From Medical Biochemistry and Biophysics Department

Karolinska Institutet, Stockholm, Sweden

OXIDATIVE REGULATION OF NCF1 IN B CELLS AND MOUSE MODELS OF ARTHRITIS

Ana do Carmo Oliveira Coelho

Karolinska Institutet

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Oxidative Regulation of NCF1 in B Cells and Mouse Models of Arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ana do Carmo Oliveira Coelho

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Principal Supervisor:
Professor Rikard Holmdahl, MD, PhD
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research

Co-supervisor(s):
Dr. Stephen Malin, PhD, Principal Investigator
Karolinska Institutet
Department of Medicine
Division of Cardiovascular Medicine

Dr. Amit Saxena, PhD
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research

Opponent:
Professor Hans-Martin Jäck, PhD
University Hospital Erlangen
Department of Internal Medicine III
Division of Molecular Immunology

Examination Board:
Professor Qiang Pan Hammarström, MD, PhD
Karolinska Institutet
Department of Biosciences and Nutrition
Division of Clinical Immunology

Docent Christopher Sundling, PhD
Karolinska Institutet
Department of Medicine
Division of Infectious Diseases

Professor Mats Bemark, PhD
University of Gothenburg
Institute of Biomedicine
Department of Microbiology and Immunology

To my parents, especially to my dear father

“A ship in harbor is safe – but that is not what ships are built for” - John A Shedd 1923

Popular Science Summary

The immune system is composed of multiple cell types, such as neutrophils and macrophages, but also antibodies, that are responsible for fighting infections as well as vigilance and prevention of the development of cancers. When one of the components of the immune system is unbalanced, multiple opportunistic infections can occur. If one part of the immune system is missing or there is a mutation in a certain gene, people can suffer from recurrent inflammation named autoinflammatory syndromes. In other cases, the immune system can have an exacerbated reaction causing allergies to animals, pollen, or medicaments. The immune system can also attack self-cells and organs which causes autoimmune diseases. These are among the most prevalent diseases of the immune system. More than 80 autoimmune diseases have been described to date and can be classified as local or systemic if single or different organs are attacked, respectively.

We all have heard about type I diabetes, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis. All these autoimmune diseases are different but share a common aspect, there is no permanent cure for any of them. Scientific advances have made it possible to improve the quality of life of patients suffering from it, however, once diagnosed patients must live with it and their consequences for the rest of their lives. Therefore, it is important to study the mechanisms of dysregulation behind such diseases.

This thesis is focused on rheumatoid arthritis, an autoimmune disease that affects 0.5-1% of the worldwide population and is characterized by joint swelling that can result in bone erosion and deformation. Both genetic and environmental factors are associated with disease development and to date, more than 100 risk loci have been reported to be associated with rheumatoid arthritis. However, the study of such genes in humans is complicated as we have different ethnicities, lifestyles, and other habits. To circumvent this, we use animal models that resemble the human immune system and disease features to study specific genetic variations while maintaining a similar background.

Here we focus on the study of B cells which are antigen-presenting, and antibody-secreting cells, and the role of reactive oxygen species in such cells. Reactive oxygen species are usually regarded as detrimental/harmful; however, they are extremely important as signaling molecules. With this thesis, we add knowledge to the importance of reactive oxygen species in B cells in the context of rheumatoid arthritis.

Ciência Popular

O sistema imunológico é composto por diferentes tipos de células, como neutrófilos e macrófagos, mas também por anticorpos que são responsáveis por combater infeções causadas por micróbios e por vigiar e prevenir o desenvolvimento de cancros. Quando um dos componentes do sistema imunológico se encontra desregulado podem ocorrer infeções oportunistas. A falta de um componente ou mutações em determinados genes pode levar a inflamações recorrentes, também designado por síndrome autoinflamatória. O sistema imunológico é também capaz de criar reações exacerbadas como é o caso de alergias ao pólen, a animais ou até mesmo a medicamentos. Em casos mais graves, o sistema imunológico pode atacar as suas próprias células originando doenças autoimunes. Este tipo de doenças é bastante comum, tendo sido identificadas mais de 80 até aos dias de hoje. Estas doenças podem ser classificadas em locais ou sistémicas, no caso de afetarem um ou vários órgãos respetivamente.

Já todos ouvimos falar da diabetes do tipo 1, esclerose múltipla, lúpus e artrite reumatóide. Estas doenças autoimunes apesar de diferentes entre si, têm um importante aspeto em comum, não têm cura. Uma vez diagnosticados, os pacientes terão de viver com a doença e suas consequências para o resto da vida, embora avanços científicos têm permitido aumentar a qualidade de vida de pacientes portadores destas doenças. Por este motivo, o estudo de mecanismos que possam estar na origem do desenvolvimento destas doenças é de extrema importância.

Esta tese foca-se na artrite reumatóide, uma doença autoimune que afeta entre 0,5-1% da população mundial. Esta doença carateriza-se por um inchaço nas diferentes articulações que levam ao seu desgaste e consequente deformação dos ossos. O desenvolvimento desta doença está associado a fatores ambientais e genéticos, tendo sido até ao momento descobertos mais de 100 locus genéticos de risco associados à artrite reumatóide. O estudo destes genes em humanos é, contudo, difícil uma vez que partilhamos diferentes etnias, estilos de vida, entre outros fatores. A utilização de modelos animais que partilham características do sistema imunológico com os seres humanos permite o estudo mais concreto destas variações genéticas e as suas consequências.

Nesta tese focamo-nos em células B, responsáveis por detetar micróbios e produzir anticorpos que os combatam, e o papel de espécies reativas de oxigénio nestas células. Espécies reativas de oxigénio apesar de serem consideradas prejudicais, desempenham importantes funções como moléculas sinalizadoras. Os resultados obtidos nos estudos desta tese permitem ampliar o conhecimento sobre a importância de espécies reativas de oxigénio em células B no contexto da artrite reumatóide.

Abstract

Autoimmune diseases (ADs) are chronic pathologies that result from a dysregulation of autoreactive B and T cells leading to autoantibody production and ultimately destruction of self.

In **paper I**, we studied inflammation associated with autoimmunity. The glycolipid alpha-galactosylceramide (αGalCer) has been used as an adjuvant for vaccines, cancer treatment, and autoimmunity regulation, and shown to be effective. We show that when combined with a sterile mouse model of inflammation by injecting the pro-inflammatory cytokine IL-18, invariant natural killer T cells (iNKT) promote the expansion of autoreactive B cells instead of regulating them. This study offers insight into the usage of αGalCer in the context of chronic inflammation and implications in autoimmunity.

Identifying genetic polymorphisms in autoimmune diseases is crucial for understanding how the immune system operates to find innovative therapies to treat human diseases. Genetic mapping of quantitative trait loci (QTL) and differentially expressed genes (DEGs) are helpful tools, however, the identification of single nucleotide polymorphisms (SNPs) is a difficult task. One of the major SNP associated with autoimmune arthritis was identified in rat models, using pristane-induced arthritis (PIA). An amino acid replacement in the NCF1 protein at position 153 from threonine to methionine (T153M) decreased the oxidative burst capacity of the cells, which led to increased arthritis severity. The NOX2 complex and its subunits are expressed in many cell types, predominantly in phagocytes (neutrophils and macrophages) but also in antigen-presenting cells (APCs). This complex is essential to produce reactive oxygen species (ROS). Several studies have pinpointed an association of SNPs in different subunits of the NOX2 complex with different autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), confirming the findings in animal models. The fact that a lower ROS response was associated with arthritis severity contradicts the prevailing dogma that ROS are detrimental, making it important to explain how ROS can shape and protect against autoimmunity.

In **paper II**, we found that a point mutation in the NCF4 subunit of the NOX2 complex, has an effect in murine models of arthritis, shaping B cell responses and consequently plasma cells that are attracted to the sites of inflammation such as the synovium, secreting pathogenic antibodies that contribute to disease progression.

In **papers III** and **IV**, we focused on the role of NCF1 in B cells and germinal center B cells (GC-B), respectively. Using conditional mice flipping *Ncf1* alleles, we show that ROS regulates the activation and differentiation of B cells, with profound effects on T cells. Generally, mice expressing the low ROS allele ($NCF1^{153M}$) exclusively in B or GC-B cells have reduced ROS levels and increased arthritis severity as compared to littermate controls

harboring the high ROS allele ($NCF1^{T153}$). In parallel, sera levels of anti-collagen type II (COL2) antibodies were increased in the $NCF1^{153M}$ mice. T cell responses were affected in both models, with increased pro-inflammatory cytokines release, immune shaping of regulatory T cells, and T follicular helper (Tfh) cells. These findings provide evidence on the pleiotropic activity of NCF1-restricted ROS and present a previously undisclosed role of NCF1 in regulating B and GC-B cells in autoimmune diseases (ADs).

It is well known that B or T cells that react against self-antigens are deleted or inactivated by central tolerance mechanisms in the primary lymphoid organs bone marrow (BM) or thymus, however, some autoreactive clones can escape to the periphery and instigate ADs. In **paper V**, we studied antigen-specific B cells that opposite to what has been postulated are positively selected in the BM and found in the periphery of mice, rats, and even humans. These cells instead of causing autoimmunity, prevent it. COL2-transgenic mouse models do not develop spontaneous arthritis but are instead protected, mainly by the induction of regulatory T cells.

List of scientific papers included in this thesis

I. Saikiran Sedimbi, Thomas Hägglof, Manasa Garimella, Shan Wang, Amanda Duhlin, **Ana Coelho**, Katrine Ingelshed, Emma Mondoc, Stephen Malin, Rikard Holmdahl, David Lane, Elizabeth Leadbetter, Mikael Karlsson. **Combined proinflammatory cytokine and cognate activation of invariant natural killer T cells enhances anti-DNA antibody responses** (2020). *Proceedings of the National Academy of Sciences*, 117 (16) 9054-9063, doi: 10.1073/pnas.1920463117.

