# Rho-GTPases in Rheumatoid Arthritis

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*“It doesn’t have to be fun to be fun.”*

# ABSTRACT

The success of alleviating rheumatoid arthritis (RA) symptoms is complicated by both heterogeneity of the disease and lack of predictive markers to guide treatment options. Deregulated Rho-GTPases, a family of hydrolase enzymes catalyzing guanosine triphospate (GTP) to guanosine diphospate (GDP), have a detrimental role in many diseases including RA. The aims of this thesis were to identify intercellular interactions and molecular pathways in RA linked to signal transducers of the Rho-GTPase family and assess the effect of anti-rheumatic treatments on these molecular pathways.

**Paper I:** Mice with a conditional knockout of Geranylgeranyl transferase type I (GGTase-I) in macrophages (*GLC* mice) develop RA due to hyper-activation of Rho-GTPases. Reciprocal expression of the Rho-GTPases Cell division control protein 42 homolog (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) in T cells, as well as suppression of caudal Homeobox A (HoxA) caused migration of thymic T regulatory cells (Tregs) into the joint-draining lymph node.

**Paper II:** We examined Rho-GTPase dependent biological processes by utilizing the transcriptome of blood $CD14^+$ monocytes and of synovial tissue macrophages at single cell resolution. These studies resulted in a metabolic gene signature which identified circulating progenitors of RA synovial antigen presenting cells. Inhibition of Janus Kinases (JAK) suppressed this progenitor population, partially explaining the anti-rheumatic effect.

**Paper III:** Examining the transcriptome of blood $CD4^+$ T helper cells, we demonstrated that Pre-B cell leukemia transcription factor 1 (PBX1) marks recent thymic emigrants. RA patients with high *PBX1* expression in $CD4^+$ T cells had favourable outcomes to anti-rheumatic treatment, predicting good response to inhibition of Tumor necrosis factor (TNF)-α and stable remission. Taken together, these studies demonstrates that Rho-GTPases mediate interplay between T-helper cells and macrophages supporting antigen presentation and IFN-γ signalling, which drives RA pathology. Moreover, we propose two approaches for endotyping RA, a metabolic signature in $CD14^+$ monocytes and expression of *PBX1* in $CD4^+$ T cells which mark recent thymic emigrants. The former approach identifies patients which may benefit from inhibition of JAK whereas the latter from TNF-α inhibition.

**Keywords**: Rho-GTPases, rheumatoid arthritis, innate immunity, adaptive immunity, RA treatment

# SAMMANFATTNING

Reumatisk artrit (RA) eller ledgångsreumatism är en autoimmun sjukdom som drabbar lederna. Med en prevalens på ca 1 procent av befolkningen utgör den en stor kostnad för samhället. Ett stort problem med RA är dess heterogenitet, vilket gör det svårt att välja rätt behandling för individen. Målet med denna doktorsavhandling var att studera intracellulära signal proteiner av familjen Rho-GTPaser och deras roll i utvecklingen och fortskridningen av RA, och även hur de påverkas av antireumatisk behandling.

I ett första steg användes en musmodell av RA, i denna specifika musstam har man tagit bort genen som kodar för enzymet GGTase-I i musen makrofager, en typ av cell i det medfödda immunförsvaret. Normalt sett interagerar detta enzym med Rho-GTPaserna för att upprätthålla korrekt funktion, och borttagandet resulterar i överaktivering av Rho-GTPaserna. Detta leder till utvecklingen av ledgångsinflammation med reaktion från det adaptiva immunförsvaret i form av T-cellsinvasion i leden och produktion av antikroppar, mycket likt RA i människor. I denna modell studerade vi T-cellernas fenotyp och migrationsmönster mellan mjälten och lymfkörteln som dränerar leden. Det vi observerade var att en viss typ av T-celler s.k. thymiska regulatoriska T-celler också aktiverade Rho-GTPaser i samband med att de inaktiverade HOX proteiner som är involverade i utveckling, omvandling och migration av celler. Dessa celler ackumulerades sedan i mjälten och lymfkörteln. Förutom detta, kunde vi genom genuttrycksanalys via RNA-sekvensering se att T-cellerna i lymfkörteln nära leden var påverkade av cytokinen IFN-γ (interferon-gamma) vilket gjorde dem inflammatoriska och därmed förvärrade ledinflammationen.

Därefter, undersökte vi patienter med RA. Från blod isolerades $CD14^+$ monocyter, immunceller som omvandlas till makrofager i vävnader. Genom att sortera patienterna i grupper med högt och lågt uttryck av Rho-GTPaser i monocyter, kunde vi med hjälp av RNA-sekvensering ta reda på vilka cell processer som är associerade med aktivering Rho-GTPaser. Vi sammanställde dessa processer i en gensignatur. Med hjälp av signaturen och oberoende ”single-cell” RNA-sekvensering på makrofager från RA-leder kunde vi urskilja en population makrofager i leden som är involverade i antigenpresentation vilket kan förvärra sjukdomen. Med andra ord, monocyterna i blod med hög aktivering av Rho-GTPaser omvandlas till antigenpresenterade makrofager i RA-leden, vilket förvärrar sjukdomen. Slutligen observerade vi att behandling med s.k. JAK-hämmare kunde reducera denna population av monocyter i blod, vilket delvis förklarar dess

antireumatiska effekt. Dessutom, kan gensignaturen appliceras för hitta patienter som möjligen borde behandlas med JAK-hämmare.

Även T celler från patienter med RA studerades med hänsyn till observationer gjorda i möss. Vi utnyttjade en molekylär partner till HOX proteiner i detta fall, en transkriptionsfaktor kallad PBX1. Likt studien av monocyter i RA patienter, delade vi patienterna i grupper med högt och lågt uttryck, denna gång av *PBX1* i $CD4^+$ T celler. Vi fann att celler med högt PBX1 uttryck urskilde olika cellpopulationer beroende på om patienterna hade aktiv sjukdom eller om de befanns sig remission. I patienter med aktiv sjukdom identifierades celler som var påväg till thymus, medan *PBX1*-uttryckande-celler i patienter som var i remission på grund av behandling sammanföll med celler som precis lämnat thymus. I båda fallen så var *PBX1*-uttryck i $CD4^+$ T celler positivt, i det första fallet resulterade det i bättre svar efter behandling med biologiska läkemedel, och i andra fallet överensstämde det med kontrollerad remission. Detta medför att mätning av *PBX1*-uttryck kan användas som hjälpmedel vid val av behandling av RA.

Sammanfattningsvis ger arbetet i denna avhandling mer kunskap om Rho-GTPasers roll i interaktionen mellan det medfödda och adaptiva immunförsvaret i RA. Dessutom föreslås två strategier för att vägleda valet av RA-behandling med antingen JAK-hämmare eller biologiska läkemedel.

# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Malmhäll-Bah E**, Andersson KME, Erlandsson MC, Akula MK, Brisslert M, Wiel C, El Zowalaty AE, Sayin VI, Bergö MO, Bokarewa MB. 2022. Rho-GTPase dependent leukocyte interaction generates pro-inflammatory thymic Tregs and causes arthritis. *Journal of Autoimmunity. 2022 Jun 02. 130; 102843*

II. **Malmhäll-Bah E**, Andersson KME, Erlandsson MC, Silfverswärd S, Pullertis R, Bokarewa MB. Metabolic signature and proteasome activity controls synovial migration of *CDC42*$^{hi}$CD14$^{+}$ cells in rheumatoid arthritis. *Frontiers in Immunology. 2023, under revision.*

III. Andersson KME, **Malmhäll-Bah E**, Oparina N, Tao W, Pandit A, Erlandsson MC, Chandrasekaran V, Silfverswärd S, Pullertis R, Bokarewa MB. Pluripotency factor PBX1 predicts treatment efficacy in rheumatoid arthritis. *Journal of Clinical and Cellular Immunology. 2022 Jul 12. 13(5).*

# ADDITIONAL PAPER NOT INCLUDED IN THE THESIS

- Chandrasekaran V, Oparina N, Garcia-Bonete MJ, Wasén C, Erlandsson MC, **Malmhäll-Bah E**, Andersson KME, Jensen M, Silfverswärd ST, Katona G, Bokarewa MI**.** 2022. Cohesin-mediated chromatin interactions and autoimmunity. *Frontiers in Immunology. 2022 Feb 10. 13.*
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- Wahlström N, Nylander F, **Malmhäll-Bah E**, Sjövold K, Edlund U, Westman G, Albers E. Composition and structure of cell wall ulvans recovered from Ulva spp. along the Swedish west coast. *Carbohydrate Polymers. 2020 Apr 1. 233; 115852.*
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# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| aCD3 | Anti-CD3 monoclonal antibody |
| ACPA | Antibody against citrullinated peptide |
| APC | Antigen presenting cell |
| ATAC-seq | Assay for Transposase-Accessible Chromatin by sequencing |
| CCL | CC chemokine ligand |
| CCR | CC chemokine receptor |
| CD | Cluster of differentiation |
| CDC42 | Cell division control protein 42 homolog |
| ChiP-seq | Chromatin immunoprecipitation sequencing |
| CII | Collagen II immunization |
| CLP | Common lymphoid progenitor |
| conA | Concanavalin A |
| CTLA4-fp | CTLA4 fusion protein (abatacept, antirheumatic drug) |
| CXCL | CXC chemokine ligand |
| CXCR | CXC chemokine receptor |
| DAMP | Damage associated molecular pattern |
| DAS28 | Disease activity score based on 28 joints |
| DC | Dendritic cell |
| DEG | Differentially expressed gene |
| dLN | draining lymph node |
| DMARD | Disease modifying anti-rheumatic drugs |
| DN | Double negative |
| DP | Double positive |
| ELISA | Enzyme linked immunosorbent assay |
| GAP | GTPase activating proteins |
| GDI | Guanosine dissociation inhibitors |
| GEF | Guanosine nucleotide exchange factor |
| GGTase-I | Geranylgeranyl transferase type I |
| HLA | Human Leukocyte Antigen |
| Homeobox | homologous alien box |
| IFN | Interferon |
| IL | Interleukin |
| IS | Immunological synapse |

| | |
|---|---|
| JAK | Janus Kinase |
| JAKi | JAK inhibitor |
| LPS | lipopolysaccharides |
| MHC | Major histocompatibility complex |
| MTX | Methotrexate |
| Mø | Macrophage |
| OXPHOS | Oxidative phosphorylation |
| PAMP | Pathogen associated molecular patterns |
| PBX1 | Pre-B cell leukemia transcription factor 1 |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative Polymerase chain reaction |
| RA | Rheumatoid arthritis |
| RAC1 | Ras-related C3 botulinum toxin substrate 1 |
| RF | Rheumatoid Factor |
| RHOA | Ras homolog family member A |
| RNA-seq | RNA-sequencing |
| RTE | Recent thymic emigrants |
| SE | Shared epitope |
| STAT | Signal Transducer and Activator of Transcription |
| TCR | T cell receptor |
| Tfh | T follicular helper cell |
| Th1 | Type 1 T helper cell |
| Th17 | Type 17 T helper cell |
| Th2 | Type 2 T helper cell |
| TNFi | TNF-α inhibitor |
| TNF-α | Tumor necrosis factor a |
| Treg | T regulatory cell |
| tTreg | Thymic T regulatory cell |

# DEFINITIONS IN SHORT

| | |
|---|---|
| *GLC* mice | A strain of mice with conditional knock out of the gene *Pggt1b,* encoding the beta-subunit of GGTase-I, in macrophages due to Cre-recombinase expression coupled to Lysozyme promoter. |
| Rho-GTPase | Hydrolase enzymes of the Rho family catalyzing GTP to GDP |

# 1 IMMUNE SYSTEM

The immune system is a whole-body encompassing and highly complex system. It protects us from various sources of pathogens like bacteria, viruses and malignant cells initiating responses to remove them, and at the same time it is controlled enough to leave non-harmful entities alone like the commensal microbiome or your own cells. It recognizes invaders quickly, and at same time it remembers those invaders for a later infraction. To achieve this, many checks and balances have sprung up over the course of evolution.

The immune system consists of different highly specialized cells, grouped together called white blood cells or leukocytes. These leukocytes develop in dedicated organs. There are primary lymphoid organs like the bone marrow and the thymus where the leukocytes develop, and secondary lymphoid organs where they can communicate with each other to gain a specific function required for the situation. However, some immune cells adopt development to the tissues they surveil, which could be anywhere in body. Speaking of surveillance, this is performed constantly and everywhere by the immune system. It is achieved by utilizing not only the blood circulation but also the lymphatic vessels, the immune system's own highways in body. The lymphatic vessels reach every organ and distant corner in body. During an immune response, leukocytes use them to travel back to secondary lymphoid organs to activate other cells, gaining a full immune response. In addition, antigens from sites of inflammation can reach the secondary lymphoid organs via the lymphatic vessel, and in that way initiate immune response.

Once immune cells are activated by encountering unwanted entities their job is to remove them. This is done in different ways, the immune cells can release highly reactive chemicals or enzymes to kill the invaders, they can "eat" them in a process called phagocytosis or if they can't do either of these, they can send out signals in the form of proteins called cytokines to attract immune cells that can.

So, the immune system has a lot on its plate, and it is amazing that things don't go wrong all the time. This is maintained due to the checks and balances, requiring a lot of communication between cells, since a single type of immune cell can't do it all. In the current paradigm, the immune system is defined as the unspecialized, quick acting, and memory lacking, innate system and the specialized, convoluted, long term adaptive system.

## 1.1 INNATE IMMUNE SYSTEM

Because of its nature, the innate immune system lends itself to be explained before the adaptive. The leukocytes that make up the innate immune system are monocytes and macrophages, as well as dendritic cells (DC), neutrophils, and Natural Killer cells (NK cells). However, the skin, GI-tract, mucosa, and blood-brain serve as barriers against foreign substances and are thereby also considered components. The main responsibilities of innate immunity are to:

1. recruit leukocytes to inflammation sites.
2. activate complement cascade to detect bacteria, activate cells, and induce clearance of antibody complexes or dead cells.
3. identify and remove foreign substances present in the body.
4. educating and activating the adaptive immune system through presenting parts of the foreign entity, so called antigens.
5. act as a physical and chemical barrier to pathogens.

In this thesis I will mainly focus on monocytes and macrophages, their recruitment to inflamed joints and antigen presentation. Recruitment of leukocytes is achieved by producing chemical factors and signal molecules, cytokines, and chemokines at the inflammation site. There are specific molecules produced for recruitment of specific cells. For instance, CCL1, a chemokine produced by different immune cells attracts cells specifically carrying the receptor CCR8. So, when CCL1 is produced at the inflammation site, it will disperse and create a gradient in the blood circulation, more CCL1 close to inflammation and less the farther away you get, guiding the wanted CCR8-bearing leukocytes to the site, a process called chemotaxis. However, chemotaxis does not explain the whole process of cell recruitment. The cell being recruited must undergo certain changes to enable entry into the inflammation site. The cell must attach to endothelial cells of the blood vessel to stop moving along with the blood flow. They must then roll along the vessel wall till they reach the site for entry into tissue where the inflammation is occurring. And lastly, they must alter their shape to squeeze between endothelial cells and reach the destination. These processes are called cell adhesion, rolling and transmigration, and Rho-GTPases, recognizable from the title of this thesis, are important regulators of these process, which I will explain in more detail later.

Before antigen presentation can occur, foreign substances must be recognized. The cells of the innate immune system achieve this by carrying pattern recognition receptors on their surface. These receptors have evolved to identify two types of molecules, pathogen associated molecular patterns (PAMPs) and

damage associated molecular pattern (DAMPs). PAMPS can be carbohydrates of bacterial origin, one commonly used during research to artificially induce a response in innate immune cells is (LPS). DAMPs are molecules released by damaged cells like ATP or uric acid. Once PAMPs or DAMPs are recognized, the innate immune cells can initiate the steps needed to remove the foreign substance, like phagocytosis, eventually leading to antigen presentation.

Antigen presentation is the central process in communication between innate and adaptive immunity. It is the way for damaged cells to signal they should be removed. Although, there are signal molecules (cytokines) which are used in cell interaction as well, antigen presentation is the process deciding what the adaptive immune system should search for. When a cell is infected with a pathogen, it can process/disintegrate that pathogen into smaller pieces. The proteins of the pathogen are broken into peptides, either by proteases or by a protein complex called the proteasome. The peptides are then loaded onto the major histocompatibility complexes (MHC) also known as human leukocyte antigen (HLA) proteins. There are two kinds of MHC, class I and class II. Class I MHC are exposed by many types of cells, not just the leukocytes in the innate system, and are recognized by cytotoxic $CD8^+$ T cells which upon binding, kills the cell. Thus, this is a system to remove infected or malignant cells.

Then, there is class II MHC, responsible for establishing a lasting immunity. These are exposed after phagocytosis, on professional antigen presenting cells (APC), including monocytes, macrophages, DCs and B cells. Antigens presented by MHC class II are recognized by $CD4^+$ T helper cells, after which the T cell will coordinate the development of memory cells and induce antibody production in B cells, all against that specific antigen. This creates a long-lasting defense against the specific antigen.

### 1.1.1 MONOCYTES

Like all the cells of the innate system monocytes are derived from the myeloid lineage of bone marrow cells. They develop in the bone marrow and then enter the blood stream. From there, they can enter tissues and differentiate into and replenish macrophage and DC populations. However, additional functions of the monocyte have been described. In fact, monocytes are avid professional APC and are thereby involved in coordinating T cells response[1]. They also produce cytokines and chemokines to recruit other leukocytes and they can become pro-inflammatory initiating an immune response.

These additional functionalities divide monocytes into 3 subsets of classical, non-classical and intermediate monocytes, based on their expression of surface

markers CD14 and CD16[2]. Classical monocytes express high levels of CD14 and no CD16. Non-classical monocytes express CD14 and high level of CD16. Intermediate monocytes express high levels of CD14 and low level of CD16. Three studies examining these subsets were reviewed by Cormican and Griffin [2], which summarized the function of each subsets by their gene expression. Classical monocytes are deemed to be patrolling and recruiting, they express high levels chemokine receptors and are avid producers of cytokines and chemokines. Non-classical monocytes are inflammatory and secrete the most pro-inflammatory cytokines. Intermediate monocytes are the APC population, they express high levels of MHC molecules and other co-receptors, which interact with T cells during antigen presentation. In summary, monocytes are not just progenitor cells of macrophages and DCs, although this function is important as well.

### 1.1.2 MACROPHAGES

Macrophages are the main phagocytosing cell of the innate immune system, and the name comes from the Greek language translating into “large eater”. Macrophages develop in tissues both pre-natal, establishing tissue-resident macrophages, and from monocytes that are recruited to tissue during inflammation. Since macrophages are present in every tissue, they acquire many different variants of phenotype, adapted to each tissue. Many of these macrophage variants have received names of their own, like the Kupffer cells of the liver, alveolar macrophages in the lungs, or microglia in the central nervous system.

