susceptible individuals [7]. An often decades long asymptomatic phase precedes the acute onset of inflammation culminating in synovitis and a faulty wound healing response leading to irreversible damage to cartilage and bone (see Fig.1) [8]. As it can also affect cardiovascular and respiratory systems, RA is considered a systemic disease.

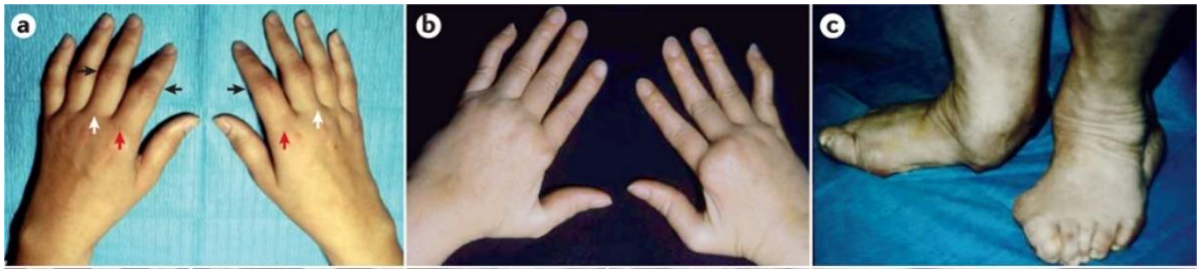


Figure 1**: Clinical manifestation of RA:** a) **early RA** with mild swelling of second (red arrow) and third (white arrow) metacarpophalangeal joints and proximal interphalangeal joints (black arrows) b) **advanced RA** with dislocation of metacarpophalangeal joints as well as swan-neck deformities (most prominent on the fifth digit c) **late-stage RA** with severe deformities of the ankle and foot joints. *Adapted from* [7] *with permission from Springer Nature.*

RA classification is based on clinical manifestations (joint involvement, symptom duration) and serological assays to measure rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPAs) and acute-phase reactants (ACR/EULAR criteria in [9]). There is no cure and the current goal for disease management is remission, with initial treatment strategies combining disease-modifying antirheumatic drugs (DMARDs) such as methotrexate with glucocorticoids (e.g prednisolone). Should this approach be insufficient, biological DMARDs such as etanercept (targeting TNFα) or rituximab (targeting CD20) may be prescribed [7].

### 1.2.1 Risk factors

RA is multifactorial in nature where disease risk is conferred by genetics, environmental triggers, and the female sex. The latter is ascribed to the effects of hormones on the immune system [10] leading to a 2 to 3-fold increased likelihood for women to develop RA.

#### *1.2.1.1 Genetic factors*

RA has a strong genetic component, with an estimated heritability of around 60% according to twin studies [11]. Detailed studies have mapped the genetic landscape of RA and identified around 100 loci conferring disease susceptibility [12]–[16]. The strongest association by far is with the HLA region where all associated HLA-DRB1 alleles share a conserved five amino acid sequence referred to as the "shared epitope". These amino acids are located in the HLA peptide-binding grove, possibly favoring the presentation of arthritogenic peptides and altering the T cell repertoire [17]. In the context of study I it should be pointed out that the single most important contributor to disease risk outside of HLA is a missense variant of *Ptpn22*; all other alleles show relatively small effect sizes [18]. Most markers of genetic heritability remain to be studied in detail. Of note, multiple genes map to signaling pathways downstream of the TCR, CD40 and TNF. The presence of various T cell related genes such as *Ptpn22*, *Ctla4*, *PKC-θ, Tnfaip3,* and *Traf6* indicate the importance of TCR signaling in the pathogenesis of this disease [18][19].

#### *1.2.1.2 Environmental factors*

The high discordance rate between monozygotic twins argues for a substantial role for environmental triggers in RA risk [20]. The strongest environmental risk factor to date is tobacco smoking where disease association is highest in seropositive individuals with at least one copy of the shared epitope [21]. While the underlying mechanisms are still up for debate, possible explanations include systemic inflammation, enhanced oxidative stress and altered DNA methylation patterns [22]. With regards to Study IV, it is interesting to note that low levels of serum vitamin D are associated with increased incidence of RA [23][24]. Finally, it is well known that infections can trigger autoimmune pathologies through molecular mimicry. One example is the Epstein–Barr virus (EBV), which shows sequence similarity to antigens relevant for RA [25]. Similarly, infections by the periodontitis-causing bacteria *P. gingivalis*, which expresses a PAD enzyme that citrullinates bacterial antigens, are proposed to lead to cross-reactivity to citrullinated self-antigens [26].

While the exact cause of RA is unknown, it is likely that genetic and environmental factors converge to ultimately result in the inflammatory response.