II. Chang He, Huqiao Luo, **Ana Coelho**, Meng Liu, Qijing Li, Jing Xu, Alexander Krämer, Stephen Malin, Zuyi Yuan, Rikard Holmdahl. **NCF4 dependent intracellular reactive oxygen species regulate plasma cell formation** (2022). Redox Biology, Volume 56, Article 102422, doi: 10.1016/j.redox.2022.102422

III. **Ana Coelho***, Mike Aoun*, Amit Saxena, Christian M. Beusch, Pierre Sabatier, Alexander Krämer, Chang He, Jaime James, Roman A. Zubarev, Stephen Malin, Rikard Holmdahl. **Bursting B cells as a regulator for autoimmunity**. *Authors contributed equally. [Manuscript].

IV. **Ana Coelho**, Mike Aoun, Christian M. Beusch, Pierre Sabatier, Chang He, Alexander Krämer, Roman A. Zubarev, Stephen Malin, Rikard Holmdahl. **Oxidative regulation of germinal center B cells by *Ncf1***. [Manuscript].

V. Mike Aoun, **Ana Coelho***, Alexander Krämer*, Amit Saxena*, Pierre Sabatier, Christian M. Beusch, Erik Lönnblom, Manman Geng, Nhu-Nguyen Do, Zhongwei Xu, Jingdian Zhang, Yibo He, Bingze Xu, Johan Viljanen, Joanna Rorbach, Gonzalo Fernandez Lahore, Inger Gjertsson, Alf Kastbom, Christopher Sjöwall, Jan Kihlberg, Roman A. Zubarev, Harald Burkhardt, Rikard Holmdahl. **Antigen-presenting autoreactive suppressor B cells**. *Authors contributed equally. [Manuscript].

Contributions to other papers and manuscripts not included in this thesis

I. Pierre Sabatier, Christian M. Beusch, Amir A. Saei, Mike Aoun, Noah Moruzzi, **Ana Coelho**, Niels Leijten, Magnus Nordenskjöld, Patrick Micke, Diana Maltseva, Alexander G. Tonevitsky, Vincent Millischer, J. Carlos Villaescusa, Sandeep Kadekar, Massimiliano Gaetani, Kamilya Altynbekova, Alexander Kel, Per-Olof Berggren, Oscar Simonson, Karl-Henrik Grinnemo, Rikard Holmdahl, Sergey Rodin and Roman A. Zubarev. **An integrative proteomics method identifies a regulator of translation during stem cell maintenance and differentiation** (2021). *Nature Communications* 12, Article Number 6568, doi: 10.1038/s41467-021-26879-4.

II. Vilma Urbonaviciute*, Laura Romero-Castillo*, Bingze Xu*, Huqiao Luo, Nadine Schneider, Sylvia Weisse, Nhu-Nguyen Do, **Ana Coelho**, Gonzalo Fernandez Lahore, Taotao Li, Pierre Sabatier, Christian M. Beusch, Johan Viljanen, Roman A. Zubarev, Jan Kihlberg, Johan Bäcklund, Harlad Burkhardt and Rikard Holmdahl. **Novel therapy targeting antigen-specific T cells by a peptide-based tolerizing vaccine against autoimmune arthritis.** *Authors contributed equally. [Manuscript].

III. Alexander Krämer, Taotao Li, Àlex Moreno Giró, Susanna L. Lundström, **Ana Coelho**, Bingze Xu, Zhongwei Xu, Roman A. Zubarev, Rikard Holmdahl. **Fc Sialylation has no effect on the pathogenicity of arthritogenic antibodies**. [Manuscript].

IV. Taotao Li, Changrong Ge, Huqiao Luo, Alexander Krämer, **Ana Coelho** *et al*. **Citrullinated type II collagen immunization breaks the physiologic T cell tolerance to immunodominant T cell epitope in P.266E mice**. [Manuscript].

Contents

List of abbreviations

ADs	Autoimmune Diseases
ACPA	Anti-Citrullinated Protein Antibody
AID	Activation-Induced Cytidine Deaminase
AIRE	Autoimmune Regulator
APCs	Antigen-Presenting Cells
ASCs	Antibody-Secreting Cells
ATP	Adenosine Triphosphate
BAFF	B Cell Activating Factor
BCR	B Cell Receptor
BM	Bone Marrow
CAIA	Collagen Antibody-Induced Arthritis
CCP	Cyclic Citrullinated Peptide
CFA	Complete Freund's Adjuvant
CGD	Chronic Granulomatous Disease
CIA	Collagen-Induced Arthritis
CLP	Common Lymphoid Progenitor
CMV	Cytomegalovirus
CNV	Copy Number Variation
COL2	Collagen Type II
CSR	Class Switch Recombination
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DEGs	Differentially Expressed Genes
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
FasL	Fas Ligand
Fc	Fragment crystallizable
FDCs	Follicular Dendritic Cells
FO	Follicular B Cells
GCs	Germinal Centers
GFP	Green Fluorescent Protein
GPI	Glucose-6-Phosphate Isomerase
GWAS	Genome-Wide Association Studies
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cells
H_2O_2	Hydrogen Peroxide

IBD	Inflammatory Bowel Disease
ICOS	Inducible T Cell Costimulator
iNKT	Invariant Natural Killer T Cells
LLPCs	Long-Lived Plasma Cells
LPS	Lipopolysaccharides
MHC (I,II)	Major Histocompatibility Complex (I, II)
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
MTX	Methotrexate
MZ	Marginal Zone B Cells
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Ncf1/2/3	Neutrophil cytosolic factor 1/2/4
NK	Natural Killer
NOX2	NADPH Oxidase 2
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PAMPs	Pathogen-Associated Molecular Patterns
PADI4	Peptidyl Arginine Deiminase Type 4
PIA	Pristane-Induced Arthritis
PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22
QTL	Quantitative Trait Loci
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
ROS	Reactive Oxygen Species
SHM	Somatic Hypermutation
SLE	Systemic Lupus Erythematosus
SLOs	Secondary Lymphoid Organs
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
STAT	Signal Transducer and Activator of Transcription
T1D	Type 1 Diabetes
TCR	T Cell Receptor
Tfh	T follicular helper cells
TNF	Tumor Necrosis Factor
Tr1	Type 1 Regulatory T Cells
Tregs	Regulatory T Cells
αGalCer	Alpha-Galactosylceramide

1 Introduction

The immune system branched into innate and adaptive, is complex, and has evolved over time to protect living beings from exogenous danger (1). The innate immune system, the first and more simplistic line of defense, is older and its origin can be dated >600 million years ago. On the other hand, the adaptive immune system is more complex and has developed mechanisms to recognize pathogens and fight them more efficiently. Its origin seems to date 450 million years ago in the first jawed vertebrates. This system is composed of B cells (that secrete antibodies), T cells with different T cell receptors (TCRs), and major histocompatibility complexes (MHC) (2). This system is not only designed to fight a wide variety of foreign pathogens but also to patrol the body's own cells for undesired mutations that can cause cancers or cells that wrongly start to recognize self as non-self, leading to the self-destruction of cells and consequently entire organs (autoimmunity).

The prevalence of any autoimmune disease (AD) is rare. However, collectively ADs affect 4-10% of the worldwide population, a number that is difficult to predict and highly dependent on the geographic area (3). Epidemiological data from a study conducted in 2009 in the United States estimates that more than 7.6-9.4% of US citizens suffer from at least one AD. However, in this study, only 29 ADs were considered, and the prevalence/incidence of the last decade was not considered, suggesting an increasingly greater occurrence of these diseases (4). ADs are characterized by the self-attack of one's own body tissues and the most common ones are Hashimoto's Thyroiditis, Type 1 Diabetes (T1D), Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), and Psoriasis. Women are three times more susceptible to ADs compared to men with a higher mortality rate (5,6), though, diseases like T1D affect both sexes equally.

Genetics heavily influences the susceptibility to develop ADs. To date, more than 100 genetic loci have been identified as risk factors for the development of ADs, but validating each of these individually has proven to be a difficult and complex task. Animal models that mimic human disease have helped dissect the functions and mechanisms of many genes, since animal models provide a controlled genetic background and housing conditions, decreasing the effects of population variability (7).

Nowadays, ADs can be ameliorated to a certain extent, but they cannot be cured. This puts a strong burden on society as the treatments are generally expensive and durable over an extensive period. Therefore, there is an unmet need to identify the underlying mechanisms of these diseases to improve treatments and potentially find a cure. RA, one of the most common ADs, is the focus of this thesis.

2 Literature Review

2.1 The Immune System

The immune system is a shield, with a network of biological processes that are meant to keep the organisms safe from external damage, like viruses, bacteria, or fungi. It protects beings not only from a wide variety of pathogens but also monitors their own cells to avoid the development of cancers and prevents attacking their own healthy cells and tissues, which can develop into an autoimmune disease (1).

2.1.1 Innate and Adaptive Immune System

The immune system can be divided into two main branches:

- Innate immune system – This is the first line of defense of an organism against any invading bacteria, viruses, or fungi, being constantly active, and providing immediate protection in the event of an invasion by exogenous pathogens. Skin and mucous membranes are part of this system, protecting the inner body from outside stimuli, however, in case of pathogen invasion, phagocytic innate immune cells such as neutrophils and macrophages will recognize patterns in the pathogens (pathogen-associated molecular patterns, PAMPs) that are not part of the body, engulf and digest them, which ultimately leads to their destruction and clearance. If cells from our body are infected, natural killer cells (NK) act by eliminating infected cells (8). The innate immune system also produces different pro-inflammatory cytokines, chemokines, and other proteins that attract other immune cells to the place of infection, which then leads to the activation of the adaptive immune system. Overall, this system serves as a quick defense mechanism that aids in managing illnesses.