As with monocytes, macrophages have been divided into subsets. Two major ones are based on their function when activated [3]. Pro-inflammatory M1 macrophages produce cytokines inducing immune response, for instance TNF-α, one of the most important cytokines in rheumatoid arthritis, is secreted in high levels from M1 macrophages. TNF-α interacts with a cell and activates NF-κB, a transcription factor present in the cytosol of non-activated cells, and induces its translocation into nucleus to the genes involved in coordinating immune response. Besides cytokine production, M1-macrophages are highly phagocytic and bactericidal.

In contrast to M1, M2-macrophages are anti-inflammatory and produce cytokines such as IL-10 or TGF-β, known to suppress immune response. IL-10 has the effect opposing TNF-α and can block NF-κB activity. M2-macrophages are required for tissue repair and wound healing in case of damage, they accumulate at wounds and phagocytose damaged tissue and

bacteria. Recent research showed that these wound recruited macrophages are actually monocyte-derived[4].

### 1.1.3 CONSTITUTIVE PROTEASOME AND IMMUNOPROTEASOME

In the cells, there is a system which facilitate production of peptides for antigen presentation called the proteasome complex. It maintains intracellular protein homeostasis by removing damaged and no longer required proteins. The proteasome is a barrel-shaped protein complex that ingest proteins in one end and spits out peptides out in the other end. The constitutive proteasome, also known as the 26S proteasome, has a proteolytic core (20S proteasome) made up of 4 rings stacked on top of each other, with a cap (19S proteasome) associated at each end. The caps serve to recognize and unfold proteins that have been ubiquitinated, making them readily degradable by the inner core. In addition, the caps constitute a lid and a base, the lids consist of non-ATPase units, involved in scaffolding of incoming proteins. The base is made up of ATPases that through an ATP-dependent motion translocate the protein to the proteolytic core for degradation. The two outer rings in the proteolytic core constitute α-subunits, which function as the gate to 2 inner β-rings. Another function of the base in 19S cap is to open the proteasome gate to allow proteins to pass though. The β-rings constitute the proteolytic site of the proteasome, 3 subunits have proteolytic activity, the β1-, β2- and β5-subunits (confusingly encoded by genes *PSMB6*, *PSMB7*, and *PSMB5*).

In response to the cytokine IFN-γ, a potent regulator of immune response, the proteasome can change its composition to form an immunoproteasome. This immunoproteasome generates peptides to load into MHC for antigen presentation. Compared to 20S proteasome, the core of the immunoproteasome replaces three proteolytic subunits with different ones, β1i-, β2i and β5i-subunits, commonly called LMP2, MECL1 and LMP7, and encoded by genes *PSMB9*, *PSMB10* and *PSMB8*. This replacement results in a proteasome core with different cleavage specificity. The immunoproteasome has greater ability at cleaving behind hydrophobic amino acid residues and lower ability to cleave behind acidic residues and renders a different result regarding basic residues[5,6]. This eases uptake of peptides by TAP transporters and facilitates translocation of peptides into endoplasmic reticulum where loading onto MHC molecules take place.

To further enhance generation of peptides for MHC loading, the 19S caps can also be exchanged by the 11S, also known as PA28. This cap comes in two forms the heterodimer PA28αβ (encoded by *PSME1* and *PSME2*) controlled by IFN-γ, and the IFN-γ-independent homomer PA28γ (encoded by *PSME3*). The PA28αβ is ATP- and ubiquitin-independent and is associated with the 20S core proteasome. Additionally, PA28αβ changes the output to more specific peptides suitable for antigen presentation by MHC[7]. The function of PA28γ homomer is more elusive. It is expressed in all tissue and its expression is not significantly changed by pro-inflammatory cytokines. However, it has been found elevated in pathologies such as cancers and autoimmunity. In fact, it has specifically been found to correlate with disease activity in RA patients[8].

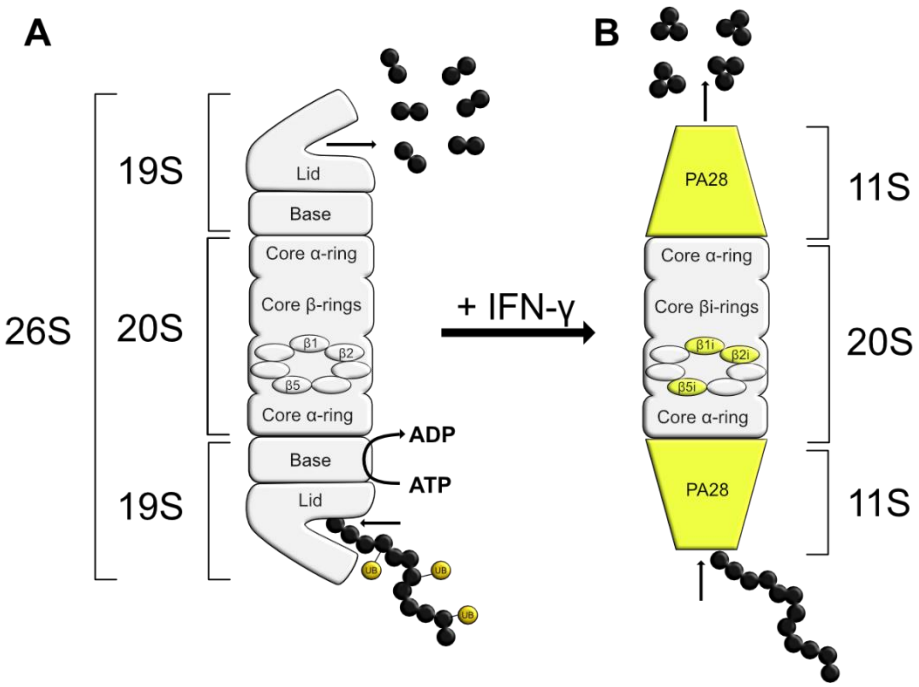


**Figure 1. Constitutional Proteasome and Immunoproteasome. (A)** Ubiquitinated proteins are recognized by 19S cap and translocated to the 20S core of the proteasome for degradation. **(B)** In response to IFN-γ, β-subunits and the 19S cap of 26S proteasome are replaced by βi-units and PA28 (11S), respectively. This facilitates generation of peptides readily presented by MHC.

## 1.2 ADAPTIVE IMMUNE SYSTEM

Complementing the innate system mammals developed what we term the adaptive immune system. The cell engaged in the adaptive immune response are T cells, which can further be divided into helper and cytotoxic, and beyond that naïve, memory, effector, or regulatory cells, and the antibody producing B cells. The function of the adaptive the immune system can be allotted into:

1. Recognition of specific "non-self" antigens in the presence of "self", during the process of antigen presentation.
2. Maintain self-tolerance by suppressing autoreactivity.
3. Generation of responses that are designed to maximally induce removal of pathogens, infected, damaged or malignant cells.
4. Development of memory B cells and T cells creating long-lasting immunological memory against encountered pathogens.

Central in achieving these objectives are the T cells, which I will explain in detail later. B cells derive from the same lymphoid progenitor as T cells, but they do not enter the thymus for further development. Instead, they migrate to the secondary lymphoid organs, lymphoid nodules and spleen, where they are flooded with antigens. B cells like T cells are antigen-specific, so when they encounter the specific or cognate antigen which activates these cells. Once activated, the B cell will either present the antigen via MHC II to T cells or differentiate into antibody producing B cells, short-lived plasma blasts producing antibodies for immediate protection, or long-lived plasma and memory B cells for long term protection. The long-lived B cells under the direction of specialized T cells enter lymphoid follicles where the form germinal centers facilitating sustained antibody production. This is a very simplified model of B cell development, B cells can also activate independently of T cells, however, this is out of scope for this thesis work and has been reviewed elsewhere[9].

Both, B and T cells develop to recognize a specific antigen. This occurs because of configuration of B and T cell receptors. These receptors are unique for each clone of B and T cells. They evolve via somatic recombination of the receptor genes called V(D)J-recombination. This occurs in bone marrow for B cells and thymus for T cells. During development, the T cells upregulate the expression of recombinases (enzymes that can shuffle DNA-sequences) RAG1 and RAG2, which allows randomization of certain DNA-sequences in the α- and β-chains of the TCR resulting in a unique heterodimeric complex. The recombination is such that the α-chain contains a variable, joining and one constant region, while the β-chain constitutes a variable, diversity and two

constant regions. This system has developed to ensure existence of a BCR or TCR for every potential pathogen.

### 1.2.1 T CELLS DEVELOPMENT

T cells are the group of immune cells with many different faces. They derive their names from where they develop, the thymus. The thymus is the organ located behind the sternum in humans consisting of two lobes, each with a central medulla, outer cortex and a surrounding capsule. Immature T cells, called thymocytes, and epithelial cells are the major cell types making up the thymus. The epithelium cells develop first and common lymphoid progenitors (CLP) immigrate into it at a later stage. The thymus continues to develop until puberty, after which it starts to degrade and thymic output decreases the rate at which T cells are produced in a process called thymic involution. This process is thought to explain why different diseases are age-related[10]. For example, newborns are susceptible to the respiratory syncytial virus, while elderly often mount inadequate immune responses to influenza. In addition, breakdown of self-tolerance causing autoimmune disorders most often happens in middle age [11].

Thymocytes undergo rigorous selection in their maturation process into naïve T cells which can enter the circulation. A simplified step-by-step development from CLP into naïve T cell could be presented as follows[12]:

1. CLP enter thymus through corticomedullary junction. At this stage CLP express progenitor cell markers c-Kit, SCA-1 and FLT3.
2. Commitment to T-cell lineage and assembly of TCR, occurs in the cortex and subcapsular region.
    a. Committed T cell is primed by Notch and IL-7 signaling and enters the double negative stage (DN1, i.e. CD4-CD8-).
    b. Downregulation of pluripotency genes marks an entrance into DN2 stage.
    c. Upregulation of genes required for the T cell receptor (TCR) assembly, *RAG1*, *RAG2* and *PTCRA (*invariant pre-TCR α-chain*)* initiates the β-chain selection, which takes place by interaction with epithelial cells. Cells passing this check-point reaches DN3 stage.
    d. Signaling through the pre-TCR results in downregulated expression of *RAG1*, *RAG2* and *PTCRA.* Cells enter DN4 stage.
3. The DN4 cells are now fully committed for T cell lineage. They migrate to corticomedullary junction and start to proliferate with simultaneous upregulation of CD4 an CD8 receptors generating DP the double-positive (DP, $CD4^+CD8^+$) cells. In addition, re-shuffling of the α-chains occurs.

4. The DP cells migrate to cortex where they undergo the positive selection via interaction of TCR with MHC-peptide complex expressed by stromal cells, epithelial cells and DCs present in thymus. Low avidity interaction promotes survival of the DP cells, and cells differentiate into single positive subsets (SP, $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$).
5. The SP cells migrate to medulla where they undergo negative selection by testing the TCR against self-antigens expressed by stromal cells and epithelial cells possessing autoimmune regulator AIRE. SP cells reacting strongly to self-antigens initiate apoptosis, while non-reacting cells survive. Intermediate reacting cells become the T regulatory cells (Tregs).
6. After this stage, the cells enter circulation as recent thymic emigrants (RTE). They are SP naïve T cells, which have not been adapted to recognize its antigen and express $CD31^{13}$, CD69, CD45RA and CD62L. These cells start surveying peripheral lymphoid organs.

Once in the periphery the T cell encounter its cognate-antigen and become activated resulting in clonal expansion and differentiation into a truly specialized cell depending on the differentiation signals.

However, I want to acknowledge that the 2 subtypes (3 if counting regulatory T cells as separate) already defined in thymus $CD8^{+}$ cytotoxic T cells ($T_c$) and $CD4^{+}$ T helpers ($T_{h)}$ cells. Tc cells are a "hunter-killer" cells, meaning they search for cells expressing its cognate antigen via MHC I, and then destroys that target by inducing apoptosis. Tc cells can induce apoptosis in various ways but essentially it relies on cell-cell contact and then either by transmitting a signal through a receptor-ligand interaction or by releasing granzymes which enters the target cell and triggers apoptosis.

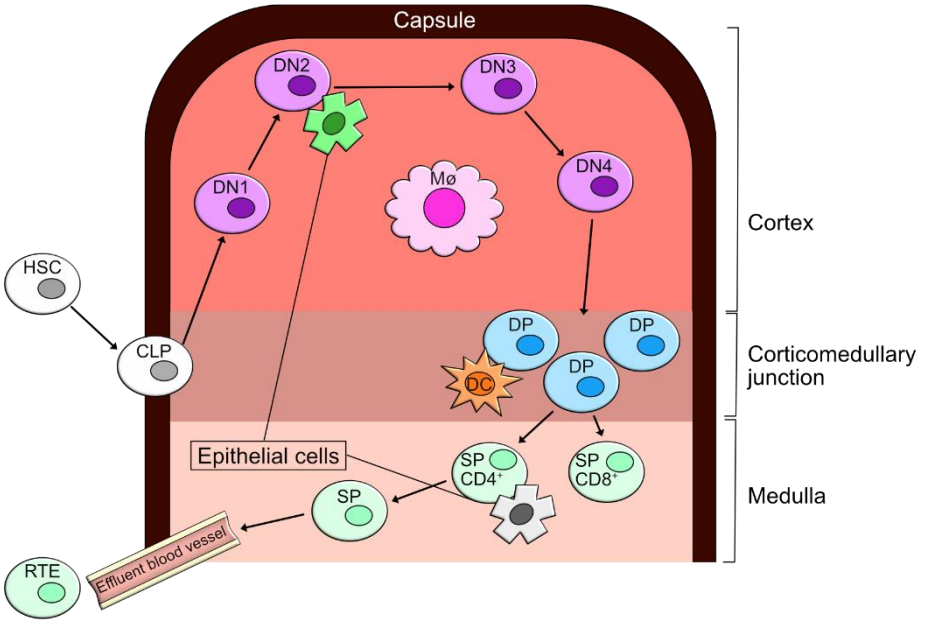


**Figure 2. T cell development in thymus.** CLP enter the thymus via the corticomedullary junction. Maturation to T cells occurs after positive selection of TCR β-chain in the cortex followed by negative selection of self-reactive cells in medulla before entering circulation as RTE.

## 1.2.2 T HELPER CELL DIFFERENTIATION

So, if Tc cells are "hunter-killers" then T-helper ($T_h$)cells are the "spider in the web" (confusingly since spider are also predators but think more about the saying) in that they are involved in coordinating most functions of immunity, like cross presentation, activating Tc cells and optimizing pathogen clearance by macrophages and neutrophils. Tregs is a subset of $T_h$ cells, so they also function in suppression and resolution of immune responses. There are 5 major subsets of $T_h$ effector cells, Th1, Th2, Th17, $T_{fh}$ and $T_{regs}$. In general, during TCR activation in the periphery and in the presence of specific cytokines, it will activate adaptor molecules called Signal Transducer and Activator of Transcription (STAT) in the $CD4^+$ T cell, which in turn promotes production of master regulator transcription factors necessary for the correct immune response. The requirements for differentiation, function, cytokine production, chemokine receptor guiding chemotaxis, and associated immune pathologies are outlined in Table 1. However, there is some plasticity in this system. Different subsets can attain characteristics of each other when subjected to another environment. Specifically, Tregs have been shown to differentiate into

the Th-subsets giving rise to so called ThX-like Treg, where ThX represents either Th1, Th2, Th17 or Tfh subsets[14]. Furthermore, thymus-derived, also known as natural Tregs (nTregs or tTregs) are not the only subset, in fact Tregs can also arise in the periphery, so called inducible or peripheral Tregs (iTreg or pTreg).

Complementing effector T cells which when activated migrate to inflamed tissue and conduct their function, some T cells transform to memory T cells (Tem). The Tem cells will go dormant and save their strength for later infractions by the same pathogen. Central memory T cells are long lasting and localize in peripheral lymphoid organs, effector memory T cells remain in circulation after inflammation has been resolved and tissue-resident T cells, which remain at the site of inflammation. These memory cells can persist for decades in the body and thereby contribute to the long-lasting protection of the adaptive immune system.

**Table 1: T helper cell subsets.**

| Subset | Differentiation (master regulator transcription factor) | Cytokine production | Chemokine receptors | Function | Associated immune pathology |
|---|---|---|---|---|---|
| Th1 | IL-12 or IFN-γ induces STAT4 or STAT1 respectively, promoting **T-BET** expression. | IFN-γ | CXCR3 | Type 1 immune response: Intracellular bacteria and viruses. | Rheumatoid arthritis, MS, insulin-dependent diabetes, IBS and atherosclerosis |
| Th2 | IL-2 and IL-4 activate STAT5 and STAT6, respectively, STAT6 induces expression of **GATA3.** | Il-4/IL-5 | CCR3, CCR4, CCR8 | Type 2 immune response: Large extracellular pathogens. | Asthma, atopic dermatitis, rhinitis, food allergies and eczema |
| Th17 | IL-1β, IL-6 and IL-23, the 2 latter activate STAT3 inducing transcription of **RORγt**. **RORγt** can also be induced by TCR signaling and NFAT/NFκB/AP-1 pathway. | IL-17A, IL-17F, IL-22 | CCR4, CCR6 | Type 3 immune response: Extracellular bacteria and fungi. | Psoriasis |
| T follicular helper cell | IL-6 and IL-21 activate STAT3, inducing **BCL-6** expression. | IL-21 | CXCR5 | Aids in B cell maturation | SLE |
| T regulatory cell | Develop in thymus due to intermediate reactivity to self-antigen during negative selection. Another subset can be induced in the periphery by IL-2 and TGF-β, promoting **FOXP3** expression by activating STAT5 and SMAD2/3 respectively. | IL-10, TGF-β | CCR4, CCR6 | Suppresses and resolved immune responses. | IPEX syndrome |

## 1.3 RHO-GTPASES

Rho-GTPases are small G-protein signal transducers involved in regulation of the actin cytoskeleton in response to environmental stimuli. They belong the to the Ras superfamily of GTPases and are highly conserved throughout the eukaryotic kingdom. Being G-proteins, Rho-GTPase have the on-and-off switches depending on bound guanosine triphosphate (GTP) or guanosine diphosphate (GDP), respectively. Currently, there are 20 described members of the Rho-GTPase family, RHOA, RAC1 and CDC42 belong to the canonical Rho-GTPases being the most studied. These proteins have a domain associated with cytoskeletal reorganization. RHOA controls the stress fiber (actin bundles) turnover and contraction. RAC1 regulates the formation of lamellipodia, a type of cytoskeletal actin projection. Lastly, CDC42 regulates filopodia, actin projections stretching beyond the lamellipodium. These structures coordinate the movement and shape of any cell, which is very important in the cell migration process, for instance, during transmigration of leukocytes from blood circulation to sites of inflammation. However, the cytoskeleton is also important during interaction between cells, for example during antigen presentation, where it coordinates clustering of receptors on the cell membrane. Hence, Rho-GTPases are heavily researched with regards to immune cell development, differentiation, and migration as well as the development of diseases.