### 1.2.2 Disease mechanism

The initial stage is characterized by the production of RF and ACPAs, with the high specificity of the latter to RA implicating them in disease pathogenesis. ACPAs constitute a heterogeneous set of antibodies reacting to a wide array of citrullinated antigens including type II collagen, vimentin and α-enolase [27]. Stratification of RA patients by ACPA-positivity allows classification into two disease subsets with distinct risk allele frequencies [28], pathogenetic mechanisms and disease outcomes [29]. The detection of ACPAs near 7 years before clinical onset [30] argues for a break in tolerance outside of the joint, possibly at mucosal sites such as the gut or lungs [31][32]. Here, environmental risk factors may trigger autoimmunity on a susceptible genetic background.

In the secondary stage autoantibody titers rise, change affinity and epitope spreading occurs until the final stage is reached, joint inflammation. Here, the synovium is a key player; it serves to lubricate cartilage surfaces and provide nutrients to cartilage which lacks its own blood supply and is composed of macrophage-like synoviocytes, fibroblast-like synoviocytes (FLS) and a sublining composed of fibroblasts, adipocytes, blood vessels and scattered immune cells. In the RA synovium both synoviocyte populations greatly expand and are activated, producing a wide array of proinflammatory cytokines such as IL-1, IL-6 and TNF-α. Central to synovitis is macrophage infiltration which acts through cytokines, ROS, phagocytosis and antigen presentation. Other innate effector cells such as neutrophils, mast cells and NK cells also contribute to the proinflammatory environment in the synovium. Importantly, adaptive immune cells infiltrate the synovial lining, either diffusely invading the tissue or forming ectopic germinal centers where mature B cells undergo class-switching and somatic hypermutation [7][33]. The efficacy of rituximab confirms the pathogenic role of CD20+ B cells [34]. Ultimately, FLS production of matrix metalloproteases (MMPs) degrades type II collagen leading to cartilage destruction and TNF-α/ IL-1/IL-6 promote osteoclast differentiation and activation leading to bone erosion [32].

### 1.2.3 Effector T cells in RA

It has long been suggested that proinflammatory Th1 cells mediate inflammation in RA. However, IFNγ receptor knock-out mice show accelerated disease progression and targeting IFNγ has failed in yielding significant results, possibly due to the pleiotropic nature of the cytokine [35]. It should be noted, however, that targeting TNF, a Th1 cytokine, has yielded a successful therapy for RA [36].

Similarly, Th17 cells have been implicated in RA pathogenesis. Th17-inducing cytokines such as IL6, IL1β and IL21 are present in the synovial joint. IL17 production was observed in synovial fluid and tissues, and $IL17^{-/-}$ mice demonstrated less inflammation in collagen-induced arthritis. Disappointingly, targeting IL17 [37] or IL17R [38] has shown limited clinical effects when compared to the outcome on psoriasis.

An increased number of regulatory T cells is found in synovial fluids and tissues from RA patients which display sufficient suppressive capacity [39] and anti-TNF treatment leads to a significant increase in CD4+CD25+ T cell in peripheral blood of RA patients. Another affected effector function is T cell help to B cells. Studies have reported increased frequencies of $CXCR5^{+}ICOS^{+}CD4^{+}$ T follicular helper cells (Tfh) specialized in promoting B cell maturation in the peripheral blood of RA patients [40].

## 1.3 REDOX SIGNALING

### 1.3.1 Reactive oxygen species (ROS)

Reactive oxygen species comprise a family of oxygen derivatives which are produced in response to a wide variety of stimuli such as growth factors, immune challenges, or exercise. The view on ROS has become more nuanced over the past decades. Traditionally known for their role in host defense and overall detrimental effects on macromolecules, ROS' role as physiological signaling mediators is, by now, indisputable. While the term 'ROS' encompasses several molecules with vastly differing reactivities (e.g. $^{1}O_2$, ROOH, …), $H_20_2$ is recognized as the major ROS in the redox regulation of biological activities due to its relative stability and ability to diffuse freely through membranes [41]. Low-level $H_20_2$ functions as a pleiotropic signaling agent which through reversible protein oxidation, acts upon a plethora of biological activities ranging from transcription, inflammation to autophagy and metabolic adaptation (see Fig.3 for an overview of redox-mediated processes). Supraphysiological levels, however, cause irreversible damage to all macromolecules, with oxidative damage to DNA particularly well studied in the field of cancer mutagenesis [42]. Therefore, intracellular $H_20_2$ concentrations in the low nanomolar range are kept tightly in check by efficient reducing systems, the major ones being the thioredoxin and glutathione systems.

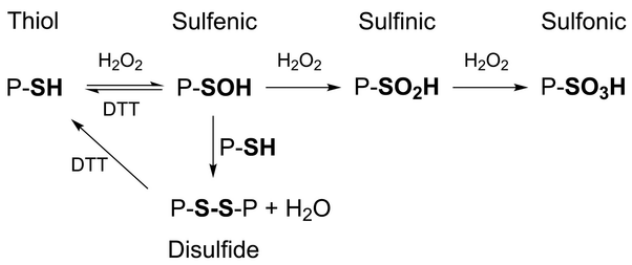


Figure 2: **Oxidation states of protein (P) cysteines**. Hydrogen peroxide induced oxidation and DTT-induced reduction of protein cysteines. *Reprinted under CC BY4.0 license from* [157].