- Adaptive immune system – Contrary to the innate immune system, the adaptive branch of the immune system is not constantly activated, however, this system is more specialized than the former one. This system comprises different cells and molecules responsible for guaranteeing a better, more specific, and stronger immune response to a given antigen. B and T lymphocytes are the main constituent cells of this system. B cells are responsible for the production of antibodies that neutralize a specific type of pathogen, but they are also antigen-presenting cells (APCs), recognizing certain antigens on the surface of pathogens, and presenting them in the context of MHCII to CD4 T cells. T cells on their hand can kill infected cells or secrete cytokines that will activate other immune cells. The adaptive immune system is extremely specialized and capable of recognizing pathogens that previously attacked the body, mounting a more efficient response

upon a second encounter with the same pathogen. This is enabled since a small percentage of antibody-secreting cells (ASCs), called long-lived plasma cells (LLPCs) will stay in circulation for some years, together with memory T cells that become effector T cells in the event of antigen reexposure (9). Vaccination operates and shapes this branch of the immune system by assuring that the immune cells of the body will recognize pathogens without having encountered them before, securing a proper immune response to fight them.

Given the fact that we live with commensal bacteria and are exposed to harmless antigens present in food, for example, the immune system has developed tolerance mechanisms that allow distinction between self and non-self while keeping immune responses effective. Tolerance is induced both centrally and peripherally, ensuring limited self-reactivity.

2.1.2 Central and Peripheral Tolerance

Autoimmunity and clonal selection theory were first described by Ian Mackay and Frank Burnet in 1962. Their hypothesis suggested that self-tolerance was achieved through the central deletion of "forbidden clones", effectively removing any clone that could potentially be self-reactive (10). Mechanisms of tolerance differ between B and T cells and between primary and secondary lymphoid organs (SLOs):

- Central tolerance – Occurs in the primary lymphoid organs bone marrow (BM) for B cells and thymus for T cells. Here, newly developed, and immature B or T cells that are self-reactive are eliminated by negative selection. By doing so, cells that could potentially recognize self-antigens are inactivated before they migrate to the periphery. Central tolerance mechanisms include clonal deletion, receptor editing, and anergy (11,12).
- Peripheral tolerance – Occurs in the peripheral tissues. In the event of cells escaping the first checkpoint in the primary lymphoid organs, B and T cells are once again tested for autoreactivity. In the periphery, this is achieved by the activation of regulatory T cells (Tregs) which prevent and control the activation of autoreactive T cells. B cells that have self-reactivity are deleted by apoptosis or are unresponsive to antigens, a condition called anergy (13,14).

2.2 Autoimmunity, Inflammation, and Infection

2.2.1 An Overview

In the late 19th century, Louis Pasteur, and Robert Koch's studies of infectious diseases such as tuberculosis, led to the creation of a new medically related discipline, immunology. Innate immunity was established based on the studies performed by Elias

Metchnikoff, where he discovered cells capable of engulfing and eliminating pathogens, today named phagocytosis (15). Antibodies that could neutralize microbial antigens were discovered around the same time by Emil Behring and Paul Ehrlich, which opened doors to the adaptive immune system concept we have today (15,16). Autoimmunity came associated with the discovery of antibodies in the early 20th century by Paul Ehrlich, considered to date the godfather of modern immunology. He introduced the term "horror autotoxicus" by observing the existence of antibodies that could be harmful to the individual, requiring the organisms to have mechanisms of self-defense against these antibodies, which he also called magic bullets (17). However, the possibility that cells would attack self was not accepted until the 1950s (18)

Autoimmunity is characterized by the activation of B, T, or both cells in the absence of an exogenous stimulus as an infection, a concept known as molecular mimicry. This results in the failure of the immune system to distinguish between self (autoantigens) from non-self. In other words, autoimmunity rises from the break of tolerance and dysregulated levels of autoreactivity (18). This leads to an immune infiltration of lymphocytes, causing inflammation and attacking either specific organs/tissues or different parts of the body systemically, consequently leading to pain, discomfort, organ/tissue destruction, loss of function, quality of life, or even death (19). All common ADs are complex, i.e., multifactorial, since under variable environmental conditions, an individual need a set of interacting genes, to allow the development of the disease. Genetic susceptibility is polygenic in nature; more than one polymorphic gene is needed to outbreak an AD. There are, however, monogenic autoimmune diseases, such as the autoimmune polyendocrinopathy syndrome that offer insights into how defects in the immune system can trigger autoimmunity (20). There are numerous ADs, varying from rare to common, together estimated to affect 5-10% of the worldwide population. Most, but not all, ADs are more prevalent in females (21). Despite the recent scientific advances, unraveling the precise mechanisms underlying ADs is a difficult task due to its complexity and multiple factors operating together, for which animal models provide important tools for the dissection and the function of each factor giving insights into the pathogenicity of such diseases (22).

Humans are subjected to a wide variety of infections throughout their lives, and it has been shown that these infections can trigger autoimmunity (23,24). Infections can contribute to the activation and expansion of autoreactive B and T cells, which are fundamental for the development of ADs, and in fact, almost all ADs are associated with at least one infection. Among the different infectious agents that have been associated with ADs are: Epstein-Barr virus (EBV), Rubella, and Cytomegalovirus (CMV) which have been shown to induce autoantibody production in systemic lupus erythematosus (SLE) (25,26); CMV, Rubella, EBV, rotavirus and *H. pylori* that have been associated with T1D (27);

and *Escherichia coli*, *Proteus mirabilis*, mycoplasma, and the oral infectious agent *P. gingivalis* that have been associated with RA (28,29).

Vaccines are designed to stimulate the adaptive immune system to combat certain pathogens and rely on the usage of adjuvants. However, adjuvants can have different facets in the context of autoimmunity, antitumor therapy, or improvement of immune responses. Therefore, it is important to study and understand how these adjuvants can shape both T and B cell responses in different scenarios, as shown in **paper I** (30,31).

2.2.2 Types of ADs

To date, there are more than 80 human diseases regarded as autoimmune. The most common ones are summarized in **Table 1**.

Table 1. List and characteristics of different ADs.

AD	Description
Type I Diabetes (T1D)	Attack of insulin-producing cells resulting in hyperglycemia (32).
Rheumatoid Arthritis (RA)	Infiltration of immune cells in the joints leading to cartilage destruction (33).
Psoriasis	Hyperproliferation of keratinocytes and the appearance of skin lesions, potentially triggering other comorbidities (34).
Multiple Sclerosis (MS)	Damage of the myelin sheath, results in low-speed transmission of messages between the brain and the spinal cord affecting the rest of the body (35).
Systemic Lupus Erythematosus (SLE)	Initially described as a skin disease, is today known to affect multiple organs like joints, the brain, the kidney, and the heart (36).
Inflammatory Bowel Disease (IBD)	Inflammation of the intestinal wall (37).
Addison's Disease	Adrenal glands are affected, potentially impairing hormones like cortisol, aldosterone, and androgen resulting in weakness, fatigue, and weight loss (38).
Grave's Disease	The thyroid gland is attacked, resulting in exacerbated production of its hormones, causing symptoms such as nervousness, fast heartbeat, and weight loss (39).
Sjögren's Disease	Lubrication glands are affected, resulting in dry eyes and dry mouth, possibly affecting joints and skin (40).
Hashimoto's Thyroiditis	Contrarily to Grave's Disease, it results in a slower activity of the thyroid gland, leading to weight gain, fatigue, hair loss and thyroid swelling (41).
Celiac Disease	Intolerance to gluten. If found in the small intestine, will trigger an immune response causing severe inflammation (42).

2.3 Rheumatoid Arthritis

2.3.1 Pathophysiology of RA

RA is one of the most common ADs and is the focus of this thesis. It is estimated that 0.5–1% of the worldwide population is affected by it, with different incidences in different geographical areas and populations, the most affected ones being Northern European and American countries (43). Even though currently this disease is better understood at present due to scientific advances, its precise immunopathogenesis remains elusive. RA is generally characterized by joint swelling, pannus formation, and synovial inflammation that can ultimately lead to bone erosion and deformity. All these symptoms cause pain and discomfort affecting the daily life of the individuals by slowly reducing their ability to perform regular tasks. It also contributes to the development of other diseases such as cardiovascular diseases or other autoimmunities and has a higher incidence in females (44,45). Both innate and adaptive immune systems play a role in the development of RA, and it is becoming clearer that genetics, epigenetics, microbiota, and environmental factors such as smoking, and dust exposure are potential disease initiators (46). RA can be stratified into four main phases: begins with a normal phase, comprising genetic factors, environmental stimuli, lifestyle, or hormones that can trigger the adaptive immune system; a second phase that constitutes the priming of the immune system, where autoantibodies start to appear in circulation; a third subclinical arthritis phase where arthritic signs and symptoms begin to present; and a last clinical inflammatory phase where arthritis disease is established, and chronic tissue destruction takes place (47) (**Figure 1**).

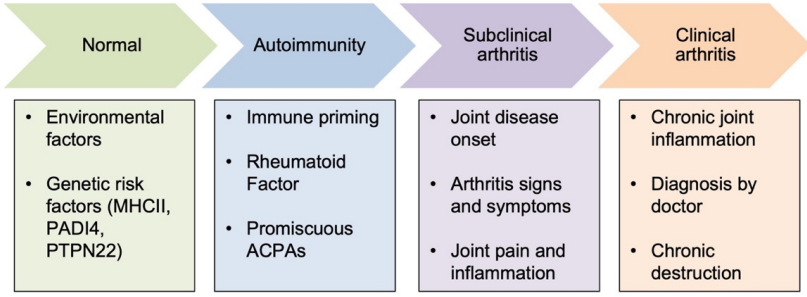

Figure 1. RA phases and characteristics. Four stages are illustrated: normal, priming, subclinical arthritis, and chronic inflammation. The arrows indicate age advancement in an individual's life. Adapted with permission from Springer Nature. Ge and Holmdahl 2019 (48).