To modulate the on-and-off state, the cell has guanosine nucleotide exchange factors (GEFs) that stimulate the release of GDP from GTPases allowing GTP to be bound, turning on the GTPase. Secondly, there are the opposite of GEFs, GTPase activating proteins (GAPs), which terminate GTPase-activity. Lastly, there are guanosine dissociation inhibitors (GDIs), which sequester the non-active GDP-bound Rho-GTPase, preventing activation by GEFs and detaching from the cell membrane. Many GEFs, GAPs and GDIs are specific for one or several Rho-GTPases, allowing the cell to pinpoint control the Rho-GTPase activity.

Beside these regulators, Rho-GTPases can undergo posttranslational modification which affect their behavior. For proper subcellular localization of RHO-GTPase, a lipid can be bound to the cysteine residue in the carboxyl-terminal “CAAX” motif. This process is called prenylation and is carried out by the enzyme geranylgeranyl transferase type I (GGTase-I). This stimulates association of Rho-GTPases with intracellular membranes and protein binding partners [15]. In this thesis work, I’ve studied the mouse model which spontaneously develops RA because of a deficiency of the GGTase-I enzyme. Turnover of Rho-GTPases is regulated by the ubiquitin-proteasome system.

### 1.3.1 RHO-GTPASES IN IMMUNE CELL MIGRATION

The Rho-GTPases are involved in leukocyte migration to inflammation sites, partly through regulating the actin projections, but also in activation and clustering of receptors required for cell migration. The process of leukocyte recruitment has been extensively reviewed[16-20]. Here, I will only highlight the Rho-GTPase/integrin mediated parts.

Firstly, when leukocytes are attracted to an inflammation site by chemokines, they adhere to endothelial cells via receptors called selectins expressed on both cells. This adhesion promotes rolling of leukocytes along the endothelial cell layer, coordinated by adhesion molecules selectins and integrins. Integrins are surface receptors functionally connected to Rho-GTPases. They exist in different states of binding capacity, and Rho-GTPases can activate their high affinity state (inside-out-signaling), and vice versa, integrins can activate Rho-GTPases (outside-in-signaling)[21] via GEFs. Integrins are heterodimeric and their specificity is regulated via pairing of α- and β- subunits. For example, to slow down leukocyte rolling, $\beta_2$-integrins are engaged as a part of LFA-1 ($\alpha_l\beta_2$) and MAC1 ($\alpha_M\beta_2$) receptors. To facilitate the complete rolling arrest, the very late antigen (VLA) 4 consisting of $\alpha_4\beta_1$ subunits binds to the vascular cell adhesion molecule (VCAM) 1 on the endothelial cells. The final step in the immune cell blood-to-tissue migration process is transmigration, where the cell moves between or through the endothelial cells. The exact molecular mechanism of transmigration has not been completely elucidated. It involves adhesion regulated by integrins that bind both extracellular matrix (ECM) components and other cells. Here, signaling via Rho-GTPase helps to reshape the actin cytoskeleton to squeeze between cells.

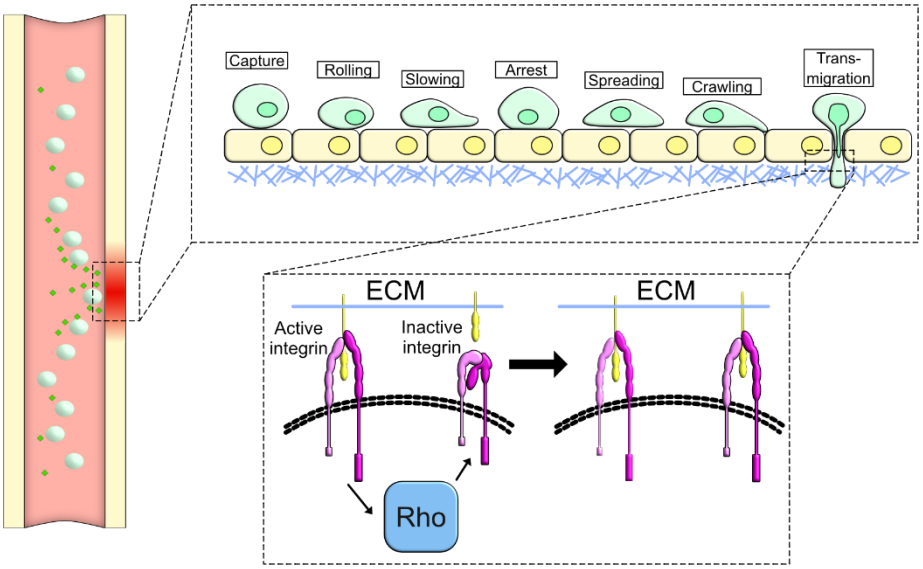


**Figure 3. Cell migration to inflammation site**. Immune cells are attracted to the inflammation site due chemokine secretion. To stop moving with the blood flow immune cells are captured by endothelial cells, after which they start rolling. When at the correct site the cells arrest and spread, which is mediated by integrins and Rho-GTPases cooperating via inside-out and outside-in signaling. The immune cells then transmigrate through the endothelial cell layer to the inflammation site in a step also mediate by Rho-GTPases and integrins.

### 1.3.2 RHO-GTPASES IN INTERACTION OF INNATE AND ADAPTIVE IMMUNITY

To gain interaction between the innate and adaptive system, there are chemokines secreted by cells to direct the movement of leukocytes and there are cytokines to induce effector differentiation. However, central process of the immune system that connects the innate and adaptive immunity is the antigen presentation. Antigen presentation is required for the cell-to-cell contact and formation of what is called the immunological synapse (IS). The IS is formed in response to a TCR recognition of an MHC bound antigen on a APC. Depending on T cell maturation stage, a specific response is induced. In thymocytes, the IS directs positive or negative selection. In $CD8^+$ T cells, the formation of the IS facilitates the killing of the antigen presenting cells. In $CD4^+$ T cells, the IS coordinates cell differentiation when in contact with DCs, and, on the other hand, it mediates to antibody production when in contact with B cells. The immunological synapse is a very complex structure with many receptor ligand pairs interacting in the space between the two cells. There is also a lot going on inside each cell regarding regulation of the actin cytoskeleton and facilitating receptor clustering resulting in a mature IS. The

mature IS constitute three supramolecular activation clusters (SMAC) creating a bull's eye shape in which different membrane proteins localize. The central (c)SMAC is enriched with TCR/MHC and CD28/CD80 co-stimulatory interactions. The peripheral (p)SMAC collects integrins and other cytoskeletal connected proteins. The distant (d)SMAC is enriched with F-actin and molecules excluded from the IS center due to steric hindrance or to their ability to negatively regulate the signaling. In the dSMAC, TCR signaling begins with TCR-CD28 assembly that eventually move to the center. Furthermore, each SMAC is characterized by a different configuration of actin networks, organized by Rho-GTPases[22]. In the dSMAC, there is the branched actin network under the control of Rac1, which promotes the branched actin nucleation via its effectors. In the pSMAC, the actin network constitutes the myosin-2 rich actin. Formation of this network is under the control of RHOA and its effectors. In the cSMAC, there is the hypodense F-actin network which regulation has not been elucidated. In both the dSMAC and pSMAC, there are actin foci formed downstream of TCR and lead to recruitment of SLP76 to LAT cluster at the membrane. SLP76 recruits the GEF VAV which can activate CDC42 promoting foci formation via its effectors. These actin structures have 4 purposes:

- facilitate assembly of TCR clusters for signaling
- center TCR micro clusters to mature IS
- activate (via inside-out-signaling) and position integrins to facilitate T cell–APC attachment
- regulate major T cell effector functions by polarized secretion

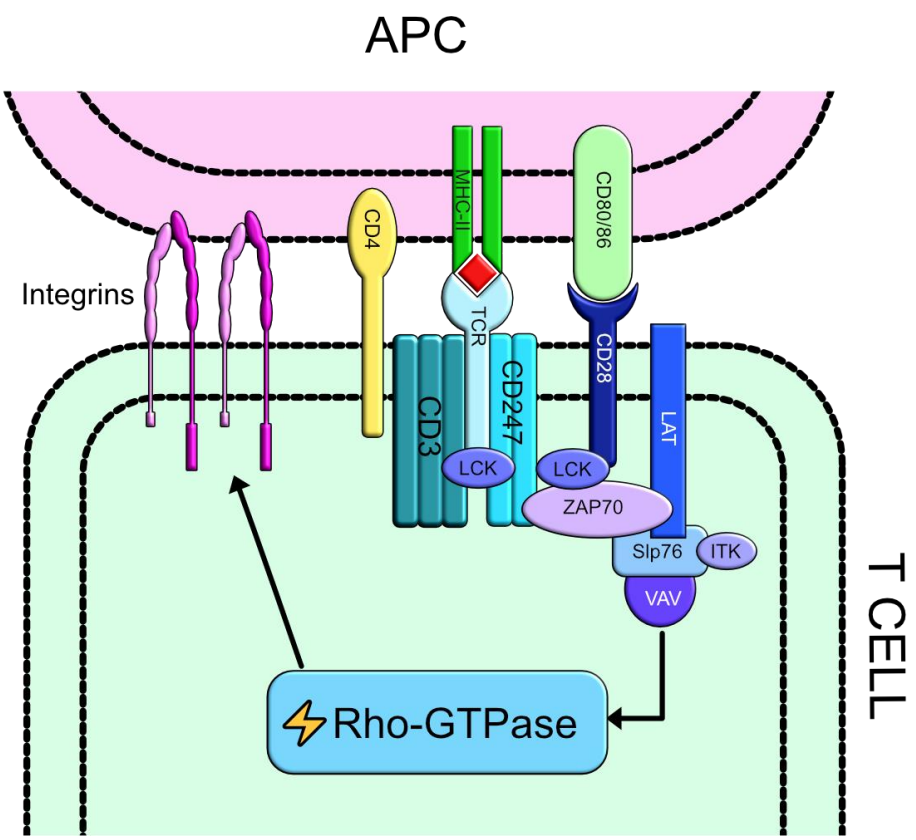


**Figure 4. TCR-signaling.** When a T cell finds its cognate antigen the intracellular components (CD3 and CD247) of the TCR are phosphorylated by LCK, this recruits ZAP70. ZAP70 is then activated by LCK enhanced by CD28 co-stimulation. This phosphorylates and recruits LAT and SLP76 to the membrane. SLP76 recruits VAV, which can activate Rho-GTPases and thereby facilitate activation of integrins via inside-out-signaling, maturing the IS.

So, Rho-GTPases play an active role in control of the IS and thereby development of leukocytes. For example, deletion of Rac1 and Rac2 paralogs in T cells blocks early thymocyte development and suppress TCR mediated proliferation[23]. Similarly, embryonic RhoA-deficiency results in thymus devoid of all moderately developed T cells, while deficiency at a later stage allows T cell maturation but at reduced frequency[24]. On the contrary, Cdc42-deficient T cells affect the activation outcome resulting in larger population of Th1 cells[25], being simultaneously required for IFN-γ secretion at the IS[26]. Also, active Cdc42 accumulates in the IS, supporting actin spreading[27]. On the innate or APC end, experiments with transformed DCs possessing constitutively active forms of RHOA and CDC42 showed increased antigen presentation capability in the form of IL-2 being produced[28]. Furthermore, DCs and T cells

use actin protrusions regulated by CDC42 called podosomes to facilitate antigen sampling and recognition respectively[29,30]. Overall, regulation of the actin cytoskeleton during IS formation by Rho-GTPases is essential for immune responses including T-cell development, antigen presentation, and migration, which makes them an attractive study object in the immune related defections.

### 1.3.3 WNT-SIGNALING

Formation of the IS allows cytokine release between cells in so called paracrine communication. Above, I have mentioned cytokines that affect T cell differentiation, but other pathways involved in T cell differentiation are also used during the cell-to-cell contact, one being Wnt-signaling. The Wnt-signaling plays a role during embryonic development and in anatomical patterning, cell fate and migration. The canonical Wnt-pathway starts with binding of a Wnt-ligand to the Frizzled receptor resulting in β-catenin activation, which translocate to nucleus and forms a complex with LEF1. This initiates transcription of genes required during T cell development and T cell differentiation[31], specifically to maintain phenotype [32]. Two non-canonical Wnt-pathways have been described. Both result in actin polymerization via Rho-GTPases and have been shown to influence T cell development[32]. In addition, Wnt-ligands specifically WNT5A, are readily produced by APC macrophages, monocytes and DC and promote differentiation to Th1 cells[33].

### 1.3.4 HOMEBOX GENES IN THE IMMUNE SYSTEM

The homologous alien box or homeobox genes encode transcription factors that bind homeobox domain in the DNA-sequences and thereby regulate transcription. The proteins containing a homeobox domain are involved in guiding the anatomical patterning of an organism, hence these genes are active during early embryonic development, controlling cell differentiation. However, HOX activity is also present in adults regulating development of leukocytes. For example, suppression of HOX proteins have been shown to regulate the T cell development [34,35] and facilitate migration in monocytes[36].

The HOX family is the most common and most studied of the homeobox genes constituting 39 members divided into 4 gene clusters arranged in tandem in humans and mice. The order of the HOX genes in the clusters corresponds to where they act along the body axis, *i.e*. HOXA1 acts cranially (closer to the head), while HOXA13 acts caudally (at the tail of the body), and the genes in between act in gradient along these extremes. Because of this distribution, different HOX proteins participate in coordinating the formation of joint structures during skeletal development making them interesting factors for

study in RA. The RA disease is very topographically distinct, symmetrically affecting small joints in the extremities. In fact, HOX transcriptome from synovial fibroblast in RA could identify joint origin[37]. Moreover, there are reports of Rho-GTPases and integrins being under the control of HOX[38,39].

HOX proteins do not act alone but form complexes with other proteins. One such protein is Pre-B-cell leukemia transcription factor (PBX) 1, a homeobox binding protein of the Three Amino acid Loop Extension or TALE family. Partnering with PBX1 enhance DNA binding and increase specificity of HOX proteins and decides whether the effect on transcription is suppressive or inducing[40,41].

# 2 RHEUMATOID ARTHRITIS

## 2.1.1 HISTORY OF RA

So, what is rheumatoid arthritis (RA)? Short and simplified, it is an autoimmune disease characterized by inflammation of synovial tissue in the joint leading to cartilage damage and erosion of the bone. Of course, there is a host of complexity behind these symptoms, which the works of this thesis and many other studies are trying to understand. But first, a short history lesson.

Entezami et al. [42] studied the history of RA in attempt to trace the major findings on the disease and described the coining of the term Rheumatoid Arthritis. Accordingly, the term "Rheumatoid Arthritis" was found in the year 1800 in a dissertation by the Augustin Jacob Landré-Beauvais. Landré-Beavauis took note of the symptoms of joint pain, which otherwise had then been thought to be gout. However, RA was mainly affecting poor women, instead of men of affluent standing. Because of this reason, he believed, that RA had been uncharacterized version of gout, which he called Primary Asthenic Gout. This spurred more research into to this new disease. In late 1800-hundreds, Alfred Garrod was able to differentiate arthritis from gout by the excess of uric acid in gout patients. Later in 1890, the fourth son of Garrod, Archibald Garrod who followed in father footsteps, conducted research on this condition and coined the term Rheumatoid Arthritis. Interestingly, Garrod the younger recognized in his dissertation that RA must had been a disease long overlooked. He referred to ancient bones found across various archaeological sites around the world. However, in the 1900-hundreds, this was challenged by Charles Short, who reviewed the paleontological reports referred to in Archibald Garrod's work and found that the erosion in the bones could not be concluded to be the result RA, as it could have been osteoarthritis, spondylitis or gout. From this, he argued that RA was a disease of modern society. Entezami *et al.* [42] ends his studies on the history RA by concluding four things.

1. RA has been present for a long time and is not a disease of modern-day society.
2. Environmental stimuli are a factor in development of RA in susceptible individuals.
3. The stimuli are of unknown origin, however, research into RA have resulted in some possible answers.
4. RA patients with a distinct genetic makeup may be more susceptible to certain stimuli. For instance, individuals who carry the MHC allele encoding the shared epitope (SE) and have antibodies against

> citrullinated peptides (ACPA), have been suggested to be at greater risk of developing RA when smoking. Hence, the historical analysis of RA requires inclusion of the possibility that the current definition of RA accounts for more than one disease.

The last sentence here reveals the reason to what makes RA so difficult to predict and treat, it's heterogeneity, legitimizing research such a my own and others. RA is not expressed identically in every patient, and every patient does not benefit from the same treatment. And this becomes more evident when we search for answers for at the genetic level.

### 2.1.2 EPIDEMILOGY AND PATHOLOGY OF RA

Despite its heterogeneity, one commonality of RA is that it is more prevalent in women. Three out of 4 patients are females. Autoimmunity in general is more frequent in women, with an estimation ratio of 2:1. There is no clear cause of this yet, but studies have shown links to hormones and defective X chromosome inactivation[43]. The RA prevalence in women is more associated to hormonal changes, due to its frequent occurrence during menopausal age.

Destruction of joints is not the only detriment of RA. In fact, it has been linked to several comorbidities, including cardiovascular disease (CVD), diabetes mellitus, infections, and cancer. The increased immune activation is hypothesized to cause the former two comorbidities, while the latter two are attributed to the immune suppressing treatment. CVD-risk in RA has been estimated to as much 46% compared to 8% in healthy individuals[44]. Similarly, diabetes mellitus has been given a 23% increased risk of occurring in RA patients[45]. To top it off, the life expectancy is also reduced in individuals with RA, with an average loss of 5 years[46].

RA is characterized by joint inflammation leading recruitment of cells into the joint synovium. This cell recruitment leads to formation of a "pannus", a tissue consisting of fibroblast-like-synoviocytes, mast cells and leukocytes, in particular macrophages which mediates bone erosion and cartilage damage. The pannus is enriched with the activated aggressive macrophages as well as fibronectin and collagen making up the extracellular matrix surrounding the inflammatory cells [47,48]. Another feature of the RA synovium is low oxygen pressure, or hypoxia, compared to healthy joints or even joints of individuals with osteoarthritis [49]. Hypoxia has been shown to aid in cell recruitment and cytokine production [50].