Redox signaling mediates its effects by reversible oxidative modifications of susceptible thiol residues with estimates showing that 10-20% of thiols in the cellular cysteine proteome are oxidized under aerobic conditions [43]. Cysteine residues that exist as thiolate anions under physiological pH values are particularly good targets for the oxidizing action of $H_20_2$. Oxidation of cysteine thiols leads to the formation of sulfenic acid which can react with another thiol to form a disulfide bond. While these reactions are reversible, further oxidation to sulfinic and sulfonic acid is considered to be irreversible [44] (Fig.2).

Only certain molecules carry such a cysteine at a critical position which allows for a specific mechanism at these redox switches. The targets of redox signaling are manifold including transcription factors [45][46], heat shock proteins [47], and especially relevant to this thesis, protein tyrosine phosphatases [48] and scaffold proteins [49].

Well-studied systems of redox biology are the NRF2-KEAP1 and NF-KB pathways. The complexity of redox regulation is illustrated in the latter where $H_20_2$, in a context-dependent manner, plays both stimulatory and inhibitory roles. Whilst cytosolic $H_20_2$ can activate the pathway via oxidation and thereby inhibition of the inhibitor ikb [50], increased nuclear $H_20_2$ can oxidize cysteines in the DNA-binding region of NF-Kb, reducing its transcriptional activity [51].

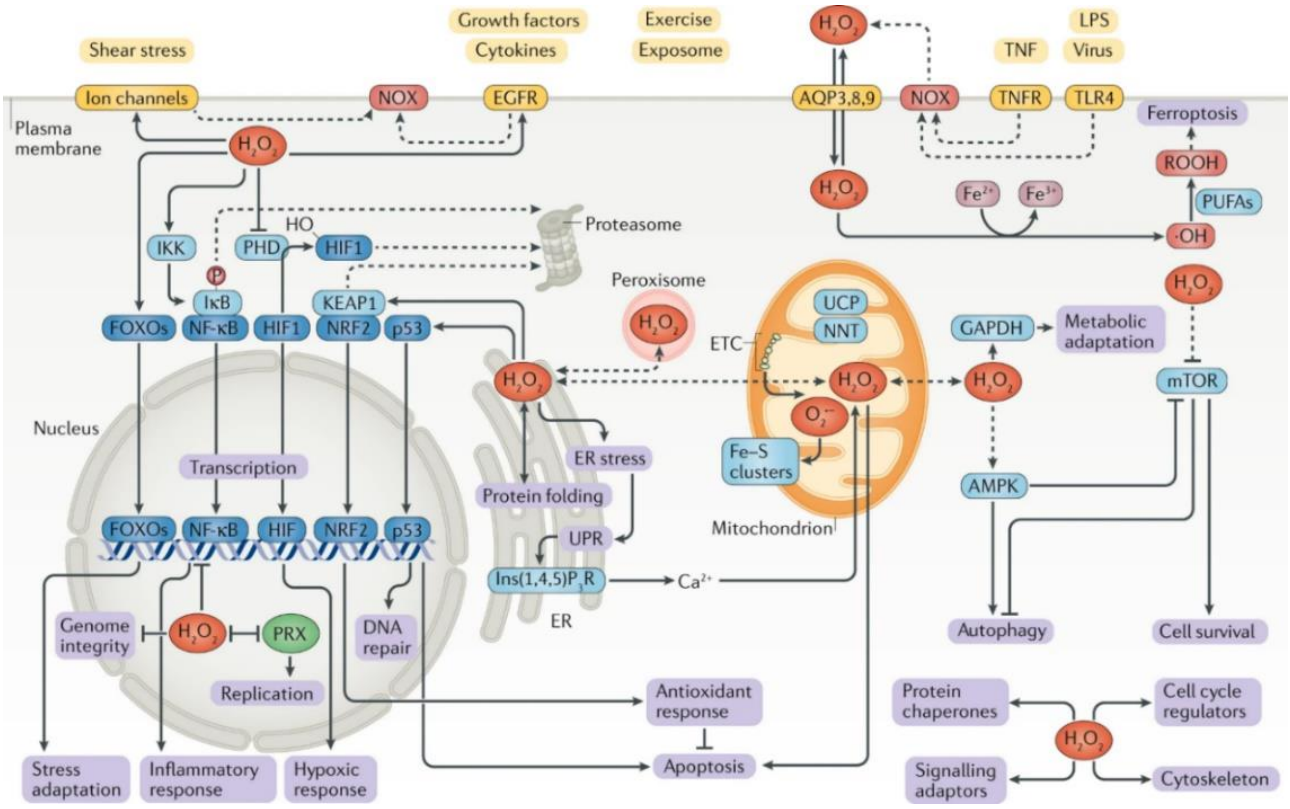


Figure 3: **Redox signaling pathways.** Exogenous stimuli (in yellow) generate the production of $O_2^-$ and $H_20_2$ which act on cellular targets (blue) and thereby regulate various biological activities (purple). *Reprinted from* [42] *with permission from Springer Nature*.