2.3.2 Diagnosis and Treatment

ADs are diagnosed based on laboratory tests and clinical manifestations, however, since different ADs share many common features, such as the presence of circulating autoantibodies, a proper diagnosis for each specific disease is typically difficult. Genetics and epigenetics play an important role in circumventing this problem, helping to predict early biomarkers, which in turn makes a better treatment possible (49). Today, RA diagnosis is made with a high degree of sensitivity and specificity. The presence of autoantibodies in the blood is one of the best RA hallmarks for its diagnosis (33). Among the different types of autoantibodies, rheumatoid factor (RF) is probably the most abundant and common. IgM-RFs bind to the Fc (Fragment crystallizable) region of IgG antibodies, exhibiting affinity maturation as opposed to the low affinity observed in healthy individuals, potentiating antigen trapping and inflammation (50). The second most common autoantibodies are anti-citrullinated proteins/peptides (ACPAs/CCP) that react against post-translationally modified self-proteins by citrullination. The presence of these autoantibodies in the serum can appear many years before the onset of the disease, making them excellent and sensitive predictors of disease susceptibility (51,52).

Even though there is no cure for RA, advances in disease treatment have improved over the years, improving the quality of life of RA patients. Previously, patients were treated with aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), which reduced the swelling of the joints and consequently the pain (53). The usage of such medications impacts however the entire immune system, lacking specificity and often presenting with adverse side effects. With a wider range of treatments in the market, the regimen usually begins with the administration of disease-modifying anti-rheumatic drugs (DMARDs), typically methotrexate (MTX), in combination with lower doses of corticosteroids to reduce inflammation and pain. There are, however, some patients that do not respond to MTX, which gave rise to the development of biological DMARDs, that consist in molecules that are antibodies or proteins that target pro-inflammatory cytokines such as the TNF-α, IL-1, or JAK inhibitors (54,55). Other biological DMARDs act in specific cells, the case of rituximab (anti-CD20) that depletes B cells, albeit, it has low efficacy in chronic RA (56). Some of the available treatments for RA at present are specified in **Figure 2**.

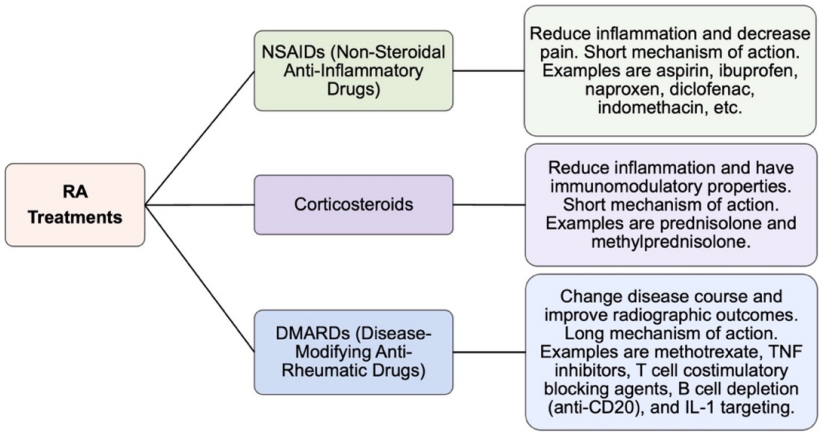

Figure 2. RA treatments. There are three classes of drugs generally used to ameliorate RA symptoms: NSAIDs, Corticosteroids, and DMARDs. They have different mechanisms and times of action.

2.3.3 Genetics and Genome-Wide Association Studies

RA has a strong genetic component associated with it (around 60%), as determined by the twin studies in a British and Finish cohort (57,58). This underscores the importance of elucidating the arthritis-prone genes, for better diagnosis, prognosis, and efficient therapies. Genome-wide association studies (GWAS) support the finding of many genetic loci that could be associated with RA or other ADs (59). Presently, more than 100 risk loci have been linked to RA and functional studies are under extensive investigation. The first locus to be identified was the MHCII, Human Leukocyte Antigen (HLA) in humans, in the late 1960s (60,61). This locus harbors different genes that are responsible for antigen presentation and therefore is involved in the presentation of arthritogenic peptides to the T cells. These findings have been supported by animal models that show the importance of certain MHCII haplotypes for collagen-induced arthritis (CIA) development (A^q and not A^b haplotype). The A^q MHCII molecules can present peptides originating from the collagen type II (COL2) protein to T cells (62). In fact, the immunodominant T cell epitope $COL2_{260-270}$ has also been associated with humans (HLA-DRA4 and DR1) (63). Many years after the discovery of MHC-locus, another locus was identified as a risk factor, containing the peptidyl arginine deiminase type 4 (*PADI4*) gene, responsible for citrullination, a post-translational modification process, recognized by ACPAs, an RA hallmark. A polymorphism in the protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), an important negative regulator of TCR signaling, was found to be associated with not only RA but also other ADs. This SNP affected not only T but also B cell responses (64,65). Interestingly, it has been shown that PTPN22 is redox-regulated and contributes to the development of autoimmune arthritis (66). Many other important loci containing genes that are of

potential importance for immune responses, such as *CD28* (67), cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4)* (68), and *CD2* (69) (reviewed in (70)) have been associated with RA.

2.3.4 Arthritis Animal Models

RA is a complex, multifactorial, and multigenic disease, making it difficult to be extensively studied in humans due to different genetic backgrounds and lifestyles. Research animals are a great solution to circumvent these problems (71,72). Today there are a wide variety of animal models, each one of them reproducing different human RA phases (**Figure 3**). Various animal species can be used to mimic human RA, such as rodents, monkeys, and dogs, however, the most used are rodents (mice, rats). Rodent models are generally low-cost, have controlled and homogenous genetic backgrounds, are relatively easy to handle, and reproduce very fast. Arthritis can be induced by using chemical agents and different RA autoantigens, and the disease progression and pathogenicity usually resemble human features quite well as the animals develop swelling joints/paws, reflecting the recruitment and infiltration of various immune cells to the synovium, triggering an RA-like disease (73).

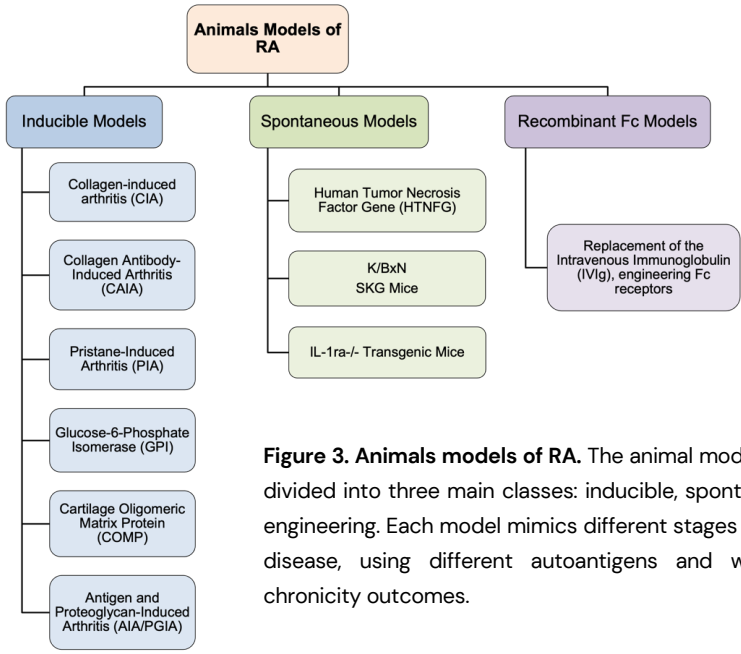

Figure 3. Animals models of RA. The animal models for RA are divided into three main classes: inducible, spontaneous, or Fc engineering. Each model mimics different stages of the human disease, using different autoantigens and with different chronicity outcomes.

The CIA model is one of the most common inducible models utilized since it resembles human disease as the animals develop severe synovial inflammation, bone erosion, and cartilage destruction. It consists of an intradermal injection (i.d.) of heterologous COL2 (e.g., bovine/rat/chicken) together with an adjuvant, typically Complete Freund's Adjuvant (CFA) in susceptible strains like the B10.Q (62,74). This model is B and T cell-dependent,

dominated by Th1 and Th17 cells, providing help to the B cells that in turn produce pathogenic autoantibodies (75).

Collagen antibody-induced arthritis (CAIA), on the other hand, represents the chronic phase of human RA. It consists of the injection of a cocktail containing monoclonal pathogenic anti-COL2 antibodies, and lipopolysaccharides (LPS) are used as a disease/severity enhancer. Arthritis signs usually appear a few days after antibody injection. It is B and T cell-independent model, relying on immune complex formation and classical complement pathway activation (76,77).

Pristane-induced arthritis (PIA) consists of the injection of a well-defined synthetic oil, pristane, and resembles human RA as joint destruction, inflammation, and pannus formation are observed in both rat and mouse models, however, the onset and severity of disease development are slightly different in these two rodent species. This is one of the very few models where T cells alone can transfer the disease to unimmunized rats (78).

Glucose-6-phosphate isomerase (GPI), an enzyme essential for the energy cycle with glycolytic activity, has been suggested as an autoantigen in RA since it can induce arthritis in animal models injected with the human GPI protein or a specific peptide ($hGPI_{325-339}$). This model is both B and T cell-dependent and highly associated with specific MHC haplotypes (79,80).

Another advantage of animal models is that they can easily be genetically modified. The generation of gene knockouts or transgenic/congenic strains makes it possible to study the role of different genes in different diseases, sometimes resulting in the generation of spontaneous models of arthritis that can be achieved naturally without the injection of proteins or chemicals (81). This is the case of human TNF-α transgenic mice that develop arthritis-like signs, mainly driven by synovial fibroblasts than immune cells (82); IL-1R antagonist mice models, that generate anti-COL2 antibodies (83); the K/BxN mouse model, achieved by the crossing of a transgenic TCR with nonobese diabetic mice (NOD) (84); or the SKG mice that result from a natural mutation in ZAP70 (85) (Figure 3). Animal models are also very useful for the testing of new treatments and therapies that can potentially be translated to humans and ameliorate disease symptoms and outcomes.