Specifying the molecular culprits in RA, the cytokine tumor necrosis factor alpha (TNF-α) has been in the limelight for a while, justly so, and successful

treatments have been employed against it. TNF-α enhances production of other pro-inflammatory cytokines including IFN-γ, IL-1β, and IL-6 perpetuating inflammation in the joint. The cytokine response recruits more leukocytes to joint enabling response from T helper cells and by extension, from the adaptive immune system. Eventually, this breaks self-tolerance and induces production of autoantibodies, particularly in RA, Rheumatoid Factor (RF) and ACPA. Citrullination is the process of replacing arginine residues with citrulline increasing the hydrophobicity of a protein.

### 2.1.3 RA RISK FACTORS

It is believed that the pre-clinical phase RA precedes years ahead the onset of disease. The exact reason for this is not clear, and may include both genetic and environmental risk factors associated with RA. The most evident genetic risk factor is possession of the HLA-DR4 expressing the SE of five amino acids in the positions 70-74 of the HLA-DRβ-chain [51,52]. Not only does this SE facilitate presentation of citrullinated peptides by antigen presenting cells, it also represents the key TCR recognition determinant. Hence, it shapes autoreactive T cell repertoire[53].

Regarding environmental factors, smoking is the one most studied and associated with increasing the risk of developing RA. The research group where I have performed this thesis work, have published several papers on how smoking negatively affects $CD8^+$ cytotoxic T cells in RA[54-56]. Another reason of attention has been put on smoking, is because of it increase in citrullinated peptides[57], which links smoking with carrying the SE. Both risk factors, due to their effect on generation of ACPA, are associated with seropositive RA. What about seronegative RA? Here, the risk factors have not been well defined. Some research depicted seronegative RA as a false claim, and suggested that it was only a matter on how you conduct the serology [58].

A third source of risk for developing RA and other autoimmune disease has been introduced in later years, the microbiome. This aspect specifically gained tractions due to more advanced sequencing technologies allowing for detection of bacteria not possible to cultivate artificially. Consequently, it has been demonstrated that the microbiome of RA patients differs from that of healthy individuals [59]. In the collagen induced arthritis, the disease severity can even be alleviated by depleting the microbiome[60]. There is also the fact that pathogenic bacteria invoke a type-1 immune response which is prevalently linked to RA. Despite all these risk factors, one thing is certain, the immune cells need to migrate into joint to make RA development possible.

### 2.1.4 MONOCYTE/MACROPHAGES IN RA

Monocytes and macrophages have a central role in the development in RA. First off, they can promote and differentiate into osteoclasts which mediate bone erosions in RA. Secondly, they produce cytokines and chemokines to activate immune response and recruit immune cells, respectively. The cytokine mainly produced by macrophages in response to pathogen pattern recognition is TNF-α, which is highly associated with RA progression. However, monocytes/macrophages also produce IL-6, IL-12 and IL-1β, all associated with increased RA severity. Lastly, monocytes/macrophages are APC, which can trigger the adaptive immune system to progress the disease.

More recently and employing new technologies, specific subsets of synovial tissue macrophages are being identified. This increases the complexity of how we view the macrophage population in the RA joint[61,62]. These studies improved the understanding of RA and presented the opportunity to study the heterogeneity of the synovial macrophage population in health and disease, potentially identifying targets suitable for therapeutics.

### 2.1.5 T HELPER CELLS IN RA

RA is associated with HLA-DRB1 (MHC-II) alleles encoding the SE. This indeed implies $CD4^+$ T helper cells play a major role in the pathology. Perhaps even more telling is the fact that T helper cells are found in joints of patients with RA. However, the $T_h$ subset responsible for RA development have been debated for a while. IFN-γ, the predominant cytokine associated with Th1, has long been known to activate APC. However, the role of IFN-γ in RA is seemingly double-edged. Some mouse studies in which IFN-γ signaling was inhibited reported decreased severity of arthritis[63,64], while others reported the arthritis-promoting role[65]. In recent years, JAK-inhibitors (JAKi) have been employed in the treatment of RA showing great efficacy. They targets Janus kinases (JAKs) downstream of the IFN-γ receptor, which speaks in favor of the arthritis-promoting role of IFN-γ[66]. However, IFN-signaling is not their only target.

Contra intuitive to the reasoning that T helper cells play a major role in RA development is the fact that RA mostly develops late in life when thymic activity has declined[67]. In agreement with this, it has been shown that RA patients develop a reduced TCR repertoire and reduce the number of RTE[68]. In addition, this thymic involution in RA is triggered by production of pro-inflammatory cytokines[69]. So, T cell involvement in RA is highly complex. The most evident genetic risk factor implicates T helper activation, while

simultaneously decrease in T cell diversity seems to be another contributing factor.

### 2.1.6 DIAGNOSIS AND TREATMENT OF RA

Currently, the diagnosis of RA is based on a combination of factors including joint swelling, joint tenderness, radiographic changes, inflammation markers (C-reactive protein, Erythrocyte sedimentation rate) as well as presence of autoantibodies (rheumatoid factor, RF, and antibodies against citrullinated peptide, ACPA) in serum. These factors can be used to calculate the disease activity. Namely, the swelling and tenderness of 28 joints (10 of each hand, both wrist, elbows, shoulder, and knees) together with either CRP or ESR and the physician's subjective opinion. This score, abbreviated DAS28, assess the current state of the disease and is widely used to determine response to treatment. Despite the difference in clinical signs of RA, these factors do not aid in treatment decision and choice of drug for treatment success. And even though treatment may be beneficial initially, resulting in remission, approximately half of patients will relapse at once dosages are tapered or treatment is ceased [70].

Drugs used to treat RA can be divided into four categories, anti-inflammatory NSAID, and 3 classes of disease modifying anti-rheumatic drugs (DMARDs), conventional (c)DMARDs, biologics (b)DMARDs, and targeted synthetic (ts)DMARDs. The list of drugs, mechanism of action, their classification, and year of FDA/EMA approval can be found in Table 2. Discernable from the table is that the Janus kinase inhibitors (JAKi) have become popular in the last decade. The previous decade were dominated by TNF-inhibitors (TNFi) and bDMARDS in general. However, first line treatment in Sweden and world-wide is still methotrexate (MTX)[71], due to its low cost and safety profile. MTX is a chemical agent that belongs to the cDMARD group. In RA, it works by inhibiting the purine metabolism, suppressing T cell activation.

If MTX fails to alleviate symptoms, physicians move onto bDMARD, often TNFi. TNF-α is the pre-dominant cytokine in RA pathology, mainly being produced by macrophages, hence the development of TNFi. The first TNFi developed was etanercept, a TNF receptor decoy acting by sequestering TNF-a and mimicking the soluble form the TNF-receptor, but with extended half-life. Etanercept was efficient, however, later it became associated with severe side effects, like serious infections, sepsis and even reactivation of latent tuberculosis and hepatitis. Following etanercept were monoclonal antibodies directly binding the TNF-α cytokine, adalimumab was the first fully human one (infliximab being chimeric between mouse and human) approved by

FDAs. A unique drug among the among the bDMARDs is abatacept, instead of targeting a pro-inflammatory cytokine, it targets the CD80 on APC which is required to initiate co-stimulatory signal in T cells during antigen presentation. Abatacept has been shown to be valid alternative to TNFi, regardless of if the patient is having developed resistance against TNFi or if they are naïve to treatment[72].

As a last resort in treatment of resistant RA, the physician can provide JAKi. JAK-inhibitors were proven to be even more effective than the TNF-inhibitors, leading reduction in swollen joints, improved DAS28 score and higher perceived physical function[73]. JAKs is a family of signal transducers, activating STATs downstream of multiple cytokine receptors, *i.e.* they can suppress pathways involved in T cell differentiation, antibody class switching and antigen presentation. JAKi are also administered in a tablet form, which is occasionally viewed advantageous over bDMARD provided by injection or infusion. In contrast, JAKi prices are for the moment very high, with an estimated 80 000-90 000 SEK per year for every patient. With a population prevalence of RA 1%, it quickly becomes evident that it would generate a huge cost to provide JAKi to all patients[71]. However, some patents have almost run out, opening the market for cheaper generic drugs.

**Table 2. Antirheumatic drugs.**

| Drug | Mechanism | Type | Brand Name | FDA Approved | EMA Approved |
|---|---|---|---|---|---|
| Methotrexate (MTX) | Purine metabolism inhibitor | cDMARD | | 1988 | |
| Hydroxychloroquine (anti-malarial) | Induce apoptosis of inflammatory cells and decrease chemotaxis | cDMARD | Plaquenil | jan-55 | |
| Azathioprine | Purine synthesis inhibitor | unknown | Imuran/Jayempi | mar-68 | jun-21 |
| Ciclosporin (Cyclosporin A) | Calcineurin inhibitor | unknown | | jan-83 | jul-18 |
| Rituximab | Chimeric monoclonal antibody against CD20 on B-cell surface | bDMARD | MabThera | nov-97 | jun-98 |
| Leflunomide | Pyrimidine synthesis inhibitor | cDMARD | Arava | sep-98 | sep-99 |
| Etanercept | Decoy TNF receptor | bDMARD | Enbrel | nov-98 | feb-00 |
| Infliximab | TNF inhibitor | bDMARD | Remicade | nov-99 | aug-99 |
| Sulfasalazine (SSZ) | Suppression of IL-1 & TNF-α, induce apoptosis of inflammatory cells and increase chemotactic factors | cDMARD | Azulfidine | aug-00 | |
| Anakinra | IL-1 receptor antagonist | bDMARD | Kineret | nov-01 | mar-02 |
| Adalimumab | TNF inhibitor | bDMARD | Humira | dec-02 | sep-03 |
| Abatacept | T-cell costimulatory signal inhibitor | bDMARD | Orencia | dec-05 | maj-07 |
| Certolizumab pegol | TNF inhibitor | bDMARD | Cimzia | apr-08 | okt-09 |
| Golimumab | TNF inhibitor | bDMARD | Simponi | apr-09 | okt-09 |
| Ustekinumab | IL-12 and IL-23 inhibitor | bDMARD | Stelara | sep-09 | jan-09 |
| Tocilizumab | IL-6 receptor antagonist | bDMARD | RoActerma | jan-10 | jan-09 |
| Tofacitinib | JAK1, JAK2 and JAK3 inhibitor | tsDMARD | Xeljanz | nov-12 | mar-17 |
| Apremilast | phosphodiesterase 4 (PDE4) inhibitor | tsDMARD | Otezla | mar-14 | jan-15 |
| Secukinumab | IL-17 inhibitor | bDMARD | Cosentyx | jan-15 | jan-15 |
| Sarilumab | IL-6 receptor antagonist | bDMARD | Kevzara | maj-17 | jun-17 |
| Baricitinib | JAK1 and JAK2 inhibitor | tsDMARD | Olumiant | maj-18 | feb-17 |
| Upadacitinib | JAK1 inhibitor | tsDMARD | Rinvoq | aug-19 | dec-19 |
| Filgotinib | JAK1 inhibitor | tsDMARD | Jyseleca | not approved | sep-20 |

### 2.1.7 RHO-GTPASES IN AUTOIMMUNITY AND RA

As Rho-GTPases are highly involved in migration and antigen presentation of immune cells, it stands to reason that their dysregulation is a factor in autoimmune diseases. Indeed, there are several reports describing improved conditions of autoimmune disease models due to inhibition Rho-GTPases or their downstream effectors. For example, RAC1 inhibition led to less production of B-amyloid in a human embryonic kidney cell model of Alzheimer's disease[74] and decreased differentiation of Th17 cells, improving symptoms in autoimmune encephalitis[75] (the experimental model of multiple sclerosis). In models of RA specifically, inhibition of Rac1 reduced paw swelling and antibody production in mice with collagen induced arthritis[76]. And a recent study by Rodríguez-Trillo et al. [77], showed that inhibition of the Rho-GTPase effector ROCK improved arthritis symptoms in mice and decreased osteoclastogenesis as well as bone resorption by monocytes isolated from RA patients. Moreover, inhibiting ROCK2 activation, led to decreased production of IL-17 and IL-22 from T cells, improving conditions in models of SLE and arthritis[78]. Lastly, inhibition of RHOA in monocytes of RA patients suppressed TNF-$\alpha$ and IL-1$\beta$ production and NF-$\kappa$B activation[79].

So, Rho-GTPases and its effectors are involved in a multitude of immune pathologies and inhibition of them can yield promising results, at least experimentally. Consequently, a mouse model in which RA is driven by hyperactivated Rho-GTPases, may prove extremely useful in finding new pathways targetable for treatment of RA.

### 2.1.8 MOUSE AS EXPERIMENTAL MODEL OF RA

Mice have served a model organism of human disease for over 100 years. Their utility to us stems from:

- Their biologic similarity to humans, pathophysiology of their diseases is similar too.
- Variations of tools developed for genetic manipulations.
- The ability to inbreed them to generate identical strains.
- Their accelerated lifespan making them easier to work with.
- They are well understood.
- Small and reproduce quickly.

For these reasons, models of several human autoimmune diseases have been generated. There are experimental autoimmune encephalomyelitis (EAE) mice which is used to study the multiple sclerosis in humans. Bcl-2 transgenic mice are used to study SLE. Nonobese diabetic (NOD) mice are used to study type-

1 diabetes. For RA, there are several canonical mouse models, which can be divided into inducible and spontaneous. The most commonly used are the inducible models of the collagen- and methylated BSA- induced arthritis[80], which works through immunization of the mouse by injecting emulsion of Freunds adjuvant and type II collagen in the second step. It has high pathological similarities with human RA, but is acute in comparison, needs male mice, and relies on the production of anti-collagen antibodies.

The spontaneous RA models are overall more chronic and slower in onset. They are achieved by manipulating part of the mouse genome. For instance, there is the hTNF-α transgenic mouse model, which produces high amounts of human TNF-α resulting in the development RA within 3-4 weeks of birth. This RA is human like with pannus formation, cartilage destruction, and production of fibrous tissue. Secondly, there is the KxB/N spontaneous RA mouse model, crossing KRN mice (mice with TCR α- and β-chains specific for a bovine ribonuclease peptide) with NOD mice (with MHC class II alleles encoding I-Ag7). This leads to the breakdown of self-tolerance and development of arthritis in 4-5 weeks[81]. One could argue that this model is in fact semi-spontaneous as another facet of the model involves injection of antibodies from the arthritic mice into naïve recipient which will develop transient arthritis. This lends the model utility in studying autoantibody-induced arthritis[82].

Thirdly, Sakaguchi et al. [83] developed the SKG RA model, which utilizes a mutation in the *Zap70* gene, a signal transducer downstream of the TCR, resulting in altered thymic selection threshold and maturity of autoreactive T cells. SKG mice develop arthritis within 2 months that progress in a symmetrical fashion and bears resemblance to human RA. The SKG mouse strain attributed arthritis development over any other autoimmune condition due to synoviocytes high sensitivity to pro-inflammatory stimuli rather than T cells destroying the tissue.

Together, these spontaneous models show that when communication between APCs and T cells is altered it can result in arthritis development.

### 2.1.9 *GLC* MICE

The model I have worked with in this thesis is the *GLC* mice. These mice present a spontaneous model of RA, due to a conditional knockout of **G**GTase-I protein in mouse macrophages guided by the **L**ysozyme M–**C**re allele (generation of *GLC* mice will be explained in detail in the methods section). This results in hyperactive Rho-GTPases (RhoA, Rac1 and Cdc42) in macrophages eventually leading to arthritis like human RA[84,85]. *GLC* mice

display severe joint inflammation detected histologically by accumulation of leukocytes in the joints and invasive growth of the inflamed synovia causing skeletal damage.

The deletions of GGTase-I both in macrophages and other cells have been examined in several studies. A conditional *Pggt1b*$^{-/-}$ model was first established in Sjogren et al. [86] and was used to study the effect on K-RAS induced lung cancer. The authors decided to cross the mice with floxed *Pggt1b* with mice heterozygous KLSL allele and the LC allele. As the LC allele mainly have been used to study myeloid cells, these cells were expected to be affected by the *Pggt1b* deficiency as well. This was indeed the case, however, normal myeloid function seemed to remain regarding viability and migration.

In a follow-up study by Khan *et al.* [85], the *GLC* RA mouse model was established, as they had noticed that the joint morphology in *GLC* mice had been affected, being infiltrated by leukocytes as well evidence of bone erosion, similar to human RA. In that study, the bone marrow derived macrophages were extracted and studied in detail. The lack of prenylation lead to accumulation of hyperactivation of Rho-GTPase, Rac1, RhoA and Cdc42. Still, migration and proliferation of the macrophages remained normal. However, pro-inflammatory programming was pronounced and supported by increased expression NF-κB activation and increased production of inflammatory cytokines TNF-α and IL-1β. Also, the *GLC* macrophage morphology was quite differentiated from WT-counterpart, being smaller and rounded. The arthritic phenotype of *GLC* mice, proved to be reversible and inducible by the bone marrow transplantation from WT mice into *GLC* mice, or *Pggt1b-/-* macrophages into WT mice. Despite overproduction of TNF-α by the *GLC* macrophages, this was determined not to be the sole contributor of the phenotype as mice treated with TNF-α inhibitor showed the reduced synovitis, but erosions remained at comparable level. This suggested that the increased TNF-α production only explains part of the phenotype. At the end, the authors concluded that prenylation is essential for Rho-GTPase regulation, and the rampant activation of macrophages led to development of arthritis.

In the follow up studies, it was demonstrated that the IL-1β production in *GLC* macrophages was the result of pyrin inflammasome activation[87]. Later on, experiments with the Rac1 GTPase were carried out [84], due to its connection with IL-1β production. Experiments were performed in *GLC* mice with heterozygous deletion of RAC1, which alleviated RA symptoms and reduced production of inflammatory cytokines TNF-α, IL-6 and IL-1β. These experiments demonstrated the involvement of the GAP Iqgap and GEF Tiam in the hyperactivation of Rac1. Furthermore, the increased activity of the

ubiquitin proteasome system in *GLC* macrophages was suggested. Lastly, the authors concluded that prenylation acted as a break on the innate immune responses, which if released leads to Rac1-induced pro-inflammatory signaling.

Prenylation was studied further in mice, not only in the macrophage driven RA model, but also in other cell types and resulted in other phenotypes. In two subsequent studies, where the *Pggt1b* and *Rhoa* gene were knocked out in intestinal epithelial cells[88] and in $CD4^+$ T cells[89], the mice developed intestinal inflammatory condition. In the latter case, the $CD4^+$ T cells expressed high amounts of the integrin $\alpha_4\beta_7$, which is a receptor for MAdCAM-1 selectively expressed on mucosal endothelial cells [90], to attract circulating lymphocytes to mucosal tissue.