### 1.3.2 NADPH oxidases

The major source of endogenous ROS are the mitochondrial electron transport chain and transmembrane NADPH oxidases (NOXs) which through their varying cellular localizations allow for compartmentalization of redox effects. Particularly relevant to the inducible production of $H_20_2$ are the multidomain NADPH oxidases comprising of seven proteins, NOX 1-5 and DUOX 1-2 which transport electrons across the membrane to reduce oxygen into superoxide [52]. Cell activation translocates the cytosolic components $p47^{phox}$ (Ncf1), $p67^{phox}$ (Ncf2) and $p40^{phox}$ (Ncf4) to the membrane where it associates with cytochrome $b_{558}$ to form an active oxidase [53].

Deficiency in Ncf1-produced ROS in rodents increases susceptibility to a wide array of autoimmune models such as collagen-induced arthritis (CIA), collagen antibody-induced arthritis (CAIA) [54], experimental autoimmune encephalomyelitis (EAE) [55] and pristane-induced lupus [56]. Additionally, conditional expression of Ncf1 in both priming and effector phases of CIA ameliorates disease symptoms [57].

The NOXs are also relevant for human disease: Loss of function mutations in any of their components result in defective ROS production causing chronic granulomatous disease (CGD) characterized by increased risk of infections and inflammatory autoimmune diseases [58]. Furthermore, a SNP in *Ncf1* leading to reduced oxidative burst is highly associated with systemic lupus erythematosus (SLE) [59].

### 1.3.3 ROS in the adaptive immune response

While the role of ROS in neutrophils and macrophages is well studied in terms of NOX-dependent oxidative burst, redox regulation of lymphocytes is less well defined. B cells express all components of the NADPH oxidase and BCR engagement stimulates $H_20_2$ production [60]–[62]. Additionally, treatment of B cells with pervanadate leads to BCR-independent phosphorylation of downstream molecules, mimicking antigen exposure [63]. In T cells, low concentrations of ROS are needed to sustain signaling by modulation of redox-sensitive targets [64]. Interestingly, primary human and mouse T cell blasts express a functional phagocyte type NADPH oxidase and absence of NADPH oxidase components leads to deficiency in TCR-induced ROS and altered T cell responses [65].

There are multiple lines of evidence for the importance of redox effects in T cell function and inflammation. Previous studies have shown that the suppressive functions of human CD8+ T regs are dependent on NOX2-derived ROS and aging-related cell dysfunction is caused by failure to upregulate NOX2 [66]. Macrophage-only expression of *Ncf1* suppresses T cell responses and mediates protection against arthritis in mice [67]. Furthermore, increasing the number of reduced thiols on the surface of T cells increases T cell reactivity, proliferation, and arthritis susceptibility [68]. In addition, RA T cells have a distinct metabolic signature that is linked to their pathogenic potential [69]–[71]. This is characterized by decreased glycolytic activity, thereby shunting glucose into the pentose phosphate pathway leading to increased production of NADPH, favoring reductive conditions in these cells.

Defective oxidative signaling prevents the activation of the redox-sensitive kinase ataxia telangiectasia mutated (ATM) creating an increased pool of inflammatory T cells [72].

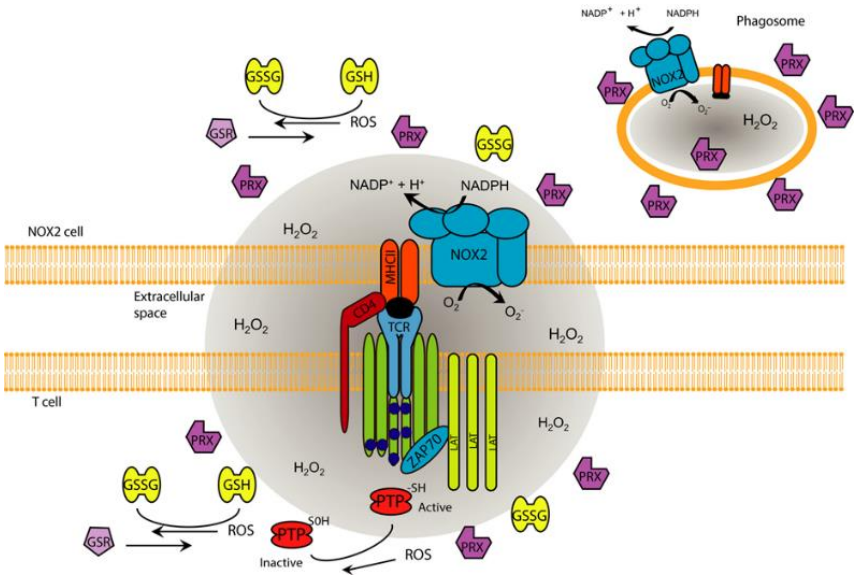


Figure 4: **ROS effects at the intersection between antigen presenting cell and T cell**. *Reprinted from* [54] *with permission from John Wiley and Sons.*