In fact, it was using animal models (in this case rats), that the *Ncf1*, one of the genes responsible for regulating autoimmune arthritis was discovered (86). This gene is part of the NOX2 complex and is crucial to produce reactive oxygen species (ROS).

2.4 Reactive Oxygen Species

2.4.1 What are they and their sources?

Reactive oxygen species (ROS) are chemical species that contain oxygen atoms, these being extremely unstable due to their lack of electrons. ROS include superoxide (O_2^-), peroxides (such as hydrogen peroxide, H_2O_2), hydroxyl radicals (OH), nitric oxide (NO), and hypochlorous acid (HOCl). Oxygen acquisition of electrons from other molecules (reduction) results in the production of superoxide, the precursor of other reactive species. The enzyme superoxide dismutase (SOD) catalyzes the superoxide to H_2O_2, which is then easier converted into hydroxyl radicals, oxygen, or fully reduced to water by catalases and glutathione peroxidases. SOD is an important antioxidant enzyme for the cells that are exposed to oxygen and has three forms in mammals: SOD1, SOD2, and SOD3, found in the cytoplasm, mitochondria, and extracellularly respectively. Biologically, ROS are important mediators of cell and calcium signaling cascades, and are crucial for clearing foreign pathogens, being induced as an antimicrobial defense. In fact, patients diagnosed with chronic granulomatous disease (CGD), which is mainly characterized by a systemic deficient ROS production, are more susceptible to a wide range of pathogens such as *Salmonella*, *Staphylococcus,* and *Aspergillus* spp (87). ROS can also be overproduced, usually under certain environmental conditions (e.g., heat, UV light, stress), leading to dramatic changes at the cellular level, which can result in their loss of function and eventually apoptosis or cell death. Therefore, physiological levels of ROS are important for the good maintenance and viability of the cells, in which the Nrf2-Keap1 pathway has a fundamental role by promoting an antioxidant transcription program (88).

A wide range of biochemical reactions originates ROS. Mitochondrial respiration is the classical one, generating ROS as a byproduct of ATP production (adenosine triphosphate). NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 2 (NOX2) is the second source responsible for the generation of ROS within a cell (89). Initially described as the enzyme responsible for pathogen killing in leukocytes (90), it is today known that the NADPH oxidase is a membrane-bound enzyme that plays an important role in virtually all cell types (91). The NOX2 complex is responsible for the reduction of oxygen to superoxide, thus forming ROS, a process known as oxidative burst. There are different isoforms (NOX1-NOX5 and DUOX1 and 2) and although these ROS sources deserve special attention, the NOX2 complex is the focus of this thesis since point mutations in different subunits of this complex have been strongly associated with autoimmunity. ROS derived from the NOX2 complex are important for the clearance of foreign pathogens, especially by phagocytic cells (neutrophils, macrophages), that can produce ROS species readily and efficiently after exogenous stimulation. Loss of function mutations in any protein of this complex lead to ROS deficiency and eventually life-threatening infections. The NOX2 complex is composed of several subunits, among them:

- NCF1, NCF2, and NCF4 (Neutrophil Cytosolic Factor 1, 2, and 4; p47phox, p67phox, and p40phox, respectively). NCF1 and NCF4 are the focus of this thesis, and their partner proteins are depicted in **Figure 4A**.
- CYBA and CYBB (cytochrome b558 alpha subunit or p22phox, and beta subunit gp91phox, respectively).
- Rho GTPase RAC.

The different subunits of the NOX2 complex are present in the cytosol. Upon exogenous stimulation, they get phosphorylated and migrate to the membrane, a process by which the NOX2 releases different reactive species (**Figure 4B**).

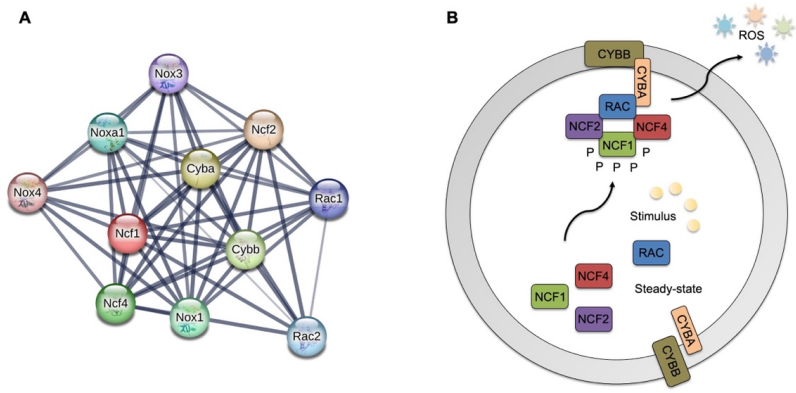

Figure 4. NOX2 complex subunits and conformation. (A) Predicted physical partners of NCF1, protein-protein interaction network obtained from STRING platform (92). **(B)** Structural interactions of the different subunits of the NOX2 complex, at a resting state and upon exogenous stimulation, which results in the phosphorylation and translocation of the different cytosolic components to the membrane making the complex operational and ultimately resulting in ROS release. Adapted with permission from Elsevier. Zhong *et al.*, 2018 (93).

2.4.2 ROS – Autoimmune Key Players and Immune Modulators

With the inducible arthritis model PIA, the genes responsible for arthritis severity in rats were identified (86). By crossing DA (susceptible) with E3 (resistant) rats and analyzing the entire genome of the resultant progeny, quantitative trait loci (QTL) were examined and their impact on arthritis incidence, onset, and severity was analyzed. Through forward genetics and SNP typing, the *Ncf1* gene was positionally cloned as a causative gene for arthritis susceptibility. DA rats harbored a natural amino acid replacement in the NCF1 at position 153 from threonine to methionine, which resulted in lower oxygen burst that possibly activated arthritogenic T cells (86). This observation changed the conventional belief that ROS accumulation is harmful to the progression of ADs, by exceeding the endogenous antioxidant capacity and triggering oxidative stress. This results in lipid, protein, and nucleic acid oxidation ultimately affecting cells of the targeted organ by

autoimmunity (94). Moreover, oxidative stress can also generate novel autoantigens, intensifying an autoimmune response (95). However, recent studies suggest that ROS may play a regulatory role by preventing the progression of chronic inflammatory responses, but the mechanisms by which ROS could be regulating autoimmunity are still not well understood (96,97). When a spontaneous mutation in the p47phox in mouse models was discovered (98), CIA models further confirmed that the deficit in ROS production due to a mutation in the NCF1 and consequently low ROS production was responsible for the increased arthritis severity and experimental autoimmune encephalomyelitis (EAE) (99). In both studies, T cells seem to be the most affected by the absence of functional ROS production. T cells have little oxidative burst capacity, but they are in close contact with different APCs during their life, where APCs are the major producers of ROS. To pinpoint which cells could be the most affected by mutations in the NCF1, conditional mouse models have been generated allowing restoration of ROS in specific cell types. Restoration of ROS in a macrophage-specific manner showed a profound effect on T cell responses, regulation, and differentiation (100,101). The CAIA mouse model (T and B cell-independent), has also been shown to develop increased arthritis in the absence of functional NCF1, suggesting a role of ROS independent of T and B cells (102). Moreover, the GPI peptide-induced arthritis ($hGPI_{325-339}$) is also aggravated in the presence of the *Ncf1* mutation (103). A recent study showed, using an inducible *Ncf1* knock-in model, the time window in which ROS play a role. Restoration of ROS levels before COL2 immunization led to decreased arthritis development and although through a different mechanism, restoring ROS after immunization also improved protection against arthritis, which points to the fundamental and potential regulatory role of ROS in controlling CIA (104).

NCF1 has however not been detected in GWAS due to its copy number variation (CNV) in the human genome, contrary to rodents (105). Nevertheless, in 2012, Jacob and colleagues performed elegant bioinformatics studies and through SNP typing of affected families they identified an association between NCF2 and SLE, both in childhood and adults (106). Based on the findings in the rat model a systematic search for SNPs among the NCF1 copies was initiated and through exon sequences, several SNPs could be identified. One of these (NCF1-339) led to amino acid replacement and reduced ROS induction capacity in similarity with the rat SNP. This SNP had a high allelic frequency and was found to be associated with SLE (107). Since then, CNV and another SNP in the NCF1 ($NCF1^{R90H}$) protein have been associated not only with SLE but also with RA (108), and potentially with a wide range of other autoimmune diseases. The SNP causing the $NCF1^{R90H}$ mutation has been introduced in the mouse, by several research groups, reproducing the susceptibility to lupus (109–111). Moreover, in **paper II** we discovered that a deficiency in NOX2-derived ROS caused by a point mutation in the NCF4 protein resulted in increased plasma cell formation, which led to increased autoantibody production and rendered animal models more vulnerable to CIA (112). Overall, important data has emerged in the past few years,

showing that ROS might play an important role as immune regulators in autoimmunity (96,97), currently investigated as a potential therapeutic approach (113). Therefore, its mechanisms of action in different types of cells deserve our attention.

2.5 B and T Cells

2.5.1 Identity, Development, and Maturation

B and T cells were previously believed to be a single type of lymphocyte. In 1961, Jacques Miller discovered T cells while studying mouse models of lymphocytic leukemia. He found that mice that had their thymus removed could not recognize foreign pathogens or fight infections (114). Max Cooper together with Robert Good and Raymond Peterson, inspired by the findings of Jacques Miller discovered B lymphocytes while studying the human syndrome Wiskott-Aldrich. This disease is characterized by few lymphocyte numbers but high levels of plasma cells and antibodies which were back them thought to derive from T cells. Using chicken, they discovered that when removing the Bursa of Fabricius (therefore B cells), there was a complete ablation of plasma cells, antibodies, and germinal centers, however, their thymus remained intact (115,116).