Lastly, a study by Du et al. [91] showed that CD4-specific deletion of *Pggt1b* in mice caused accumulation of mature T cells in thymus and thereby lymphopenia in the periphery. By deeper analysis of pathways affected in *Pggt1b*-deficient CD4 cells, they showed that accumulation of CD4 cells in the thymus was caused by defective Cdc42 activation and impaired chemotaxis towards the relevant molecules, in this case, S1P, Ccl19, Ccl21 and Cxcl12.

So, by modulating prenylation in immune cells and thereby Rho-GTPase dependent pathways, different inflammatory phenotypes occur stemming from increased pro-inflammatory signaling and altered migration.

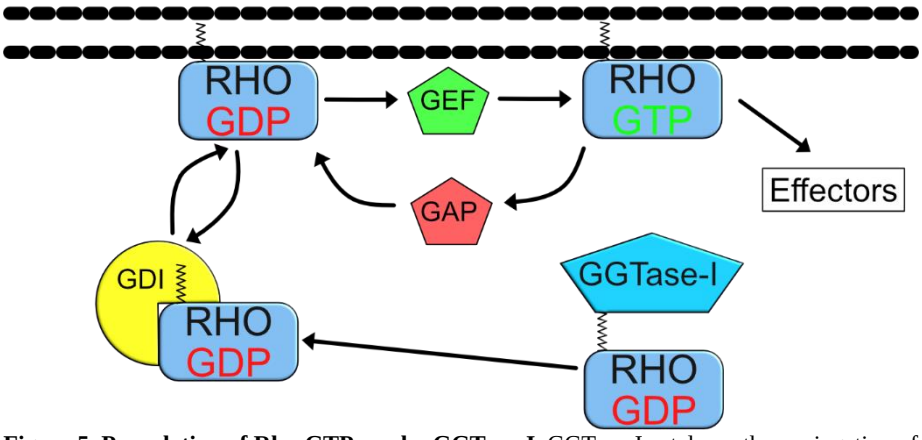


**Figure 5. Prenylation of Rho-GTPases by GGTase-I.** GGTase-I catalyzes the conjugation of a lipid-chain to Rho-GTPases. This facilitates sequestering of Rho-GTPases with GDI or attachment to cell membranes where they can become activated by GEFs.

# 3 AIM

The purpose of this project is to aid in the choice of anti-rheumatic treatment by better understanding pathogenic interaction between $CD4^+$ T cells and macrophages triggering RA disease. In translational setting of clinical material and experimental models of arthritis, I attempt to identify pharmacologically traceable molecular pathways behind RA development and realize how they are affected by current therapies used against RA. This leads to further goal of tailoring treatment for the individual.

This will be achieved by:

1. To determine the how macrophages with overactive Rho-GTPase affect $CD4^+$ T cell phenotype resulting in arthritis development in the *GLC* mice.
2. To define the Rho-GTPase dependent signature of $CD14^+$ cells in RA patients and to understand how it is affected by different RA treatments.
3. To investigate molecular signature of the recent thymic emigrants and their role in response to anti-rheumatic treatment.

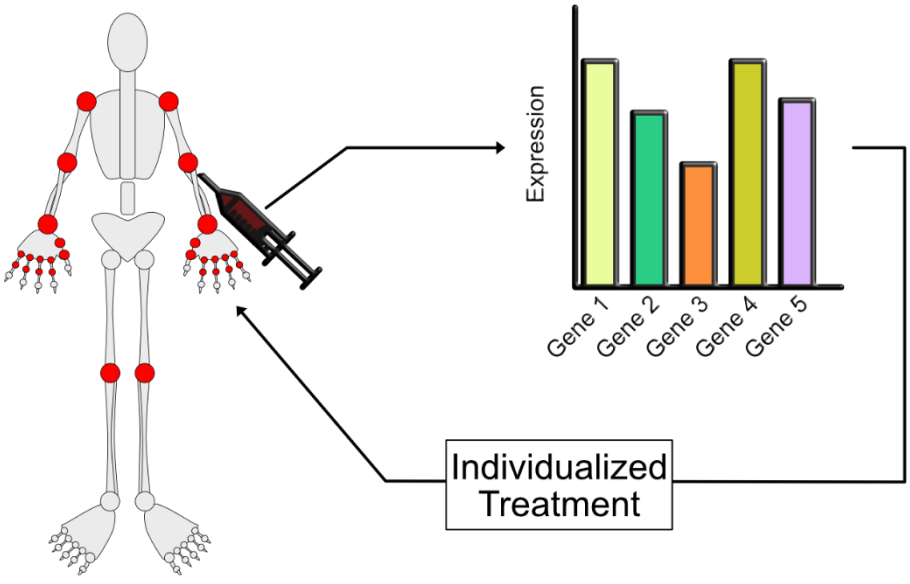


**Figure 6. Individualized treatment.** With the right knowledge measuring gene expression in patients may guide treatment choice in RA. This way, treatments can be tailored to the current disease state of the patient.

# 4 MATERIALS AND METHODS

This thesis is done in a translational setting, findings in RA mouse model guided the research questions in patients with RA. In general, I attempt to use gene expression similarities in different tissues obtained from mice experiments, patient material collected at Sahlgrenska University Hospital or scavenged from publicly available databases to track phenotypically like cells. With the aid of various data analysis tools, I drew conclusions on how these phenotypically similar cells behave in RA and respond to treatment.

## 4.1 RA MICE MATERIAL

### 4.1.1 GENERATION OF *GLC* MICE

In **paper I,** we utilized the *GLC* RA mouse model, lacking the gene encoding the beta subunit of GGTase-I selectively in macrophages[85,86]. We crossed it with other strains detailed below, in addition to performing intervention experiments. The *GLC* mouse strain was generated accordingly:

The mutant allele *Pggt1b*$^{fl}$ was introduced between loxP sites flanking exon 7, which is critical for enzymatic activity of GGTase-I [92], using vector technology. Two targeted clones (*Pggt1b*$^{fl/+}$) were used to generate chimeric mice, which transmitted the mutation to their offspring. *Pggt1b*$^{fl/+}$ and *Pggt1b*$^{fl/fl}$ mice had healthy joint morphology. Cre recombination converted the *Pggt1b*$^{fl}$ allele to a null allele (*Pggt1b*$^{\Delta}$), which produced a transcript lacking exon 7. *Pggt1b*$^{fl/fl}$ mice were bred with LC mice (The Jackson Laboratory) to produce *Pggt1b*$^{fl/+}$*LysM-Cre*$^{+/0}$ mice (designated *GLC*$^{fl/+}$ in this thesis). Cre recombinase was found in more than 90% of the bone marrow derived macrophages, in 87% of osteoclasts and in 40% of neutrophils, while no Cre recombinase was found in lymphocytes[85]. *GLC* mice were born at Mendelian ratios and had no obvious phenotypes. Control group comprised littermate *Pggt1b*$^{fl/+}$*LC, Pggt1b*$^{+/+}$*LC, Pggt1b*$^{fl/+}$ and *Pggt1b*$^{fl/fl}$ mice, collectively called wild type (WT).

#### Sorting of CD4 cells

$CD4^+$ cells were isolated from spleen and the joint draining LNs by a positive selection using Dynabeads™ Untouched™ Mouse $CD4^+$ Cells Kit. The purity of the isolated cells was controlled by flow cytometry and indicated 78-93% of $CD4^+$ T cells. The cells were resuspended in the Iscove's medium enriched with 10% FBS, 4mM L-glutamine, 50 μM β-mercaptoethanol, 50 μg/ml

gentamycin sulphate. Cells were activated with concanavalin A (1.25 µg/ml) for 2 h prior to harvest. The harvested cells were used for RNA-seq and the supernatants were used in ELISA analysis.

### 4.1.2 CROSSING OF *GLC* MICE

We bred *GLC* mice on a background of *Rac1*$^{fl/fl}$ [84,93], *Cdc42*$^{fl/fl}$ [94], *RhoA*$^{fl/fl}$ [95], and *Rag1*$^{-/-}$ [96] alleles to obtain double knock-out (DKO) mice *Rac1*$^{fl/fl}$*Pggt1b*$^{fl/fl}$*LC* (*GLC-Rac1*$^{fl/fl}$), *RhoA*$^{fl/fl}$*Pggt1b*$^{fl/fl}$*LC* (*GLC-RhoA*$^{fl/fl}$), and *Cdc42*$^{fl/fl}$*Pggt1b*$^{fl/fl}$*LC* (*GLC-Cdc42*$^{fl/fl}$), and *Rag1*$^{+/-}$*Pggt1b*$^{fl/fl}$*LC* (*GLC/Rag*$^{+/-}$); and *Rag1*$^{-/-}$*Pggt1b*$^{fl+}$ *LC (WT/Rag1*$^{-/-}$), respectively. Mice were genotyped by PCR-amplification of genomic DNA isolated from tail clips as described [85,93,95,96]. All mice were 18 weeks old when experiment was terminated. Spleens were harvested for gene expression analysis and joint morphology was determined histologically and by µCT scan.

### 4.1.3 ABATACEPT INTERVENTION

Testing if arthritis in *GLC* mice is alleviated by disrupting interaction between macrophages and T cells, the *GLC* mice were treated by intra-peritoneal injections of 10 mg/kg abatacept, CTLA4 fusion protein. Injections were given once weekly, starting at week 5 and continued until experiment termination at week 25. Spleens and dLNs were harvested for gene expression analysis and joint morphology was determined histologically. Control group of *GLC* mice received intra-peritoneal injections of PBS.

### 4.1.4 COLLAGEN IMMUNIZATION

To understand if compromised prenylation increased susceptibility to arthritis development, the twelve-week-old *GLC*$^{fl/+}$ and WT mice were immunized with collagen. 100 µg chicken collagen II, emulsified in complete Freund's adjuvant, was subcutaneously injected in the tail root with as described [80], and boosted 55 days later. The experiment was terminated on day 70. Spleens and dLNs for gene expression analysis were harvested and joint morphology was determined histologically.

## 4.2 PATIENT MATERIAL

### 4.2.1 NAÏVE RA COHORT

In **paper II and III,** we used a publicly available RNA-seq data of CD14$^{+}$ and CD4$^{+}$ cells purified from PBMC of 78 RA patients naïve to biological treatment. These samples present a subset obtained from the Biologicals and

Outcome Compared and predicted Utrecht region in Rheumatoid Arthritis, in this thesis referred to as BiOCURA cohort, collected between June 2009 and October 2012[97,98]. Aridaman Pandit and Weiyang Tao at the Center for Translational Immunology, University Medical Center Utrecht, The Netherlands, kindly shared the clinical data of these individuals. In addition, patients of this cohort started treatment with adalimumab or etanercept and were followed for 3 and 6 months after the start of treatment. Disease activity and subsequently the treatment response compared with baseline was calculated [99]. All RA patients fulfilled the EULAR/ACR classification criteria 32.

### 4.2.2 TREATED RA COHORTS

In **paper II,** we used RNA-seq data of $CD14^+$ cells purified from PBMC of 59 randomly selected RA patients at the Sahlgrenska University Hospital, Gothenburg enrolled between October 2019 and October 2020, referred to as the NeumRA cohort. Additional RA patient samples were drawn to perform in vitro experiments in hypoxic environment and in the presence of tofacitinib, these samples were collected between April-May 2022.

In **paper III,** we used RNA-seq data of $CD4^+$ cells purified from PBMC of 87 randomly selected RA patients at Sahlgrenska University Hospital, Gothenburg enrolled between September 2018 and October 2020, referred to as the RÖK cohort. In the RÖK cohort, a follow up of anti-rheumatic treatment was performed through medical records 2 years later by pre-defined key evaluation questions. All cases and time point for change of medication were registered. At inclusion, patients were clinically examined, and the disease activity score, global health assessment, and the patient's pain experience were recorded. All RA patients fulfilled the EULAR/ACR classification criteria 2010 (32).

#### Isolation of $CD14^+$ and $CD4^+$ cells

Human peripheral blood mononuclear cells were isolated from the venous peripheral blood by density gradient separation on Lymphoprep. CD4+ cells were isolated by positive selection using in wells coated with anti-CD3 antibody in RPMI medium supplemented with 50µM b2-mercaptoethanol (Gibco, Waltham, Massachusetts, USA), Glutamax 2mM (Gibco), Gentamicin 50µg/ml (Sanofi-Aventis, Paris, France) and 10% fetal bovine serum (Sigma-Aldrich) at 37°C in a humidified 5% CO2 atmosphere for 48 hours. $CD14^+$ were subsequently purified from the remaining cell mixture by positive selection (17858; Stem Cell Technologies, Vancouver, Canada) and cultured in the same medium and conditions as the $CD4^+$ cells but stimulated with LPS

(5 µg/ml; Sigma-Aldrich). Culture supernatants were collected for analysis of cell products by ELISA.

### 4.2.3 SINGLE CELL RNA SEQUENCING

In **paper II & III,** we used the single-cell RNA sequencing (scRNA-Seq) technique to find the tissue cells phenotypically identical to the blood $CD14^+$ and $CD4^+$ cells of our RA cohorts. In **paper II,** we identified the recently published data set of $CD11b^+CD64^+HLA\text{-}DR^+$ synovial tissue macrophages [62] and used it to match the RA $CD14^+$ cells. These cells were collected from knee synovial tissue of 25 individuals, 17 with RA, 4 with undifferentiated peripheral arthritis (UPA) and 4 healthy individuals. The 17 RA patients fulfilled the 2010 revised EULAR/ACR criteria for RA.

In **paper III,** we used the recently published atlas of thymic development[100] to match the $CD4^+$ cells of RA patients collected in the RÖK and and BiOCURA cohorts. The cells used to generate this atlas were sampled from 15 embryonic and fetal thymi spanning thymic developmental stages between 7 and 17 post-conception weeks, as well as 9 postnatal thymi from pediatric and adult individuals.

### 4.2.4 CELL STIMULATION

The cell material used in these studies were stimulated to enhance gene expression or cell products, which is outlined in Table 3. Mice tissues were homogenized to single cell suspension prior to stimulation or downstream analysis.

**Table 3. Cell Stimulation and sample analysis.**

| | Paper I | | | | | | Paper II | | | | | Paper III | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Experiment** | GLC vs WT | DKO | Abatacept Intervention | | Collagen Immunization | | BiOCURA | NeumRA | Hypoxic (in vitro) | JAKi (in vitro) | STM | BiOCURA | RÖK | Thymic Atlas |
| **Cells** | CD4+ from Spleen and dLN | Spleen | Spleen | dLN | Spleen | dLN | CD14+ cells | CD14+ cells | CD14+ cells | CD14+ cells | $CD11b^{+}CD64^{+}HLA\text{-}DR^{+}$ cells | CD4+ cells | CD4+ Cells | Thymys |
| **Stimulation** | conA, 2h | conA, 2h | conA, 2h | No stim. | aCD3, 2h | No stim. | No stim. | LPS, 48 h | LPS, with and without IFN-γ, 48 h | LPS, with and without tofacitinib 10 µM, 48 h | No stim. | No stim. | conA + LPS, 72h | No stim. |
| **Analysis** | qPCR, RNA-seq, ELISA | qPCR | qPCR, ELISA | qPCR | qPCR, ELISA | qPCR | RNA-seq | RNA-seq, ELISA | qPCR | qPCR | scRNA-seq | RNA-seq | RNA-seq | scRNA-seq |

# 4.3 SAMPLE ANALYSIS

## 4.3.1 EVALUATION OF ARTHRITIS SEVERITY

### Histology and immunohistochemistry

In **paper I** arthritis severity in mice was evaluated histologically. Briefly, one front and one hind paw were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Interphalangeal, metacarpal, wrist, metatarsal, and ankle joints were analyzed microscopically in coded sections for signs of arthritis. Histological scoring system between 0 and 3 was used to define synovitis and joint damage severity. The total arthritis score was calculated as the sum of all inspected joints, as previously described [101,102].

### Micro-computed tomography of paws

In **paper I**, joints of *GLC* mice and generated *GLC-Rhoa*$^{fl/fl}$ *GLC-Rac1*$^{fl/fl}$ *GLC-Cdc42*$^{fl/fl}$ were analyzed by micro-computed tomography (µCT). Left hind paws were scanned with SkyScan1176 micro-CT (Bruker, Antwerp, Belgium) as previously described [103]. The analysis was performed with focus on the MTP II–IV joints chosen as regions of interest. Measurements of tissue volume (TV) and bone volume (BV) were calculated by the marching cubes method and presented as the bone volume fraction (BV to TV ratio).

### DAS28

In **paper II & III** disease activity in RA patients was calculated by the DAS28 score, an index commonly used within rheumatological practice. This score is based on the assessment of 28 joints for tenderness and swelling as well as the erythrocyte sedimentation rate (ESR). The global health and pain were measured by a 100 mm visual analogue scale.

## 4.3.2 FLOW CYTOMETRY

In **paper I**, splenocytes were analyzed by flow cytometry. Cells were Fc-blocked and stained using primary monoclonal antibodies to analyze surface proteins. Intracellular proteins were analyzed by permeabilizing cells with Cytofix/Cytoperm™ Solution Kit. For extracellular analysis and when needed the fluorochrome minus one setting was used. Fluorochrome compensation was performed using CompBeads. Cell volume was defined by forward cell scatter (FSC-A). Data was analyzed using the software FlowJo.

## 4.3.3 RNA-SEQ PREPARATION

### RNA Extraction

For the mice experiments and the samples collected at the Sahlgrenska University Hospital, cell RNA was prepared using the mini spin-column RNA extraction kits. Pre-qPCR RNA quality was controlled by the dual absorbance ratios 260:280 and 260:230 spectrophotometrically. Pre-RNA-seq RNA quality was analyzed by Bioanalyzer.

### Transcriptional sequencing (RNA-seq)

Illumina Sequencing was used to sequence the extracted RNA. The sequencing of RNA consists of 4 steps, converting RNA to DNA, amiplification, sequence and analyses. Briefly, the extracted RNA was converted to complementary DNA (cDNA) by the reverse transcriptase reaction. The cDNA is then fragmented followed by the addition of adapter sequences that act as reference points during amplification. The DNA with adapters is then loaded into a flow chamber were the reaction takes place. The flow chamber contains oligonucleotides that bind the adapters. Following, random primers and nucleotides contain a reversible fluorescent blocker are washed into the flow cells. Each type of nucleotide contains a specific fluorescent blocker. The blocker restricts the DNA polymerase from adding more than 1 nucleotide at a time onto the DNA fragment. After each round of adding, a camera snaps a photo recording the current nucleotide added to the fragment. The blocker is then chemically removed to allow a second round. This process is repeated until the DNA fragment is fully sequenced. This reaction occurs simultaneously for many DNA fragments at a time.