Where and how ROS exerts its effects is still a matter of investigation. Its relatively long half-life and uncharged nature afford $H_20_2$ greater stability and the ability to freely diffuse through membranes. However, the scavenging functions of reducing enzymes most likely limit the reaction radius of $H_20_2$, thereby compartmentalizing the oxidative effect. This allows for transient, localized accumulation of $H_20_2$ around membranes, particularly within signaling domains [73]. An attractive place where this could occur is the immune synapse, the interphase between antigen-presenting cell and lymphocyte. Here, locally produced $H_20_2$ may regulate key membrane proximal targets such as PTPN22 and LAT which are the subject of this thesis (Fig.4).

## 1.4 PTPN22

An attractive target for redox regulation is *Ptpn22* **(**Protein tyrosine phosphatase, non-receptor type 22), a cytoplasmic tyrosine phosphatase that is expressed in all hematopoietic cells. Interest in this protein has exploded since the discovery that a single-nucleotide polymorphism in *Ptpn22* (C1858T) predisposes to a variety of autoimmune diseases such as type 1 diabetes, rheumatoid arthritis, and SLE [74]–[76]. Minor allele frequencies vary greatly among populations: Northern Europeans of Caucasian descent show the highest frequency (15% in Finland) with prevalence following a north-to-south gradient (2% in Italy; very rare in African and Asian populations) [77][78].

### 1.4.1 PTPN22 signaling

Generally, C1858T shows higher association with autoantibody-associated diseases such as RA, T1D, lupus, with no effect observed on MS or psoriasis, hinting at anomalies in B cell signaling or T cell help [79]–[81].

While the autoimmune variant of PTPN22 has been shown to impair binding to the kinase CSK [82], it is still hotly debated how this translates into a pathophysiological mechanism with a wealth of evidence behind both loss-of-function and gain-of-function hypotheses [83][84].

Much effort has been invested in discovering the pathways underlying aberrant immune cell activation and delineating the relative contribution of various cells to the immune response using systems where the protein has been either knocked out or mutated into the autoimmune variant (R620W).

#### *1.4.1.1 T cells*

PTPN22 is well established as a negative regulator of TCR signaling. *Ptpn22* $^{-/-}$ mice show enhanced TCR-induced signaling and calcium mobilization after TCR triggering leading to increased positive selection and expansion of effector/ memory T cells [85]. Knockdown of *Ptpn22* in human peripheral T cells shows increased phosphorylation of TCR-CD3ζ, Zap-70 and Slp-76 [82]. The *Ptpn22* promoter is a target of Foxp3 [86], and knocking out the gene expands peripheral Treg numbers and improves their suppressive qualities [87][88]. PTPN22 also aids in the discrimination of weak versus strong ligands, restraining damaging inflammatory responses to weaker agents such as autoantigens [89]. Substrate-trapping experiments have identified the primary substrates of PTPN22: Fyn, LCK, Zap70, Vav, TCR-CD3ε and CD3ζ [90][91].

#### *1.4.1.2 B cells and myeloid cells*

*Ptpn22* appears to be dispensable for B cell signaling and development as *Ptpn22* $^{-/-}$ mice show no differences in BCR signaling and B cell compartments [85][92]. However, this contrasts sharply with findings related to mice and humans expressing the autoimmune variant where the T allele allows the escape of autoreactive B cell clones due to defective central tolerance preceding the onset of symptoms [93]. Inhibition of *Ptpn22* succeeded in resetting central tolerance in a mouse model [94]. In myeloid cells, *Ptpn22* functions independently of its phosphatase function: PTPN22-TRAF3 interaction potentiates type I interferon signaling whilst PTPN22-PAD4 binding inhibits citrullination; both functions are disturbed by the R620W variant [95]–[98].

#### *1.4.1.3 Autoimmunity*

*Ptpn22* $^{-/-}$ mice do not develop spontaneous autoimmunity, and the various pathogenic/ protective effects on inflammation depend on genetic context and experimental model. PTPN22 deficiency on the arthritis-susceptible KBxN background worsens disease [99], but decreases disease severity in the EAE model [88]. Both silencing and overexpression of *Ptpn22* lead to protection in T1D models [92][100].

#### *1.4.1.4 Oxidative regulation of PTPs:*

The activity of PTPs such as PTPN22 can be regulated through the reversible oxidation of their catalytic cysteine. The unique environment of the PTP active site renders the pKα value of the sulfhydryl group extremely low which makes it susceptible to oxidation. Treatment of various PTPs with $H_20_2$ *in vitro* has shown to abrogate their activity as the modified Cys residue can no longer function as a phosphate acceptor [101][102]. A crystal structure of PTPN22 revealed a disulfide bond formation between the catalytic cysteine (C227) and a "back-door" cysteine at position 129 [103]. This presented a possibility for redox regulation of PTPN22 which was the starting point for study I.