B cells derive from hematopoietic stem cells (HSCs), which become multipotent progenitors, and later common lymphoid progenitors (CLPs). From here on, these cells go through rearrangement of their heavy and light chains as well as the variable region, a process known as VDJ recombination. This results in the formation of a pre-B cell receptor (BCR) that is then tested for functionality and autoreactivity which decides the fate of a B cell, continue, or cease development (117). T1 immature B cells then migrate from the BM to the SLOs such as the spleen to fully develop and mature. In the spleen, they become T2 transitional B cells that will differentiate into follicular (FO) or marginal zone (MZ) B cells, depending on the signals received. In the SLOs, B cells are constantly exposed to antigens that circulate in the lymph (118). If B cells recognize the antigen they will be activated, a process that could be T cell-dependent, mostly conducted by FO B cells; or T cell-independent, preferentially driven by B1 and MZ B cells, involving extrafollicular responses and generating less efficient immune response compared to the former (119).

T cells are specialized lymphocytes that such as the B cells have origin in HSC in the BM, where they differentiate into multipotent progenitors and later in CLPs which will originate B, T, or NK cells. These CLPs will migrate to the thymus to rearrange their TCR and become mature. T cells are a fundamental component of the adaptive immune system and have a close relationship with B cells for mounting a proper immune response. There are different types of T cells: helper, cytotoxic, memory, and regulatory (120).

2.5.2 B and T Cell Dysregulation and Autoimmunity

B cell selection is a tightly controlled process, consisting of different checkpoints that occur both in the BM as well as in the periphery, to avoid undesired reactivity against self-antigens. A B cell's major goal is the production of high-affinity and specific antibodies against extrinsic antigens without the destruction of self. Apoptosis during clonal deletion, receptor editing, anergy, or ignorance are the processes that a B cell can go through if autoreactivity is detected (121). Improper activation of autoreactive B cells or inhibition of regulatory B cells can occur, resulting in an unbalanced B cell activation/proliferation, which can result in the production of pathogenic autoantibodies triggering autoimmunity. Interestingly, in **paper V** we discuss a new function for autoreactive B cell in the context of RA. Autoantibodies, the result of the adaptive immune system, are directed to self-tissues causing their destruction. Even though their role is well accepted in the scientific community as the responsible agent of many autoimmune diseases, their specific function remains to be clarified in many human disorders (122). High levels of circulating autoantibodies, mainly RF and ACPAs are detectable in the serum years before the clinical evaluation of RA making B cells an important cell type to be investigated (123).

B cells are not only antibody-producing cells but also efficient APCs, presenting antigens to the T cells in an MHCII-restricted manner to generate an efficient immune response. If B cells are autoreactive, they will present autoantigens to the T cells which can lead to its activation and contribution to autoimmunity development (124). T cells on their hand also need to go through central and peripheral tolerance mechanisms to check for autoreactivity. Poor regulation by the autoimmune regulator (AIRE) or lack of some tissue-specific genes in the thymus, might let autoreactive T cells escape during thymic selection. In the periphery, these T cells can recognize self-peptides and become activated if they receive proper signals for that. This in turn will activate B cells and trigger autoantibody responses (125).

2.5.3 NCF1 in B cells

B cells are non-phagocytic cells, but they also have the NOX2 complex, and therefore NCF1 and other subunits of this complex at different amounts in different life stages (**Figure 5**). They can produce and release ROS upon BCR engagement (126); however, the exact mechanisms of how ROS operates in B cells remain elusive. It has been shown that mice protected against CIA (127), had this resistance wrecked when a systemic point mutation in the *Ncf1* gene was introduced. This caused enhanced germinal center (GC) formation, pronounced T cell responses, and epitope spreading. This fact pointed to the important role of ROS in B cells (128), which was explored in **papers III** and **IV**. Altogether and considering the important role of B cells in autoimmune diseases as antibody-producers and antigen-presenting cells, it is important to clarify if defected B cell-derived

ROS affects other cell types (T cells, macrophages) and how in turn this affects the development of RA, for example.

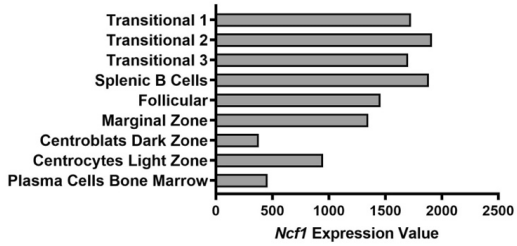

Figure 5. *Ncf1* expression in different B cell subsets. Data extracted from the Immunological Genome Project (ImmGen) (141).

2.6 Germinal Centers

Germinal centers (GCs) were first described by the German biologist Walter Flemming in 1884 as anatomical structures where mitosis of large lymphocytes occurs (129). These are sites where oligoclonal B cells gain high specificity towards a T cell-dependent antigen by mutating their antibody genes, a process known as somatic hypermutation (SHM) and class-switch recombination (CSR), where the enzyme activation-induced cytidine deaminase (AID) plays an important role (130). GCs are formed in secondary lymphoid organs such as inguinal lymph nodes (iLNs) or the spleen after B cells encounter a certain antigen and become activated. In the GCs, B cells endure intense clonal expansion, and isotype switching of their Ig classes, ultimately undergoing different rounds of SHM or exiting the GCs as memory B cells or LLPCs. GCs are highly compartmentalized structures and are commonly divided into two different sites; the dark zone (DZ), where B cells (centroblasts) hyper proliferate and undergo SHM after proper antigen recognition and T cell help; and the light zone (LZ), where follicular dendritic cells (FDCs) present the antigen to B cells and only the ones capable of binding to it with high specificity will receive help from T follicular helper cells (Tfh) and will be rescued from cell death through apoptosis (131,132). A bunch of cytokines such as IL-6 and IL-21 are also important during a GC reaction, helping in the control of the Ig class switch as well as B cell fate after exiting the GC. Interestingly, GC-B cells also express *Ncf1*, which was explored in **paper IV**. This part of the adaptive immune system enables the host to mount long-lived responses to certain foreign pathogens, and it is through GCs that vaccines also create a memory. T cell help is crucial for the tolerance to self-antigens. If no help is given from T cells, the B cells with self-specificity (autoreactive and probably autoimmune) will be eliminated. Importantly, the formation of GCs is essential for producing pathogenic B cells critical for the development of CIA (133). Over the past few years, our knowledge about GCs has increased significantly, and many studies are pointing toward its correlation with the development of arthritis, not only in animal models (134) but also in RA patients (135,136).

3 Research Aims

This thesis focuses on B cells, germinal center B cells, and reactive oxygen species (ROS) in the context of the autoimmune disease rheumatoid arthritis (RA).

To understand the role of ROS in B cells we used two subunits of the NOX2 complex harboring a point mutation, the *Ncf1,* and *Ncf4*, but we also developed new mice strains, allowing the manipulation of ROS production in a B or germinal center B cell-specific manner.

Lastly, using a collagen-specific epitope knock-in mouse model, we characterized a new subset of B cells that have a suppressive phenotype.

The aims of each individual study were:

Study I – To study the role of αGalCer and iNKT cells in murine models of inflammation and autoimmunity, as B cell helpers or regulators of autoreactive B cells.

Study II – To study the effect of a point mutation in the *Ncf4* subunit of the NOX2 complex and its consequences in B cells and arthritis mouse models.

Study III – To study the role of NCF1 restored to similar levels of those of the wild-type in B cells specifically using the mouse model BQ.*Mb1-Cre.Ncf1*$^{*/*}$.

Study IV – To study the role of NCF1 restored to similar levels of those of the wild-type in GC-B cells specifically. This study is very similar to study III, however here we used the BQ.*AID-Cre.Ncf1*$^{*/*}$ mouse model that allowed ROS restoration in cells that express *Aid*, mainly prior to and during a germinal center reaction.

Study V – To study antigen-specific B cells and their protective role in autoimmunity since such cells are mostly regarded as pathogenic.

4 Materials and Methods

4.1 Ethical Considerations

For all the studies described in this thesis animal models were used. Therefore, ethics should be discussed and properly considered. RA is a multifactorial multigenic disease, with environmental and genetic factors involved in its onset. The use of animal models is essential to dissect the mechanisms behind the disease, however, this leads to various ethical issues. Is it morally correct to use small animals in the name of science? Personally, I think no researcher likes to use animals for research, but we know how precious they are to answer scientific questions. We could of course obtain human samples; however, humans are very heterogenous, which at the first line of research makes the results difficult to interpret. But what makes animals more suitable for this purpose? Are we superior beings that just decide they can be used? Do animals have fewer moral rights than humans, or should they be equal? But with these questions, a more important one rises: would the scientific field be as advanced as it is nowadays without the usage of animals during the past few years? Would we have found that animals have "feelings/emotions" if we would not perform experiments with them before? Many small things that we know today come from research animals and we cannot deny that we have developed valuable information with them. This is why we have so tight rules when it comes to the use of laboratory animals. We cannot do whatever we want with them, without a purpose and without respecting them. Ethical committees, permits, and considerations play a crucial role. Also, nowadays as science advances other models are being developed and with time we might be able to control and research many more things with computers (modeling) for example rather than using animals. And even when we apply for such ethical permits to perform our research, questions like: could this be addressed using small organisms? Could cell lines for instance be used instead of real/complete animals? The 3R's rule comes up here: reduction, refinement, and replacement, and this rule need to be followed and justified when researchers apply for ethical permits. I think it is important to not only follow ethical permits but also use our awareness, that is what distinguishes us from animals, our intellectual level. We do need to treat these animals correctly, cause as less pain as possible and not use them without a purpose. Some people also argue that such animals are born with scientific determination, they are born to die. I do not think that is an excuse. They are born with this specific goal, but this can't allow us to do whatever we want with them. Overall, I think there are no correct answers to such issues, and we will not stop using animals any time soon. It is fundamental to be as human and responsible as possible and always try to consider the interests and welfare of the animals, considering the different available options and taking good care of them.