## 4.3.4 QUANTITATIVE PCR

In addition to RNA-seq, we measured gene expresison by quantitative polymerase chain reaction (qPCR) accordingly. Extracted RNA was converted to cDNA by the reverse transciprtase reaction. This DNA was later used for amplication in the PCR reaction utilzing primers designed for specfic gene sequences. The PCR reaction was held for 40 cycles alternating between 60 and 95 °C followed a melt-curve analysis slowly reaising the temprature from 60 to 95 °sC over 15 min. Amount of amplicons and the melting point was determined using SYBR green chemistry. The relative quantity of the product between samples was then determined using the ddCT method against a reference gene.

### 4.3.5 ELISA AND FCAP ARRAY

To determine protein products in supernatants of cell cultures, we used two separate tools, the conventional ELISA and the FCAP array, a flow cytometry-based tool.

## 4.4 DATA ANALYSIS

### 4.4.1 RNA-SEQ ANALYSIS

The raw sequence data were obtained in bcl-files and converted into fastq text format using the bcl2fastq program from Illumina. Transcripts were mapped with the UCSC Genome Browser using the annotation set for the hg38 human genome assembly and analyzed with the core Bioconductor packages in R-studio allowing for gene expression quantification. Differentially expressed genes (DEGs) were identified with DESeq2 R package[104]. The input to DESeq2 is a matrix of raw counts, i.e. number transcripts mapped to gene from the Illumina sequencing. The count table was also associated with sample data defining the experimental groups. The DE-Seq can then perform after the internal normalization, where a geometric mean is determined for each gene across all samples. This mean is then used to divide each count of the gene for every samples. This normalization results in several ratios, the median of which is used as a size factor for that particular sample. It corrects for the library size and RNA composition bias. Furthermore, a dispersion value is calculated for each gene to estimate the variance. DESeq2 then fits negative binomial generalized linear models for each gene and uses the Wald test for significance testing. The genes were considered differentially expressed (DEG) if the nominal p-value<0.05. the ComplexHeatmap R package was used to construct heatmaps with clusters using the K-means algorithm to gain clusters that are closely correlated within the identified DEG. The K-means algorithm works by an iterative process after selection of number of clusters desired.

### 4.4.2 ENRICHMENT ANALYSIS

To annotate function to a set of genes we used the enrichment analysis. For example, this applied to the cluster of genes generated by K-means algorithm. This tested our gene set against databases such as Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genome (KEGG) containing other gene sets already attributed a function. We also used this function to determine potential upstream regulators of gene sets that utilizes the data bases on results of chromatin immunoprecipitation sequence (ChIP-seq) technique. For this type of analysis, we used separate web clients, g:Profiler[105] to find pathways , GSEA

[106] to find the upstream transcriptional regulators and TRRUST database[107] to find transcriptional networks.

### 4.4.3 REGRESSION MODEL

In **paper II,** we constructed a model to select samples based on their ratio between 2 variables. A linear equation between the point representing the maximums and the point representing the minimums of the variables of interest. Using the equation, the predicted value was calculated, and samples were considered inside the model if the absolute value of the difference between the predicted and real value was less than 1.

### 4.4.4 PRINCIPAL COMPONENT ANALYSIS

Principal components analysis (PCA) was performed using the R package FactorMineR and visualized using factorextra. In **paper I,** I performed iterative PCA to find and visualize the parameters to define the mouse strains of the DKO experiment. In **paper II,** the aim of PCA was to visualize the effects of the treatments on identified pathways of interest.

### 4.4.5 STATISTICS MOUSE EXPERIMENTS

In **paper I**, the mouse experiments produced few samples in every experiment and therefore we did not assume anything about the distribution. Hence, statistical analysis was performed non-parametrically. Difference between groups was determined using the Mann-Whitney t-test, and correlations were calculated using the Spearman´s rank test. The p-values below 0.05 were considered significant.

### 4.4.6 PREDICTION OF PBX1 REGULATED GENES

In **paper III** PBX1 target genes in $CD4^+$ T cells were predicted by extracting data of PBX1 chromatin immunoprecipitation (ChIP) sequencing from human A549 pulmonary epithelial cell line, RCH-ACV lymphoblastic leukemia cells and 697 acute lymphoblastic leukemia cell line of the ReMAP database[108]. The PBX1 peaks from this data was used in conjunction with an assay of transposase-accessible chromatin (ATAC) using sequencing from activated $CD4^+$ T cells, thereby projecting PBX1 binding sites on the open chromatin in activated $CD4^+$ T cells. Sequences of overlapping PBX1 binding sites and open chromatin were annotated with GREAT allowing annotation of both sequences in known loci and sequences near known loci. Another set of PBX1 targets were extracted from the CHEA database according to Harmonizome data source [109].

### 4.4.7 TISSUE ORIGIN ANALYSIS

In **paper III** tissue origin of PBX1 targets in T cells was analyzed in the Human Bone Marrow Atlas, Immgen cell populations within the Immunological Genome Project consortium database, and Immune Signatures Database within GSEA project [110] through the ToppFun tool.

### 4.4.8 SINGLE CELL RNA SEQUENCING

In **paper II** we extracted the scRNA-seq dataset obtained by Alivernini *et al.* [62] followed by clustering of cells by Unsupervised Uniform Manifold Approximation and Projection (UMAP). *CDC42* expressing macrophages in synovium were identified using the aggregate expression of *ATP5BP*, *COX7A2*, *PSMB6*, *PSME3*, *GTF3C6*, and *GTF2E2* together termed the metabolic signature (MetSig). Gene markers of the *CDC42*$^{hi}$MetSig$^{hi}$ STM cluster were determined using the default Wilcoxon rank sum test method with the remaining cluster as comparison. Genes were considered markers if |log2FC|>0.25 and minimum feature percentage detection at 25 %.

In **paper III** PBX1 expressing cells in thymus were identified in Atlas of human thymic development[100]. The whole cell census was used for co-clustering analysis based on EBI scRNA-seq. UMAP was used to combine PBX1 expression with known cell pattern.

# 5 ETHICAL COMMENTARY

**Paper I**. All animal experiments were approved by the Animal Ethics committee at the University of Gothenburg (Dnr: 122-21010, 100-2013 or 104-2015).

**Paper II**. The samples collected at Sahlgrenska University Hospital were part of a study approved by the Swedish Ethical Review Authority (2019-03787) and collection was performed in accordance with the Declaration of Helsinki. The trial is registered at ClinicalTrials.gov with ID NCT04378621. All RA patients fulfilled the EULAR/ACR classification criteria 32 and gave their written informed consent prior to the blood sampling.

**Paper III**. The samples collected at Sahlgrenska University Hospital were part of a study approved by the Swedish Ethical Review Authority (659-2011) and was performed in accordance with the Declaration of Helsinki. The trial is registered at ClinicalTrials.gov with ID NCT03449589. All RA patients fulfilled the EULAR/ACR classification criteria 32 and gave their written informed consent prior to the blood sampling,

Publicly available material was deemed ethically collected based on statements by authors of the papers initially publishing the material.s

# 6 RESULTS AND DISCUSSION

## 6.1 MACROPHAGE CO-ORDINATE T CELL MEDIATED AUTOIMMUNITY VIA CDC42

RA is a chronic autoimmune disease with development of autoantibodies requiring the involvement of lymphocytes. In **paper I**, one of first things we confirmed is the dependency on lymphocytes for the development of RA in *GLC* mice. When crossing *GLC* mice with Rag1-deficieint mice, which lack mature lymphocytes due to inability to assemble a TCR, the histologically measured arthritis severity was reduced. With this fact known, we could conclude that **macrophages must confer a phenotype to the cells of the adaptive immune system which aids in RA development in *GLC* mice**, as the genetic modification had been restricted to macrophages[85]. Next, we focused the attention on $CD4^+$ T helper cells, due to their central role in interfacing with innate immunity and activating adaptive immunity. We analyzed their gene expression in spleen and joint draining lymph nodes (dLN). This gave us a pattern on transcriptional changes in the T cells approaching the joint. Notably, Rho-GTPases Cdc42, and Rac1, which are hyperactivated in macrophages of *GLC* mice, were also overexpressed in spleen $CD4^+$ cells of *GLC* mice compared to their WT counterparts. Furthermore, flow cytometry analysis revealed enrichment of $\beta_1$-integrins and $\beta_2$-integrins on the surface of T cells, both essential for their migration and interfacing with APC. Accordingly, we demonstrated that spleen $CD4^+$ cells of *GLC* mice activated a mechanosensory complex of $\beta_1$-intergrin, vimentin and Nrp-1 suggesting that these T cells were invasive. Nrp-1 has been shown to promote $\alpha_5\beta_1$-integrin mediated adhesion to extracellular matrix[111] and vimentin promotes clustering of $a_5b_1$-integrin [112], both important for proper migration towards fibronectin. Furthermore, we observed an upregulation in expression of β-catenin (encoded by *Ctnnb1*) known to be stabilized by vimentin, and *Lef1*, complex partner of β-catenin.

Hox proteins and integrins are in close chromosomal proximity and the former has been shown to control expression of latter [39,113]. Thus, we investigated the HoxA expression in spleen $CD4^+$ cells of *GLC* mice. Caudal *Hoxa10* was downregulated and we observed an inverse correlation of *Hoxa10* with *Cdc42*, *Rac1*, *Itgb1*, *Ctnnb1* (encoding b-catenin) and *Lef1*. Indicating a regulatory connection between Rho-GTPases, mechanosensory complex, β-catenin/Lef1 signaling and caudal HoxA.

Interestingly, *Lef1* and β-catenin expression are essential to maintain Treg phenotype [114]. Hence, we focused on this population with flow cytometry analysis and demonstrated high frequency of $Foxp3^+$ T regulatory cells within the splenocyte $CD4^+$ population. Furthermore, Nrp-1 of the mechanosensory complex is a known marker of thymic Tregs (tTregs)s. This indicated that the mechanosensory complex in cooperation with **Rho-GTPases control the peripheral migration of tTregs**.

Comparing gene expression analysis of the spleen and joint dLN $CD4^+$ T cells, we were able to track a strong similarity in expression pattern *i.e.*, the upregulated Rho-GTPases, mechanosensory complex and tTreg markers with downregulated caudal *Hoxa10*. This gene combination identified a specific subset of T cells that migrated and accumulated in joint draining lymph node (dLN) during arthritis. RNA-sequencing of $CD4^+$ cells isolated from dLN provided an additional insight into potential molecular mechanisms.

1) Rho-GTPase activation occurred downstream of integrins or TCR and was facilitated by GEFs *Tiam1* and *Vav2* which were upregulated in *GLC* mice. In addition, we observed indications of enhanced TCR signaling and APC interaction as genes responsible for palmitoylation and aggregation of lipid rafts which functions to enhance TCR activation[115,116] were upregulated. Furthermore, Nrp-1 which is part of the mechanosensory complex has been shown enhances Treg interaction with APC[117]. This enhanced interaction **prolong signaling within the immunological synapse[118], ultimately leading to loss of tolerance and auto-antibody production seen in *GLC* mic**e[85].

2) **Tregs are of thymic origin** as *Foxp3* expression strongly correlated with *Nrp1*, *Lrrc32* (encoding by Garp), *Runx2, Cd73* (encoding by Nt5e) and *Ikzf2* (encoding by Helios), defined markers of murine Tregs[119-122] required for effector function.

3) Pathway enrichment analysis demonstrated an enriched **IFN signature** present among the upregulated genes, suggesting a **skewness to Th1 pro-inflammatory phenotype**.

4) The T cells were ready to **egress from dLN and migrate to joint** this was evident by upregulation of a chemokine receptor repertoire (*Cxcr3*, *Ccr4*, *Ccr5*) associated with cells of the rheumatic joint[123]. In addition, these cells downregulated *Ccr7,* known to mediate lymph node homing and *Pggt1b* expression was increased to facilitate egress of $CD4^+$ cells, which have been shown to be required in egress thymus[91].

We connected the thymic Treg accumulation, IFN signature and readiness to migrate with arthritis and *Foxp3* expression by correlation analysis. This analysis revealed a selectivity in connection between these facets. tTreg markers correlated stronger with *Foxp3* expression, while the arthritis severity had stronger correlation with the IFN response genes and readiness of $CD4^+$ T cells to migrate. Hence, Rho-GTPase mediated activation of mechanosensory complex favor accumulation of tTregs with Th1-phenotype, which migrate to joint and contribute to arthritis development.

This led us to the conclusion that **macrophages acting via Rho-GTPases confer accumulation of thymic Tregs with activated mechanosensory complex. These tTregs gain an egressing Th1 phenotype in dLN and enhanced TCR signaling lead to breakdown of self-tolerance contributing to arthritis development.**

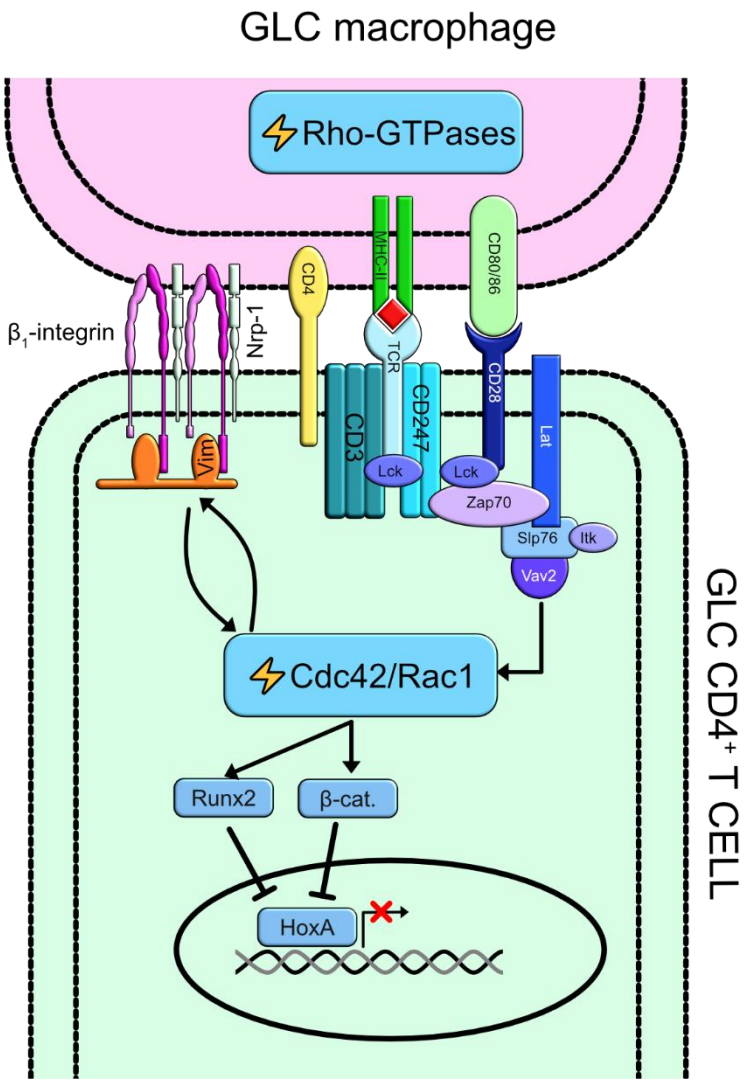


**Figure 7. Potential mechanism of HoxA repression via Rho-GTPases in *GLC* mice.** Downstream of TCR signaling, Cdc42 and Rac1 is activated by Vav2. This in turn leads to activation of mechanosensory complex consisting of $\beta_1$-integrin, Nrp-1 and vimentin, which in a feedback loop leads to signaling via Runx2 and β-catenin, suppressing caudal HoxA and counteracting homing.

Next, we wanted to determine which of the canonical Rho-GTPases was responsible for promoting the T cell phenotype. By crossing *GLC* mice and mice with *Cdc42*$^{fl/fl}$, *Rac1*$^{fl/fl}$, or *Rhoa*$^{fl/fl}$, we obtained mice deficient in GGTase-I and one of the canonical Rho-GTPases. Here, the mice deficient in *Cdc42* stood out. The gene expression analysis in splenocytes of *Cdc42*$^{fl/fl}$ *GLC*

mice demonstrated that the pattern of activated Rho-GTPases, mechanosensory complex around $\beta_1$-integrin was diminished. In accordance, β-catenin (*Ctnnb1*) and *Lef1* expression was reduced, and this yielded a reduced population of tTregs in spleen, evident by reduced expression of *Nrp1* and Helios (*Ikzf2*). In addition, caudal *Hoxa9* was upregulated and transcription factors defining other T cell subsets (*Tbx21*, *Gata3* and *Rorc*) as well as the overall frequency of $CD4^+$ T cells was reduced in *GLC* $Cdc42^{fl/fl}$ mice compared to *GLC* mice. Size analysis by flow cytometry in the forward scatter range, pointed towards increased activation status of $CD4^+$ cells. And surface expression of $\beta_1$-, $\beta_2$-, $\alpha_2$-, $\alpha_5$-integrins as well as accumulation $Foxp3^+CD25^+$ Tregs had increased. Hence, we concluded that ***Cdc42*** **was essential in macrophages to promote the migration of tTregs** to spleen**.**

We wanted to know if anti-rheumatic specifically targeting T cell co-stimulation would alleviate the observed pattern tTreg accumulation in spleen and dLN. Hence, we treated *GLC* mice with CTLA4-fp (abatacept), it reduced activation of mechanosensory and thereby accumulations of tTregs in spleen and dLN, evident by downregulation of tTreg markers. This strengthened the notion that **macrophages are responsible accumulation of this subset of Tregs within a tissue and that direct cell-cell interaction is required**. However, Rho-GTPases remained equally expressed in treated and non-treated mice.

In a last experiment we aimed to induce arthritis by forcing antigen presentation via collagen-II immunization (CII) in non-arthritogenic $GLC^{fl/+}$ and WT mice to observe how tTreg migration and arthritis development is affected by compromising prenylation. Again, we demonstrated that *Cdc42* expression regulates the migration of tTregs to spleen. In the immunized mice, *Cdc42* expression in spleen was suppressed compared to *GLC* mice. This was combined with the increased population of thymic $Foxp3^+CD25^+$ and peripheral $Foxp3^+CD25^-$ Tregs. However, differentiating CII $GLC^{fl/+}$ and CII WT, was the accumulation of tTreg markers Helios, Nrp1 and Runx2 in spleen of CII WT mice contrary to CII $GLC^{fl/+}$ where they situated in dLN. In addition, *HoxA10* was downregulated in spleen of CII $GLC^{fl/+}$ counteracting homing and allowing migration to dLN according to what we previously seen. This suggested **that compromised prenylation in macrophages promoted migration of Rho-GTPase expressing tTregs to dLN** in response to induced immune response. An outline tTreg migration in *GLC* mice in response to Rho-GTPase and mechanosensory complex activation is give Figure 8. summarizing the finding of **paper I**.