## 1.5 LAT

LAT (Linker for Activation of T cells) is a transmembrane protein that is rapidly phosphorylated upon TCR activation. It nucleates the assembly of a multiprotein signaling complex, serving as a molecular scaffold that propagates the TCR signal [104]. Important interactors include PLC-γ1, Grb2, Gads and SLP-76 which associate to LAT via SH2/SH3 domain binding of phosphorylated tyrosine residues (see Fig.5 for an overview of the LAT signaling complex).

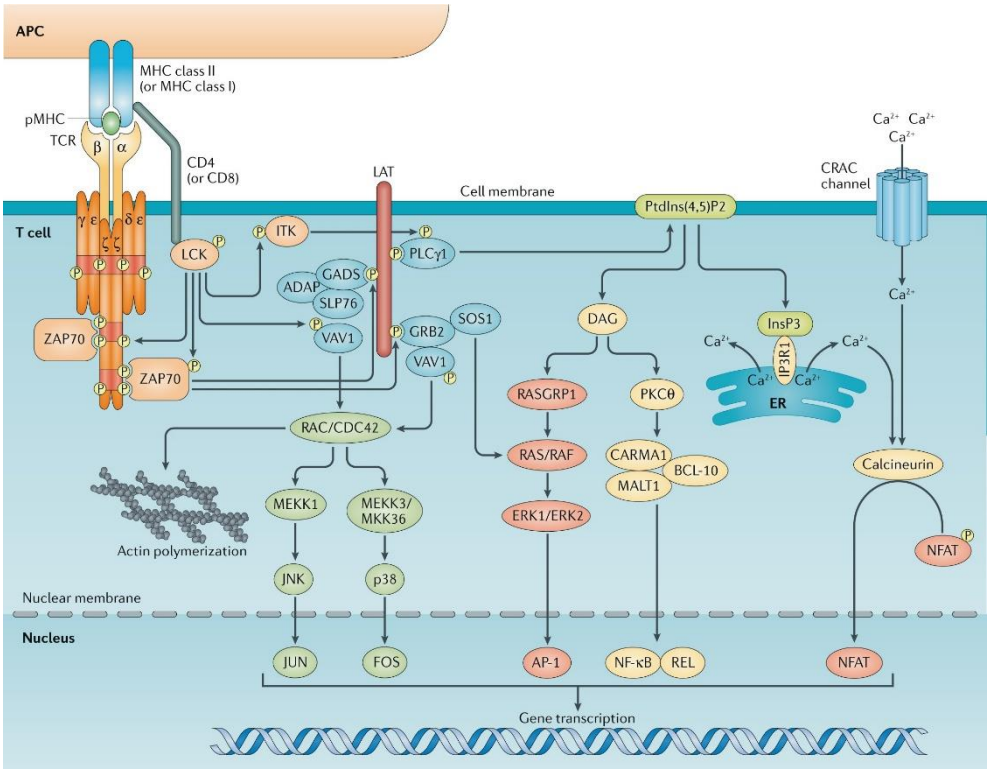


Figure 5: **LAT as central mediator of T cell signaling.** Binding of the TCR to peptide-MHC complexes triggers T cell signaling transduction. LCK phosphorylates ITAM motifs on the CD3 chain, allowing the binding and activation of ZAP70 which in turn phosphorylates LAT. Various effector proteins are recruited to LAT forming the LAT signalosome which mediates $Ca^{2+}$–calcineurin, mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways. *Reprinted with permission from Springer Nature from* [156].

### 1.5.1 LAT signaling

The role of LAT in T cells is well established. LAT deficient Jurkat T cells show reduced phosphorylation of PLC-γ1 and SLP-76 and fail to activate the IL2 transcriptional machinery. Importantly, reintroduction of LAT rescues TCR signaling [105].

Extensive work has been performed to identify the role of various amino acid residues on the cytoplasmic tail of LAT. By mutating distal tyrosines to phenylalanine, Zhang et al., could pinpoint the binding preferences of various LAT interactors to different tyrosine residues [106]. Palmitoylation of the membrane-proximal cysteine residues C26 and C29 is essential for LAT insertion into the plasma membrane and anergic T cells show defective immune synapse localization of LAT due to defective palmitoylation [107][108].

LAT is involved in pre-BCR signaling [109] and LAT-deficient mast cells show defects in the phosphorylation cascade downstream of FcεR1 [110]. Interestingly, the cytoplasmic region of LAT has intrinsically disordered properties which is characteristic of signaling hubs as the unstructured nature improves accessibility for its binding partners [111].