4.2 Genetics of the Animal Models

The strains used in the studies of this thesis were: BQ.*Ncf1*^{m1j} (99), BQ.*Mb1-Cre*$^{+/-}$.*Ncf1*^{m1j} (137), BQ.*AID-Cre*$^{+/-}$.*Ncf1*^{m1j} (138), BQ.*TN3* (104), BQ.*ACB* (127), BQ.*ACB.Ncf1*^{m1j} (128), BQ.*ACB.R360Q*, BQ.*QCII24* (139), BQ.*ACB.QCII24,* and BQ.*ACB.Mb1-Cre*$^{+/-}$.*Ncf1*^{m1j}. For several generations, they were crossed with C57BL/10.Q (hence named BQ) to introduce the MHC class II H2-A^{q} that licenses arthritis development (62). Age and sex-matched littermates were used in all experiments. Mice were housed in the KMA facility (Karolinska Institutet, Stockholm) under SPF conditions. All animal experiments were approved by the local ethical committee under the permit N35/16 or 2660-2021 and performed accounting for the animal welfare and humane endpoints. Animals were anesthetized by isoflurane breathing and sacrificed with CO_2.

4.3 CIA - Collagen-Induced Arthritis Model

For all the studies, 8 weeks old animals were immunized intradermally (i.d.) at the base of the tail with 100 μg of bovine collagen type II (COL2) in 0.1 M of acetic acid, emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, IL, USA). Boosting was conducted on day 21 or day 35 with 50 μg of COL2 in incomplete Freund's adjuvant (IFA; Difco, Detroit, IL, USA). Clinical scoring was performed for up to 90 days and wound formation from immunization was monitored. In brief, each inflamed toe or knuckle scores one point, whereas an inflamed wrist or ankle scores five points, resulting in a maximum score of 15 (five toes plus five knuckles plus one wrist/ankle) for each paw and 60 points maximum for each mouse. Mice were scored two or three times per week, except during the first 25 days when they were scored once a week.

4.4 Anti-COL2 Antibody Titers - ELISA

Mice were anesthetized and bled from the sub-mandibular vein at the indicated time points post-COL2 primary immunization. Blood was centrifuged at 12.000 rpm, the serum was harvested and used for ELISA to detect anti-COL2 antibodies. Briefly, bovine COL2, used for immunization, was diluted in PBS to a final concentration of 10 μg/mL; 50 μL were added to a 96-well MaxiSorp plate and incubated overnight at 4°C. Plates were blocked with 1% BSA for 1 hour at room temperature. Serum samples typically diluted 1:1000 or 1:10.000 in PBS were incubated at room temperature for 2 hours. Detection antibodies HRP coupled were diluted 1:4000 (Southern Biotech) in PBS and samples were developed either with ABTS reading the absorbance at 405 nm or TMB reading the absorbance at 450 nm.

4.5 Organs Single-Cell Suspensions

Single-cell suspensions were obtained from the spleen, lymph nodes, and bone marrow. Cells were spun down 300g for 8 minutes. When necessary red blood cells (RBCs) were lysed with ammonium chloride-potassium (ACK) buffer (homemade) for 5 minutes on ice. Cells were washed with PBS, counted on a Sysmex KX-21 cell counter, and incubated with 10 µg/mL Fc-block (2.4G2, homemade) for 10 minutes at room temperature (RT) followed by 30 minutes of surface markers staining at 4°C. For intracellular staining, cells were stained with surface markers followed by a fixation/permeabilization with a kit (BD Bioscience). For intranuclear staining eBioscience Foxp3/transcription factor fixation/permeabilization kit (Thermo Fisher Scientific) according to the manufacturer's recommendations.

4.6 Flow Cytometry and Antibodies Used

All the antibodies used in the studies of this thesis were purchased from BD Bioscience, Biolegend, or eBioscience. Anti-mouse/human antibodies and clones used were the following: anti-CD19 (1D3), anti-CD45R (B220, RA3-6B2), anti-IgM (II-41), anti-IgD (11-26c.2a), anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-H-2, I-A/I-E (MHCII) (2G9), anti-CD138 (281-2), anti-GL7 (Ly-77), anti-CD95 (15A7), anti-CD184 (CXCR4) (L276F12), anti-CD86 (GL1), anti-CD38 (HIT2), anti-Ki-67 (16A8), anti-Ly-6A/E (Sca-1) (D7), anti-pSTAT3 (pS727, 49/pSTAT3), anti-STAT3 (M59-50), anti-ICOS (C398.4A), anti-Bcl6 (7D1), anti-CXCR5 (L276F12), anti-PD-1 (29F.1A12), anti-CD49b (DX5), anti-Lag3 (C9B7W), anti-pSYK (I120-722), and anti-human CD2 (RPA-2.10). Dead cells were excluded using a fixable near-IR dead cell stain kit (Thermo Fisher Scientific, catalog number L10119). Samples were acquired with Attune NxT flow cytometer (Thermo Fisher Scientific) and analyzed with FlowJo version 10.7.2.

4.7 Intra and Extracellular ROS Measurement

For intracellular detection of ROS in **studies II, III**, and **IV**, cells were incubated with 3 µM of dihydrorhodamine (DHR) 123 (Invitrogen) resuspended in incomplete DMEM for 10 minutes at 37°C and stimulated with 200 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 20 minutes at 37°C. The geometric mean fluorescence intensity (MFI) of Rhodamine 123 was determined using the BL-1 FITC channel in the Attune NxT flow cytometer. Extracellular ROS measurement in **study II** was done by chemiluminescence assay. Cells were stimulated with 100 ng/mL of PMA for 60 minutes in HBSS solution (Thermo Fisher Scientific) with 150 µM isoluminol (Sigma-Aldrich) and

18.75 units/mL of HRP-II (Sigma-Aldrich). Data was measured in relative light units (RLUs).

4.8 B and GC-B cell Isolation, and *in vitro* Plasma Cell Differentiation

For **studies II, III, IV,** and **V**, CD19+ B cells or untouched mouse B cells were isolated using magnetic-activated cell sorting (MACS) following the manufacturer's recommendations (Miltenyi Biotec, order no. 130-052-201 and 130-090-862, respectively). Briefly, spleen cells were resuspended in MACS buffer (containing 2% FBS and 2 mM EDTA) and incubated with CD19+ MicroBeads for 15 minutes at 4°C or biotin antibodies targeting all no B cells for 5 minutes at 4°C. After that CD19+ B cells were passed through a pre-rinsed LS column and untouched B cells were incubated with anti-biotin beads for 10 minutes at 4°C and passed through a pre-rinsed LS column. For **study IV**, mouse GC-B cells were MACS isolated (Miltenyi Biotec, order no. 130-110-479). Briefly, inguinal lymph node cells from 3 pooled immunized mice were resuspended in MACS buffer and incubated with PNA-biotin antibodies for 5 minutes at 4°C. After that anti-biotin beads were added for 10 minutes at 4°C, and cells were passed through a pre-rinsed LS column. The positive fraction containing the GC-B cells was eluted in 5 mL MACS buffer, spun down, and resuspended in MACS buffer. GC-B cells were then stained with Live Dead Dye, CD19, B220, IgD, CD38, and GL7, and sorted by fluorescence-activated cell sorting (FACS).
For *in vitro* plasma cell differentiation, untouched B cells were cultured in 96-well plates (Sarstedt) in complete RPMI media (10% FBS and penicillin/streptomycin) supplemented with 10 µg/mL lipopolysaccharides (LPS, Sigma), at 37°C and 5% CO_2 incubator. After 3 days supernatants were collected for antibody secretion (IgG, IgM) and cells were subjected to flow cytometric analysis.

4.9 SDS Page and Western Blot

CD19+ B cells were lysed in 100 µL of RIPA buffer (Thermo Fisher Scientific), freshly supplemented with protease inhibitors (Roche). The total amount of protein was quantified using a Pierce BCA kit (Thermo Fisher Scientific). SDS-PAGE (NuPAGE 4-12% Bis-Tris Gel, Thermo Fisher Scientific) run in MOPS buffer for 50 minutes at 200V. PVDF membrane (Millipore) was used for blotting, 1.5 hours at 35V. Membranes were blocked with BSA 5%, RT, 1 hour. Anti-mouse primary antibodies were incubated overnight at 4°C and secondary HRP-coupled antibodies for 1 hour at RT. Membranes were washed and developed with ECL substrate (GE, Healthcare) on a ChemiDoc XRS+ (Bio-Rad).

4.10 ELISpot

For T cell recall assays, $1x10^6$ cells/well from inguinal lymph nodes were plated in anti-IFN-γ (AN18, 10 μg/mL, in-house produced), anti-IL-17A (TC11-18H10.1, 5 ug/mL, in-house produced) and IL-2 (10 μg/mL, 1A12, Mabtech) coated plates (Merck Millipore, #MSIPS4W10), stimulated with native peptide (25 μg/mL) or gal peptide (GalHyK264) (25 μg/mL) and incubated for 24 hours at 37°C. Bound cytokines were detected with biotinylated anti-IFN-γ (R46-A2, 4 μg/mL, in-house produced), anti-IL-17A (TC11-8H4, 1 μg/mL, in-house produced), IL-2 (5 μg/mL 5H4, Mabtech) followed by streptavidin-conjugated alkaline-phosphatase. The spots were developed using the substrate BCIP/Nitroblue Tetrazolium (Sigma). Scanned wells (ImmunoScan) were analyzed with ImmunoSpot software (Cellular Technology).