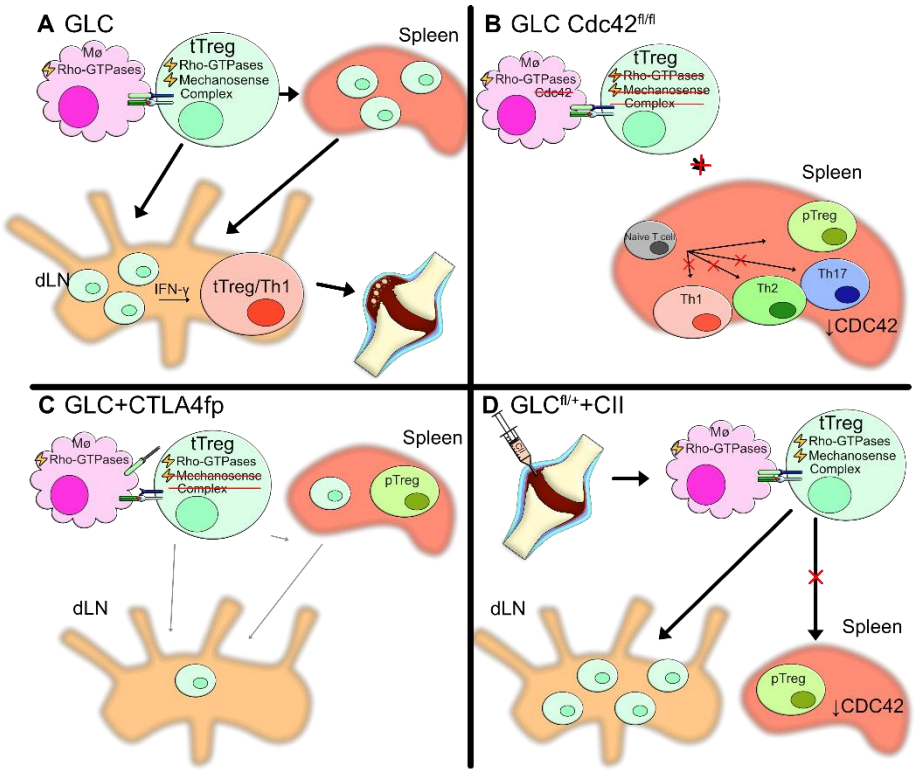


**Figure 8. tTreg migration in *GLC* mice. (A)** In *GLC* mice macrophages with hyperactivated Rho-GTPases confer a reciprocal upregulation of Rho-GTPases in tTregs, resulting in the activation of a mechanosensory complex around $\beta_1$-integrin. These tTregs accumulate in both spleen and joint draining lymph node (dLN). In addition, in dLN tTregs are skewed towards Th1 pro-inflammatory phenotype evident by upregulation of *Stat1*, *Eomes*, *Tigit* and *Irf5*. Downregulation of *Hoxa10* and *Ccr7* cancels homing to lymph node and upregulation of *Cxcr3*, *Ccr4* and *Ccr5* denotes joint destination. **(B)** Simultaneous knock-out of *Cdc42* in *GLC* macrophages abrogates tTreg migration to spleen, results in lymphopenia and inhibits T cell differentiation into Th1, Th2, Th17. However, Tregs not expressing thymic markers accumulate, suggesting an increased peripheral Treg differentiation. **(C)** Disrupting co-stimulation in *GLC* mice with CTLA4 fusion protein suppressed migration of tTregs to spleen and dLN, however, this lack of tTregs was compensated, presumably by pTreg differentiation as $Foxp3^+$ population remained unchanged. **(D)** Forcing antigen presentation by CII-immunization in prenylation compromised mice ($GLC^{fl/+}$) caused accumulation of Rho-GTPase expressing tTregs in spleen and dLN. In contrast, WT counterparts (not shown) only accumulated tTregs in spleen, which suggest that dysregulation of Rho-GTPases in macrophages is required to recruit Rho-GTPase expressing tTregs to dLN.

## 6.2 *CDC42* EXPRESSION FACILITATES MONOCYTE INFILATRATION TO JOINTS

Our findings that *Cdc42* expression in macrophages was important for regulation of T cell trafficking in *GLC* mice, set the starting point for the analysis of human $CD14^+$ cells. In **paper II**, we investigated $CD14^+$ cells in two independent RA patient cohorts. At our disposal, we had RNA-seq data of the publicly available BiOCURA cohort consisting of 77 individuals with active disease, naïve to treatment, and the in-house NeumRA cohort with RA patients in remission consisting of 59 individuals on different RA treatment. To mirror *GLC* mice in RA patients, we stratified the cohorts by *CDC42* expression in $CD14^+$ derived from PBMC, resulting in a $CDC42^{lo}$ and $CDC42^{hi}$ group in each cohort

Like the *GLC* macrophages, activity of the canonical Rho-GTPases CDC42, RAC1 and RHOA were linked in human $CD14^+$ cells. In *GLC* mice, we observed this as accumulation of activated Rho-GTPases as a result of the GGTase-I deficiency[85]. Accordingly, in $CD14^+$ cells from RA patients the gene expression of the canonical Rho-GTPases was highly correlated and this was evident in both RA cohorts. Hence, it was reasonable that biological processes of $CDC42^{hi}CD14^+$ in the two cohorts would be comparable as well. To find the most conserved CDC42-depdent biological processes, we extracted the intersecting DEG between $CDC42^{hi}$ and $CDC42^{lo}$ of each cohort. We performed unsupervised division of these DEG into 5 cluster of highly correlated genes using the K-means algorithm, separately for each cohort. We iteratively overlapped the gene set from each cluster of one cohort to the gene sets in the clusters of the other cohort. This yielded robust co-expressed gene sets, which we could explore using pathway enrichment analysis. Furthermore, in these gene sets, we searched for enriched transcriptional regulators. The analysis of the enriched biological pathways and transcriptional regulators led us **to construct a metabolic signature (MetSig) of $CDC42^{hi}CD14^+$ cells**. The MetSig was based on expression of genes involved in oxidative phosphorylation (OXPHOS), proteasome complex and RNA transcription, as these processes were highly enriched and regulated by the same transcriptional regulators. Using the MetSig, we constructed a linear model between the standardized aggregate expression of the signature genes and *Cdc42* expression.

Almost all $CD14^+$ cells of the remission cohort fell within close proximity of the model. However, the treatment-naïve RA cohort presented two groups of $CD14^+$ cells, one within the model boundaries showing strong correlation

between *CDC42* expression and MetSig score. However, a substantial number of samples formed another group, which were outside the model but also had a strong correlation between *CDC42* and MetSig Score. Re-grouping the samples based on their proximity to model, resulted in groups of samples included and rejected by the model. Notably, the MetSig differed between the groups, but not *CDC42* expression. The included samples were MetSig$^{hi}$ and the rejected samples were MetSig$^{lo}$. Differential gene expression analysis between the groups revealed that MetSig$^{hi}$CD14$^{+}$ cells were dedicated to antigen presentation as evidenced by 1) Upregulation of certain immunoproteasome subunits (*PSMB8*, *PSMB10*, *PSME1*), 2) increased expression of MHC genes, both MHC I *(HLA-A, -B, -E -F*) and II (*HLA-DMA, -DMB, DOA, DPA1, DPB1, DRA, DRB1*), 3) Overexpression of *TAP1, CD74* and *B2M* regulators of peptide loading onto MHC. Moreover, MetSig$^{hi}$CD14$^{+}$ upregulated a plethora of chemokines, increasing transcription of *CCL2, 3, 4, 4L2, 8, CXCL1, 2, 3, 8, 9 and 10*. This marked cells with high recruiting ability. Hence, this analysis revealed a potential mechanism of how ***CDC42$^{hi}$*MetSig$^{hi}$CD14$^{+}$ cells contribute to immune response in RA via recruitment of other leukocytes and activating adaptive immune response via antigen presentation**.

We hypothesized that this population of CD14$^{+}$ may be present in the synovium of RA patients. To examine this, we utilized a recently published scRNA-seq dataset of CD11b$^{+}$CD64$^{+}$HLA-DR$^{+}$ synovial tissue macrophages (STM)[62]. By searching this data set **using the MetSig, a cluster of STM with high expression of *CDC42*, dedicated to antigen presentation was identified.** These cells overexpressed the complete immunoproteasome (*PSMB8, PSMB9, PSMB10, PSME1, PSME2*), several MHC molecules (*HLA-A, -B, -C, E, -DMA, DPA1*), peptide loading regulators (*TAP1, TAP2, TAPBP*), CDC42 effector WASp[124] (encoded by *WAS*) mediating filopodial protrusions required in antigen presentation and genes indicative of response to IFN-γ (*IFNGR1, STA1, IRF1, IRF8*). Enrichment of upstream transcriptional regulators was also similar to circulating *CDC42$^{hi}$*CD14$^{+}$. In a way this was surprising, as we expected that the hypoxic condition of the RA joint would suppress the OXPHOS-dependent metabolic signature. Indeed, genes involved in OXPHOS-pathway were divergent in the STM cluster. However, there was a clear division between downregulated mitochondrial genes and upregulated non-mitochondrial OXPHOS-genes. We validated a decrease in MetSig by hypoxia through cultivation of freshly isolated CD14$^{+}$ cells in hypoxic environment. This suggested that the *CDC42$^{hi}$*STM cluster comprised the most recent immigrants in the hypoxic joint. In addition, we looked at expression of the genes which define tissue-resident and tissue-infiltrating macrophages[61]. This revealed the *CDC42$^{hi}$*STM cluster were characterized by a suppression of

the tissue-resident markers *FLOR2*, *ID2*, *TREM2*, while the IFN-sensitive genes *ISG15*, *CLEC10A* and *CD47* were highly expressed. In contrast to blood MetSig$^{hi}$CD14$^{+}$ cells, chemokine expression was downregulated in *CDC42$^{hi}$*STM. The chemokine expression in MetSig$^{hi}$CD14$^{+}$ may reflect a readiness to start producing chemokines in response to the appropriate trigger, which was not present in the rheumatic joint. In fact, IFN-γ have been shown to inhibit chemokine production by LPS stimulated macrophages[125], explaining the downregulation of chemokines *CDC42*$^{hi}$STM. Hence, this led us to conclude that **CD14$^{+}$ cells with the CDC42-dependent metabolic signature perpetuate RA disease by infiltrating joints, and presenting antigens, thereby coordinating autoimmunity**.

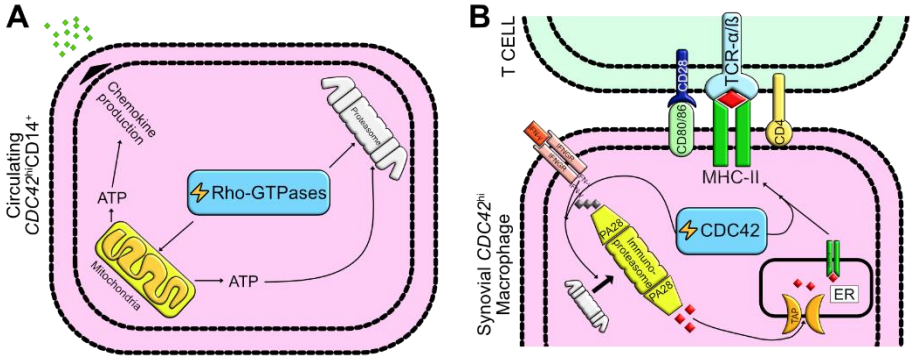


**Figure 9. *CDC42$^{hi}$*CD14$^{+}$ and *CDC42$^{hi}$*STM. (A)** *CDC42$^{hi}$*CD14$^{+}$ showed increased activity of oxidative phosphorylation and proteasomal complex. The increase in OXPHOS supply the cell with energy to facilitate the proteasomal activity and to sustain the chemokine guided migration of *CDC42$^{hi}$*CD14$^{+}$ cells in circulation. In addition, sustained Rho-GTPase activity explain the increased proteasome activity, as Rho-GTPases turnover is regulated by the proteasome. **(B)** Entering the synovial compartment, IFN-γ rich milieu initiates expression of antigen presentating machinery *i.e.,* immunoproteasome, MHC and TAP. CDC42 aids in phosphorylation of STAT1 downstream of IFN-γ and coordinates MHC-II antigen presentation via WASp.

## 6.3 IFN-GAMMA PERPETUATE ANTIGEN PRESENTATION IN THE JOINT

In **paper I** and **paper II,** we show the implication of IFN-γ in the Rho-GTPase associated mechanisms. In *GLC* mice, we demonstrated that tTregs recruited to joint dLN were imprinted with IFN-dependent Th1 phenotype. This took shape as enrichment of upregulated genes involved in cellular response to IFN-γ and IFN-β. For example, the transcription factor *Eomes* known to induce Th1 programming in Treg/Th17 phenotype[126,127], but also *Irf5*[128], *Stat1* and *Tigit*[129].

The expression of these IFN-sensitive genes strongly correlated to arthritis severity, implicating IFNs in *GLC* mice pathology. Moreover, in **paper II** we showed that the STM cells identified by the *CDC42* and the metabolic signature were heavily embossed by IFN-γ. This was evident by activation of the JAK/STAT-pathway and further downstream increased expression of immunoproteasome subunits, TAP peptide transporters and MHC, all to facilitate antigen presentation. Notably, it's been argued and reported that T cells and not monocytes nor macrophages are the main producers of IFN-γ[130]. However, in **paper I** we didn't detect any superfluous IFN-γ from the $CD4^+$ T cells. And in **paper II**, we showed that production of IFN-γ is miniscule from $CD14^+$ cells in RA patients, regardless of treatment. This underscores **the need of an extrinsic trigger for IFN-γ production to perpetuate RA.**

We hypothesized that the RA joint which is known to be hypoxic[131] would promote IFN-γ production and thereby explain the expression of immunoproteasome and other components facilitating antigen presentation. We tested this in **paper II** by cultivating $CD14^+$ cells in hypoxic environment. Indeed, this increased IFN-γ secretion by $CD14^+$ cells. However, these hypoxic conditions were not sufficient to induce immunoproteasome transcription. However, we confirmed that stimulation of $CD14^+$ cells with exogenous IFN-γ did indeed induce expression of the immunoproteasome subunits, independently of oxygen availability. This points to IFN-γ and not the hypoxic environment being the main driver of antigen-presentation in $CD14^+$ cells, but if hypoxia is grave enough it might stimulate sufficient IFN-γ production. Interestingly, a study by Lee et al. [132] showed that deletion of ubiquitin ligase regulating the hypoxia induced transcription of HIF1α led to a sustained activation of this transcription factor and in turn promoted IFN-γ production in Tregs and culminating in autoimmunity. This could explain our finding in *GLC* mice of Tregs gaining Th1 phenotype and propose a potential source of IFN-γ in hypoxia.

In contrast to the evidence that IFN-γ is associated with increased activity of Rho-GTPases and arthritis severity in RA patients and *GLC* mice, the *GLC*-CTLA4-fp experiment of **paper I** presented contradictory evidence. Here, we managed to suppress to splenocytic production of IFN-γ in *GLC* mice by treatment with CTLA4-fp. Also, it reduced the expression of tTreg markers in spleen and dLN, suggesting a reduced population of tTregs which would contribute to arthritis development. Despite this, arthritis severity remained unchanged in these mice, which may indicate that suppression was insufficient to yield any detectable changes. But it also introduces the dichotomy of IFN-γ being protective and disease progressing, previously shown arthritis[133-135].

## 6.4 LIFTING SUPPRESSION OF HOMEBOX PROTEINS IN T CELLS CONTRIBUTES TO RA DEVELOPMENT

In **paper I**, we demonstrated that thymic Tregs accumulated in the joint dLN during *GLC* arthritis. This went hand in hand with the decreased caudal HOXA expression. In regulating body planning HOX proteins forms complexes with PBX1[136,137]. In **paper III,** we hypothesized that the PBX1 expression pattern and related biological pathways will improve our understanding of the $CD4^+$ T cell development in RA.

We utilized RNA-seq data obtained on $CD4^+$ cells of two independent cohorts. the naïve to treatment BiOCURA cohort and the RÖK cohort of RA patients in remission receiving different treatments. In a approach similar to **paper II**, we stratified samples by *PBX1* expression, and analyzed the presence of the differentially expressed genes. Notably, there were similarities in the biological pathways enriched in $PBX^{hi}CD4^+$ in both cohorts. For example, $PBX^{hi}CD4^+$ cells were engaged in the immune response related pathways. In both cohorts, the $PBX1^{hi}CD4^+$ cells expressed more of the stem cell markers *SOX4*, *KIT* (encoding c-KIT), *CAT* and *FLT3*. However, the more detailed analysis of T cell maturation identified key differences between the cohorts. In active disease, the $PBX1^{hi}CD4^+$ cells were enriched with *CD34*, a marker tagging the pre-thymic common lymphoid progenitors (CLP). While in RA remission, the $PBX1^{hi}CD4^+$ cells were enriched with *PECAM1* and *CR2* tagging them as recent thymic emigrants (RTE). Hence, **we concluded that $PBX1^{hi}CD4^+$ cells denote the pre-thymic and post-thymic population of $CD4^+$ T cells, which had a connection to the disease state**. This is in accordance with thymic output being implicated in autoimmune diseases[10] and specifically RA[68,69].

Using the approach analogous to the one applied in **paper II**, we used the human thymic atlas[100], providing thymocyte transcriptional data at a single cell resolution which could be searched for clusters of cells with high *PBX1* expression. We managed to identify several *PBX1* expressing clusters. Interestingly, one of those clusters had high expression of *PECAM1*, *CD34*, *KITLG* (encoding KIT ligand) and *STAT3* as well as absence of *IL7*, denoting the progenitors of RTE. Which suggest that **PBX1 expression is maintained throughout T cell development**.

We also investigated the genes potentially regulated by PBX1, using the ReMAP database we built a list of PBX1 peaks from ChIP-seq data of various

cancer cell lines. These targets were integrated with the open-chromatin in activated $CD4^+$ cells using a publicly available ATAQ-seq dataset. This allowed prediction of PBX1 gene targets by GREAT analysis. The resulting predictions from this analysis were overlapped with DEG in the independent RA cohorts, which identified the transcription factors and cytokines regulated by PBX1 and involved in T cell differentiation. Among these targets we found *IFNG*, *TNF*, *IL6*, *TBX21*, *PRDM1*, *BATF* and *FOXP3* to be down regulated in *PBX1*$^{hi}$$CD4^+$ T cells. These genes are specifically involved in the differentiation Th1, Th2, Th17 and Treg subsets. This result resembled the one we observed in splenocytes of *GLC* mice deficient in *CDC42*. The *CDC42*$^{fl/fl}$ *GLC* splenocytes had high expression of caudal HOXA and low transcription of *Tbx21*, *Gata3* and *Rorc*, which regulated differentiation in T helper subset. **Taken together, these observations indicate that maintaining expression of homeobox proteins suppress differentiation of $CD4^+$ T cells.** Despite the maintained expression of *Hoxa9* in *GLC CDC42*$^{fl/fl}$ mice, arthritis disease activity was not reduced compared to *GLC* mice.