#### *1.5.1.1 Autoimmunity*

LAT is essential to the functioning of the pre-TCR: $LAT^{-/-}$ mice show thymocyte development arrest at the double positive stage, with no mature T cells present in the periphery [112][113]. In humans, a loss-of-function mutation in *LAT* leads to a severe combined immunodeficiency phenotype characterized by a significant lack in T cell development [114]. Targeted loss-of-function mutations of *Lat* in mice have resulted in a series of phenotypes collectively termed the "LAT signaling pathology" which are characterized by aggressive hyperproliferation of Th2 effector cells [115].

In cells that were made deficient in LAT, LCK- and ZAP70- induced phosphorylation patterns were nearly comparable with LAT-sufficient signaling [116]. This seemingly paradoxical result can be attributed to the lack of the negative feedback loop that keeps the TCR signaling module and LAT independent signaling pathways in check [117].

#### *1.5.1.2 Oxidative regulation of LAT in RA*

$CD4^+$ T cells from RA patients exhibit a peculiar phenotype: while they are hyporesponsive to TCR engagement when stimulated *ex vivo* as evidenced by IL2 production and $Ca^{2+}$ mobilization [118], they hyper-proliferate and differentiate into effector T cells that drive the disease [119][120]. Interestingly, synovial fluid T cells from RA patients have been proposed to exhibit an oxidative milieu due to significant reduction of the anti-oxidant glutathione [121]. The observation that this changes LAT localization [122][123] sparked the idea for study II.

# 2 RESEARCH AIMS

The overarching theme of this thesis is: mechanisms contributing to T-cell dependent autoimmunity. Specifically, Studies I and II focused on the role of redox regulation on T cell signaling proteins whilst Studies III and IV investigated arthritis-regulating quantitative trait loci (QTL) and thereby discovered two important regulators of T cell function in autoimmunity. Thus, our aims were as follows:

- Identify if and how redox regulation of PTPN22/LAT affects T cell function and arthritis development.
- Investigate the mechanisms behind the Cia37 [124] and Pia43 [125] QTLs.

# 3 METHODS

## 3.1 MOUSE MODELS OF INFLAMMATION

**Delayed-type hypersensitivity (DTH):** This model is essentially an *in-vivo* recall assay that is dependent on Th1 responses by $CD4^+$ T cells. In the sensitization phase, animals are subcutaneously immunized with antigen emulsified with an adjuvant and 5-14 days later are re-exposed to the antigen by dermal injection into the skin, leading to expansion of antigen-specific T cells as well as production of inflammatory cytokines. The model can be evaluated by assessing localized swelling, immune cell infiltration as well as cytokine profiling [126].

**Collagen-induced arthritis (CIA):** Considered the gold standard model for RA, CIA is induced by immunization with heterologous collagen type II (CII) emulsified in Complete Freund's Adjuvant. This elicits autoreactive T cell responses [127] and production of arthritogenic autoantibodies [128] leading to synovitis, pannus formation, and finally culminating in cartilage and bone destruction [129]. Enhanced reactivity against CII is also detected in RA patients, in terms of both autoreactive T cell and antibody responses [130][131].

**Glucose-6-phosphate isomerase-induced arthritis (GIA):** This murine model of arthritis is based on the observation that chronic, spontaneous polyarthritis in K/BxN T cell receptor (TCR) transgenic mice is mediated and can be transferred by antibodies specific to the ubiquitously expressed glucose-6-phosphate isomerase (GPI). GPI may also be an important autoantigen in RA as anti-GPI IgGs are elevated in serum and synovial fluid of RA patients as compared to controls [132]. Here we immunize mice with the immunodominant T cell epitope of human GPI (hGPI 325-339) which has been shown to induce disease with high severity and incidence on the MHC II q haplotype [133]–[135].

# 4 RESULTS AND DISCUSSION

## 4.1 STUDY I - REDOX REGULATION OF PTPN22 AFFECTS THE SEVERITY OF T CELL DEPENDENT AUTOIMMUNE INFLAMMATION.

PTPN22 is a regulator of T cell receptor signaling and major susceptibility factor for a wide array of autoimmune conditions [81]. It belongs to the class of PTPs which are well-known to be regulated by oxidation of their catalytic cysteines [136]. However, this had hitherto not been studied *in vivo*. By mutating the redox-sensitive cysteine residue C129 in mice we could analyze the impact of altered sensitivity to redox changes on PTPN22 effector functions. We show here that PTPN22 can indeed be oxidatively regulated via this non-catalytic residue and that lower resistance to oxidation promotes downstream signaling and arthritis development.

Though the relevance of *PTPN22* in autoimmunity and therefore as a therapeutic target is undisputed, the nature of the PTP active site makes it notoriously difficult to target [84][137]–[139]. An alternative approach may be to focus on redox-sensitive effects. Recent advances in the field of PTP1B, a promising target for the treatment of Type 2 diabetes, may serve as an example: conformation-sensing antibodies have been shown to successfully stabilize the oxidized form thereby targeting phosphatase function [140][141].

## 4.2 STUDY II - REDOX REGULATION OF LAT ENHANCES T CELL MEDIATED INFLAMMATION.

In a similar vein to the prior project, Study II investigated redox regulation of another key T cell protein, LAT. Previous evidence suggested the presence of redox-sensitive cysteines [122][123] which we mutated to generate a mouse model that is insensitive to redox changes. Using this *in vivo* approach, we show that redox insensitivity affects LAT phosphorylation/localization and further impacts thymic selection, peripheral T cell populations and autoimmune inflammation.

Dysregulation of LAT function is heavily associated with autoimmune phenotypes in mice [115][142][143] and the hypoactive behavior of synovial fluid T cells from RA patients is attributed to the effects of redox imbalance on LAT [123]. As LAT surface levels correlate with the magnitude of the T cell response this study provides further insight into fine-tuning LAT expression through redox regulation and thereby altering downstream signaling and the autoimmune response.

## 4.3 STUDY III - ENDOPHILIN A2 DEFICIENCY PROTECTS RODENTS FROM AUTOIMMUNE ARTHRITIS BY MODULATING T CELL ACTIVATION.

This study was based on the observation that a subset of DA rats which are usually highly susceptible to chronic inflammatory diseases [144], was suddenly resistant to arthritis. Further analysis revealed a spontaneous mutation in the *Sh3gl1* gene leading to a loss of *Sh3gl1*-encoded EA2 expression. Indeed, knock-out mice confirmed that resistance to arthritis could be attributed to loss of *Sh3gl1* which in turn impacted TCR internalization and T cell signaling. Additionally, *Sh3gl1* expression was upregulated in RA patients, implicating a role for *Sh3gl1* in RA disease pathogenesis [125].

Curbing the expansion of autoreactive T cells is key in targeting autoimmune diseases. However, most efforts in restraining T cell activation have, with mixed results, focused upon modulation of co-stimulators or cytokine pathways rather than the TCR signal itself [145]–[147]. Therefore, targeting and fine-tuning important elements of the TCR machinery may represent a novel treatment approach. Here we have characterized *Sh3gl1* as a key signaling molecule in the TCR signaling cascade and thus identified an attractive new target for the treatment of RA and other T-cell mediated diseases.

## 4.4 STUDY IV - VITAMIN D3 RECEPTOR POLYMORPHISMS REGULATE T CELLS AND T CELL-DEPENDENT INFLAMMATORY DISEASES.

In this study we identified polymorphisms in the vitamin D receptor (*Vdr*) promoter controlling *Vdr* expression and T cell activation. Overexpression of *Vdr* in activated T cells led to increased susceptibility to T-cell dependent autoimmunity. We also found increased *Vdr* expression in synovial tissues of RA patients suggesting a crucial function for *Vdr* in human disease pathology [148].

The importance of vitamin D as a dietary supplement has attracted a great deal of attention in popular culture. However, whether and how vitamin D exerts its immunoregulatory functions is contested. Whilst vitamin D itself has been reported to have anti-inflammatory properties [149][150] and vitamin D deficiency has been associated with several autoimmune conditions [151][152], clinical trials have not revealed convincing benefits [153]–[155]. Here we have made use of naturally occurring genetic variants to further elucidate the mechanisms behind vitamin D signaling in a physiological context and identified the Vdr as a proinflammatory mediator of T cell effector function.

# 5 CONCLUDING REMARKS

In conclusion**, Studies I** and **II** highlight the physiological importance of ROS regulation veering away from the prevalent view of ROS as mere mediators of cell damage and exemplifying their rather subtle workings within signaling systems. They provide a glimpse into how redox pathways impact immunoregulation. Further insight into these processes may reveal additional opportunities for therapeutic intervention.

**Studies III** and **IV** demonstrate the power and elegance of mouse models in identifying and functionally characterizing causal variants of complex traits. Both add to the understanding of the complex genetic makeup of autoimmune diseases. Study IV addresses the importance of vitamin D receptor polymorphisms and Study III identified a novel immune regulator. The latter may even be of therapeutical interest, not just for RA, but also for autoimmune diseases in general.

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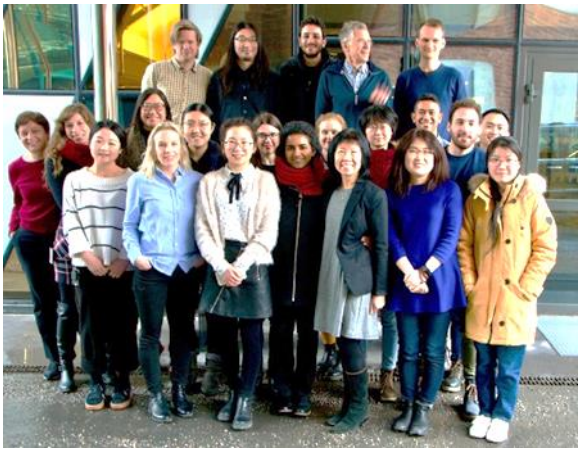

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