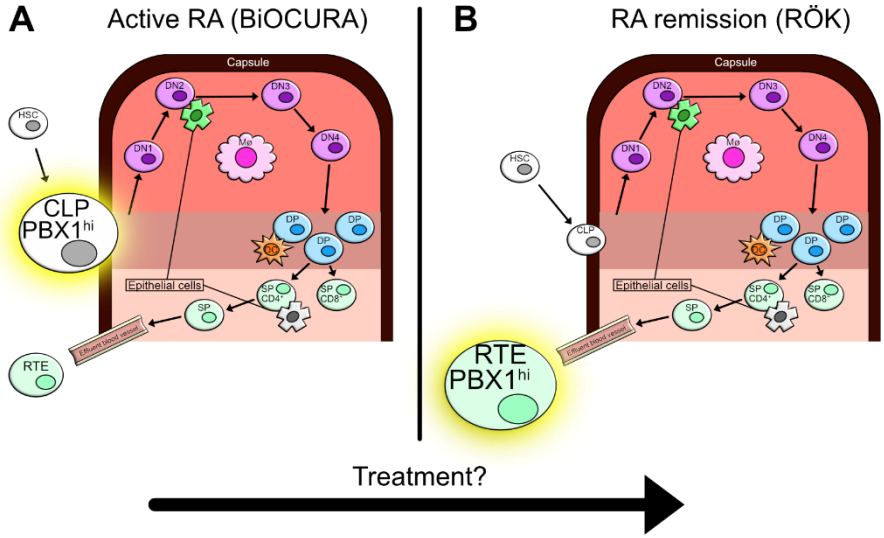


**Figure 10. PBX1 denotes pre-thymic and post-thymic population depending on disease state. (A)** *PBX1*$^{hi}$$CD4^+$ cells were enriched with CLP markers in active disease, the high *PBX1* expression was associated with favorable treatment. **(B)** In remission *PBX1*$^{hi}$CD4+ marked RTEs and high PBX1 expression this was associated lower treatment demands. A question that arises is if treatment increases thymic output and at the same time maintains *PBX1* expression to yield a higher naïve T cell pool.

## 6.5 GENE EXPRESSION GUIDING TREATMENT CHOICE

Through gene expression analysis of *CDC42*$^{hi}$CD14$^{+}$ in **paper II** and *PBX*$^{hi}$CD4$^{+}$ in **paper III**, we attempted to find mechanisms and markers to predict response to specific DMARDS and thereby allow for a more informed drug choice in treatment of RA. Treatment of RA goes through stages starting with traditional cDMARDS like Methotrexate, if that fails to alleviate symptoms patients are offered a type of bDMARD, which type is up the clinical rheumatologists, and as a last pharmaceutical resort patients may receive JAK-inhibitors. The reason for this staging is not only due price and proven safety, but also because it is difficult to determine choice of drug with the current diagnostics.

In **paper II,** we show that a substantial subset of patients has a proportional correlation between the CDC42-related metabolic signature and disease activity (DAS28). By confirming that this signature could be found in a specific cluster STM, we argue that MetSig identifies CD14$^{+}$ cells dedicated to joint infiltration. Additionally, these cells represent a population of cells that will perpetuate disease by antigen-presentation, induced by the IFN-γ rich milieu in the RA joint. In the NeumRA cohort we demonstrated that patients, who were MetSig$^{hi}$DAS28$^{hi}$ were treated less intensively, *i.e.* with TNFi or MTX, while patients on the other end of spectra received JAKi. Thereby, we propose that patients with high MetSig and higher disease activity may mark individuals in need of intensified treatment with JAKi. Notably, JAKi selectively reduced expression of the MHC II and not MHC I genes in CD14$^{+}$ cells compared to CD14$^{+}$ cells of MTX and TNFi-treated patients. This would indicate that **communication between CD14$^{+}$ and CD4$^{+}$ is perturbed and this results in beneficial effects on disease activity**. Downregulation of MHC-II in response to JAKi is in stark contrast to a recent study Mizuno and Yanagawa [138] which showed that tofacitinib raised the levels of MHC-II but decreased CD86. The authors concluded that tofacitinib treatment led to T cell anergy due to lack of co-stimulatory signal. Our results indicated that JAKi-treatment resulted in upregulation of the genes active in the IS of CD4$^{+}$ T cells and created a disbalance in communication between the two cell types. This disbalance was mirrored by Rho-GTPase expression, low in CD14$^{+}$ and high in CD4$^{+}$ cells. To add to the complexity, a recent report of Li et al. [139] showed that *CDC42* expression in CD4$^{+}$ cells of RA patients was inversely correlated with

DAS28, which attributed the increased disease activity with increased population of Th1 and Th17 cells. The same phenomenon is seen in mice with *Cdc42* deficient $CD4^+$ cells [25], suggesting that the increased *CDC42* expression we observe in $CD4^+$ of RA patients may be beneficial by reducing pro-arthritogenic Th1 and Th17 phenotypes.

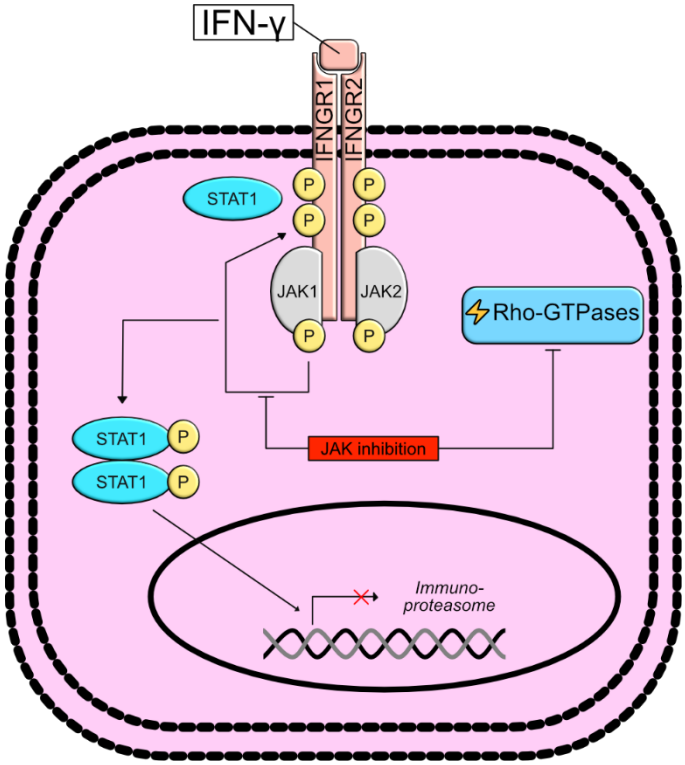


**Figure 11. JAK-inhibition suppress immunoproteasome expression**. In patients treated with JAKi we observed a clear suppression of IFN-γ signaling, resulting in suppression of immunoproteasome expression. Through unknown mechanism Rho-GTPase expression was suppressed as well, with concordant down regulation of the metabolic signature.

In addition, IFN-γ promotes production of cytokines TNF-α, IL-6, and CXCL8 [140]. In **paper II**, we observe a discrepancy between the anti-rheumatic treatments regarding these cytokines, with JAKi suppressing production of all them compared to treatment with TNFi or MTX. From one perspective this can be seen as JAKi mainly suppressing IFN-γ and thereby inhibits productions of the other pro-inflammatory cytokines. From another, this can be interpreted a JAKi having a broad effect suppressing immune response overall.

In **paper III,** we investigated how *PBX1*$^{hi}$CD4$^{+}$ cells were associated with RA disease measured by activity, treatment response and requirement of treatment intensification. We found that in the RA remission (RÖK cohort), *PBX1* expression in CD4$^{+}$ cells were associated with a lower degree of treatment. The patients with high *PBX1* expression were likely to receive cDMARDS while patients with low *PBX1* expression received either TNFi or JAKi. As cDMARD are the first line of RA treatment, we deduced that the patients with high *PBX1* expression in CD4$^{+}$ cells reached remission earlier than those with low *PBX1* expression. However, individuals with high *PBX1* expression were also more likely to require intensified treatment at a later stage, suggesting that autoreactive CD4$^{+}$ T cells may be hidden in the naïve T cell pool. Utilizing the BiOCURA cohort with active RA, which also contained the information about response to TNFi treatment, we examined if *PBX1* expression in CD4$^{+}$ cells predicted the treatment outcome. Notably, we found that *PBX1*$^{hi}$ group had a significantly more pronounced drop in DAS28, indicating a better treatment response. **Hence, our results points to beneficial effects on RA disease activity when maintaining the pool of CD4$^{+}$ cells with high *PBX1* expression**.

Together, **paper II** and **paper III** showed that gene expression analysis in CD4$^{+}$ and CD14$^{+}$ cells can be utilized to guide treatment choice. Patients with high CDC42-related MetSig in CD14$^{+}$ cells may benefit from early intervention with JAKi, which led to suppression of antigen presentation, thereby alleviating disease. On the other hand, enrichment with *PBX1*$^{hi}$CD4$^{+}$ cells may identify patients who need less intensive treatment. The replenishment of CD4$^{+}$ cell pool with *PBX1*$^{hi}$ cells from the bone marrow is achieved through treatment with cDMARDS or TNFi.

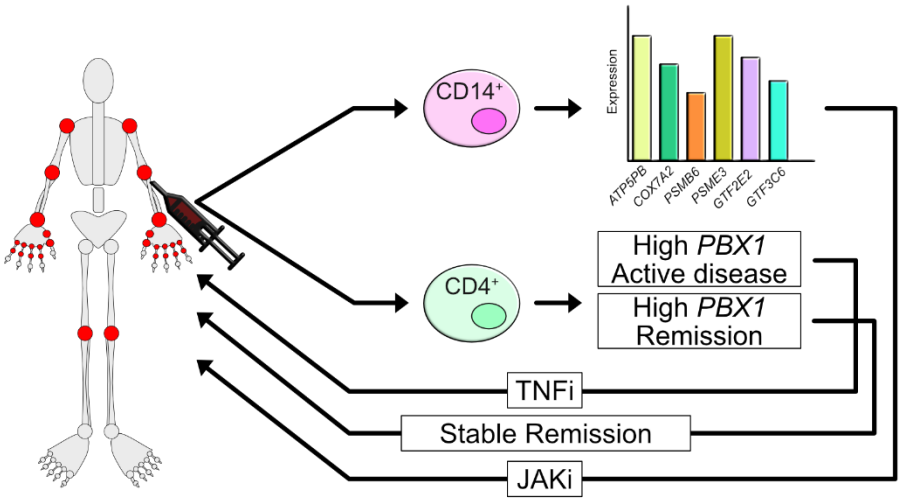


**Figure 12. Predicting treatment response.** Two approaches for guiding treatment of RA, a metabolic signature in $CD14^+$ cells may predict patients requiring JAKi. Expression of *PBX1* in $CD4^+$ cells predict better response to TNFi, or patients in stable remission.

# 7 CONCLUSIONS

**Paper I: Rho-GTPase dependent leukocyte interaction generates pro-inflammatory thymic Tregs and causes arthritis.**

*GLC* macrophages control egress of Rho-GTPase expressing tTregs to the periphery via Cdc42, where they invade joints and contribute to arthritis progression.

Findings that led to this conclusion:

- Genetic manipulation only of macrophages.
- *GLC* arthritis is T cell dependent.
- Abrogation of T cell co-stimulation with CTLA4-fp reduced expression of tTreg markers and RA-specific cytokines.
- High expression of Treg thymic markers in the joint draining lymph nodes.
- IFN-γ embossed $CD4^+$ cells in dLN.
- Cdc42 in macrophages conferred T cell recruitment to spleen.
- *GLC* macrophages induced suppression of caudal *Hoxa genes* in $CD4^+$ cells, which triggered differentiation and migration.

**Paper II: Metabolic signature and proteasome activity controls synovial migration of *CDC42*$^{hi}$CD14$^+$ cells in rheumatoid arthritis**

The CDC42-related MetSig identifies the antigen-presenting $CD14^+$ cells that migrate to joints to coordinate autoimmunity. Accumulation of $MetSig^{hi}CD14^+$ cells in blood identify patients perceptive to JAKi treatment.

Findings that led to this conclusion:

- MetSig identified IFN-γ embossed cluster of *CDC42* expressing $CD11b^+HLA\text{-}DR^+$ macrophages in RA synovia. These *CDC42*$^{hi}$STM were equipped with the immunoproteasome complex and MHC receptors.
- IFN-γ, rather than hypoxia, confers a switch to immunoproteasome in $CD14^+$ cells.
- MetSig and immunoproteasome expression in $CD14^+$ cells was suppressed by JAKi treatment.

**Paper III:** Pluripotency factor PBX1 predicts treatment efficacy in rheumatoid arthritis.

RA patients with high expression of *PBX1* in $CD4^+$ cells had favorable outcomes of anti-rheumatic treatment. *PBX1* controls thymic egress and differentiation of $CD4^+$ cells.

Findings that led to this conclusion:

- RA patients, who respond to TNFi treatment and those with low treatment demands had higher *PBX1* expression in $CD4^+$
- $PBX1^{hi}CD4^+$ cell in RA remission showed characteristics of the naïve T cells that recently emigrated from thymus.
- $PBX1^{hi}CD4^+$ cells in active RA showed characteristics of the common lymphoid progenitors prior to thymus entry.
- Predicted PBX1 gene targets constitute genes involved in $CD4^+$ T cell development and differentiation.

# 8 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis advances the understanding of interaction between the innate and adaptive immune system. More specifically, the understanding of how Rho-GTPases are associated with the processes of cell recruitment, differentiation, homing and direct contact interaction between $CD14^+$ and $CD4^+$ cells during antigen presentation. Furthermore, it strengthens the notion that the autoimmunity of RA is conferred by interaction between these two parts of the immune system. It provides new perspectives to how to use gene expression analysis in clinical work to guide treatment choice of RA. Lastly, it shows that findings in the *GLC* mouse RA model can be used to identify mechanisms involved in RA development in patients.

Further studies in *GLC* mice are required and should focus on how *GLC* mouse macrophages confer the thymic Treg phenotype we observe. Methods of visualizing the macrophage/T cell direct contact are improving[141] and since Rho-GTPases are crucial in regulating cell shape via actin regulation I believe studies such as these would reveal valuable insight into how the innate immune system shapes the adaptive. In general, there is lack of knowledge of the molecular processes regulating the APC during antigen presentation, as most studies has been conducted in model system where T cells interacting with planar lipid bilayers[142].

In addition, the understanding about the arthritis in *GLC* mice can likely be deepened by examining the cells of the joint. Khan *et al.* [85] of course confirmed the presence of $CD4^+$ T cells and macrophages ($F4/80^+$ cells) in the joints by histological analysis and we further strengthened this notion by demonstrating upregulation of a chemokine receptor repertoire associated with joint destination in $CD4^+$ cells of the joint dLN. However, the exact phenotype of these cells remains to be confirmed. The same can be said of the thymus, we show the accumulation of Rho-GTPase expressing thymic derived Tregs in spleen and dLN**,** however the cellular dynamics between T cells and macrophages in thymus remains unexamined, are T cells already predestined to migrate to joints after interaction with thymic macrophages? Also, it would be interesting to investigate if *GLC* mice suffer from progressed thymic involution as RA patients[69]. In these two cases, scRNA-seq coupled together with analysis software examining interactions between cells, such as the R package CellChat[143] would prove useful as it might reveal crucial receptor-ligand pairings between T cells and macrophages involved in the pathogenesis.

Regarding implementation to a clinical setting, lets address gene expression measurements to improve treatment of RA. Kurowska-Stolarska and Alivernini [61] suggests that analyzing the gene expression of the synovial compartment on a single cell level in RA patients may be the way forward in predicting treatment response. This is now more easily achieved with improved biopsy techniques that are less invasive[144]. However, they also show that cells perpetuating RA are of the tissue-infiltrating subset[62] while the tissue-resident population are largely protective maintaining synovial homeostasis. Our results, finding precursors of tissue-infiltrating population in blood in RA patients, suggests synovial tissue biopsy may be circumvented. Intuitively, utilizing blood samples rather than synovial biopsies to predict treatment response seems far easier as most health care staff can perform the sampling procedure and it is probably less anxiety inducing for the patient. Furthermore, once cells have entered the synovium, they may already be causing damage to the joints, whereas cells from circulation may be detected before, at an even earlier stage, so correct treatment can be predicted and initiated ahead of relapse. However, an unknown needing an answer is: Which one comes first? Or rather what can be detected first? Does the inflammatory joint give rise to tissue-infiltrating pre-cursors or is tissue-infiltration required to initiate RA?

Disregarding the specific targets of analysis to predict treatment response or disease. There are also challenges within the RNA-seq technology that needs to be resolved to optimize its usage in a clinical setting. Downstream data analysis must be determined and stream-lined to ensure quality and reproducibility of results. Currently there are many options on how to treat RNA-seq data, in this thesis I used the DESeq2 algorithm for the differential expression analysis, but there are many other alternatives and they tend to produce different results[145]. Reference genome used during gene annotation will impact the results and therefore needs to be standardized for each disease phenotype. There are technical and biological biases when assessing expression. Genes vary in length and in GC-content, however, methods to normalize against this have been suggested, but determining the best approach for the application in question may be difficult. These are just some technical difficulties that need to be ironed out. Otherwise, I believe RNA-seq is an excellent approach to complement full genome sequencing, both to diagnose and to predict optimal treatment approach, as it may provide a current picture of the disease state in the individual.

Anti-rheumatic treatment seems to restore the naïve T cell pool, which is otherwise perturbed in RA patients. However, this benefit may be somewhat restricted to a subset of patients primed for thymic activity *i.e.* patients accumulating CLP. This directs my thoughts towards the difficult to treat

RA[146] and if *PBX1* expression in $CD4^+$ cells may predict patients likely to develop this condition. A common feature of difficult to treat RA is immune senescence, so this does indeed align, since thymic output is a hall mark of increased immune senescence[147,148]. Hence, the patients with low expression of *PBX1* in $CD4^+$ may benefit from therapeutics targeting immune senescence. Drugs targeting senescent cells are called senolytics and research of their efficacy in RA is scarce and warrants further study, but it is plausible that *PBX1* may predict response to these drugs as well, identifying patients with pronounced immune senescence. Of note, our study only examined patient response to TNFi, hence the value of prediction by *PBX1* expression may be limited to this drug.

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