4.11 A^q-Gal Tetramer Enrichment

For studies **III, IV**, and **V**, biotinylated MHCII monomers (A^q-Gal, homemade) were conjugated with Streptavidin-PE (Thermo Fisher Scientific) to form PE-conjugated MHCII tetramers that were used to stain the samples for 1 hour at room temperature. A^q-Gal-PE tetramers were enriched magnetically using anti-PE beads and according to the protocol described in (140).

4.12 RNA Isolation and Gene Expression

For **study III,** RNA was isolated with a RNeasy kit (Qiagen), and for **study IV,** RNA was isolated using Dynabeads mRNA purification kit (Thermo Fisher Scientific), both following the manufacturer's recommendations. cDNA was synthesized using iScript (BioRad) following the manufacturer's recommendations. iQ-SYBR green supermix (BioRad) was used for quantitative real-time PCR amplification of $NCF1^{153M}$ (Fw: ACATCATGGGCCCCATCAT and Rv: CGCTCTCGCTCTTCTCCACAA).

4.13 Statistical Analysis

Graphs were plotted as mean ± SD and statistical analysis were done with GraphPad Prism version 9.0. The Mann-Whitney test was used when comparing two groups and 2-way ANOVA was used when comparing multiple variables. The significance level was set to 0.05 and the p-values are indicated with asterisks (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001).

5 Results and Discussion

5.1 Dual role of invariant natural killer T cells (iNKT) in sterile inflammation and autoimmunity

In **paper I** we investigated the role of iNKT cells in the context of autoimmunity and inflammation. This T cell subset is involved in innate immune responses and can shape B cell responses by providing them help or avoiding autoreactivity.

5.1.1 B cell responses are enhanced in αGalCer-mediated iNKT activation

By combining a model of chronic inflammation by injecting the pro-inflammatory cytokine IL-18 for 10 consecutive days with an injection of the glycolipid alpha-galactosylceramide (αGalCer) on day 1 we evaluated B cell responses. Our results showed increased levels of IgE, IgM, and IgG3 when IL-18 was co-administered with αGalCer, however, total IgG levels were slightly higher in the IL-18 group alone (**Figure 6A**). Double-stranded DNA reactive antibodies were also increased in the IL-18+αGalCer group but not for IgG or IgG1, however the ratio between IgG1 and the pro-inflammatory antibody IgG2b was increased, pointing to expanded B cell responses (**Figure 6B**).

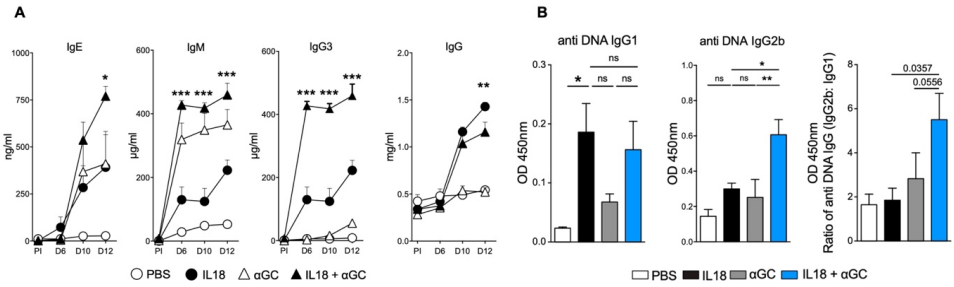

Figure 6. Antibody levels after injection of PBS, IL-18, αGalCer, or a combination of both. (A) Increased levels of IgE, IgM, and IgG3 in the IL-18+αGalCer group, but increased IgG in the IL-18 group alone. **(B)** Increased pro-inflammatory antibody IgG2b in the IL-18+αGalCer group. Figure reused and adapted from (31).

5.1.2 Germinal Center formation is promoted in αGalCer chronic inflammation

It has been implicated that iNKT regulation of B cells happens before a GC reaction. Here we showed that injection of IL-18+αGalCer induced more GC formation than IL-18 or αGalCer alone without affecting plasma cell formation (**Figure 7A**). However, class-switched plasma cells (IgE and IgG1) coming primarily from a GC reaction were increased in the IL-18+αGalCer group (**Figure 7B**), pointing to the regulation of GC formation

accompanied with isotype switch when iNKT cells are activated with cognate lipid antigen together with IL-18.

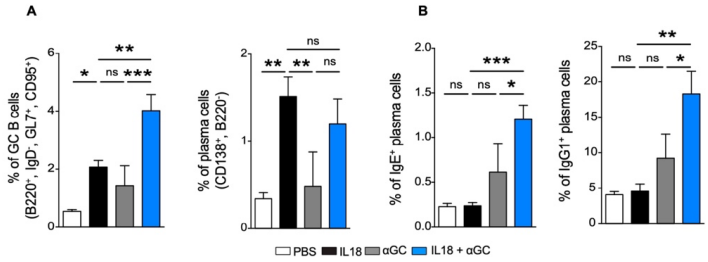

Figure 7. Plasma cell, GC formation, and isotype switching. (A) Increased GC formation with unaffected plasma cells in the IL-18+αGalCer group. **(B)** Increased levels of IgE and IgG1 secreting plasma cells in the IL-18+αGalCer group alone. Figure reused and adapted from (31).

5.1.3 Neutrophil licensing of iNKT is abrogated by IL-18+αGalCer inflammation

It has been shown that neutrophils license iNKT cells to regulate B cell responses. We observed increased neutrophil recruitment in the IL-18 group but even more in the IL-18+αGalCer group (**Figure 8A**). Serum levels of the B cell-activating factor (BAFF), mostly produced by neutrophils, correlated with these findings (**Figure 8B**). Neutrophil licensing of iNKT is regulated by the transcription factor GATA3. When injected with an haptenated form of αGalCer (NP-αGalCer), GATA3 levels were downregulated compared to the IL-18 group, accompanied by decreased levels of Fas ligand (FasL) (**Figure 8C**).

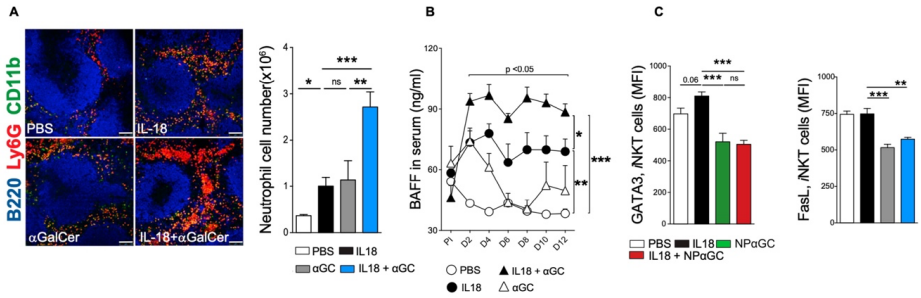

Figure 8. Neutrophil recruitment and GATA3 expression. (A) Increased neutrophil recruitment in the spleen in the IL-18+αGalCer group was visualized by immunofluorescence and flow cytometry cell counts. **(B)** Increased serum BAFF levels in the IL-18 and IL-18+αGalCer groups. **(C)** Decreased GATA3 and FasL. Figure reused and adapted from (31).

5.1.4 iNKTfh cells derived from IL-18+αGalCer provide B cell help

Using the *AID-Cre* mouse model that turns on a green fluorescent protein (GFP) if AID is being expressed, we could see that the IL-18+αGalCer had higher numbers of plasma cells expressing GFP. This mouse model is engineered to express hCD2 when AID is activated. Interestingly, we observed higher numbers of GC-B cells expressing hCD2 in the αGalCer-treated groups, supporting prolonged GC reactions and higher antibody production (**Figure 9A**). To test how αGalCer affected mouse models of autoimmunity we took advantage of CIA. We could see that mice injected with αGalCer developed arthritis scores much faster than the IL-18 group alone, accompanied by increased anti-COL2 antibodies in the sera, and immune cell infiltration in the hind limbs (**Figure 9B**).

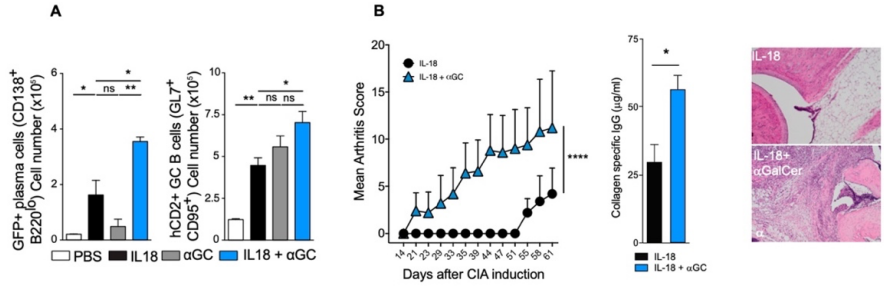

Figure 9. IL-18+αGalCer exacerbates CIA model. (A) Increased GFP+ plasma cells and GC-B cells expressing hCD2. **(B)** Robust development of arthritis in the IL-18+αGalCer group. Figure reused and adapted from (31).

5.2 The role of NCF4 in plasma cells and arthritis

It has been demonstrated that lower levels of ROS affect autoimmunity. In **paper II** we studied the role of a mutated subunit of the NOX2 complex, the NCF4 protein.

5.2.1 *Ncf4*^{R58A} affects intracellular ROS, disease severity, and anti-COL2 antibodies

A mutation in the Ncf4 subunit of the NOX2 complex (*Ncf4*^{R58A}) led to decreased intracellular but not extracellular ROS production capacity in both neutrophils and B cells (**Figure 10A**). A single injection of COL2 led to increased disease severity and incidence rather than with the normal protocol that consists of a COL2 boost 21/35 days post-immunization, accompanied by increased anti-COL2 antibodies (**Figure 10B**).

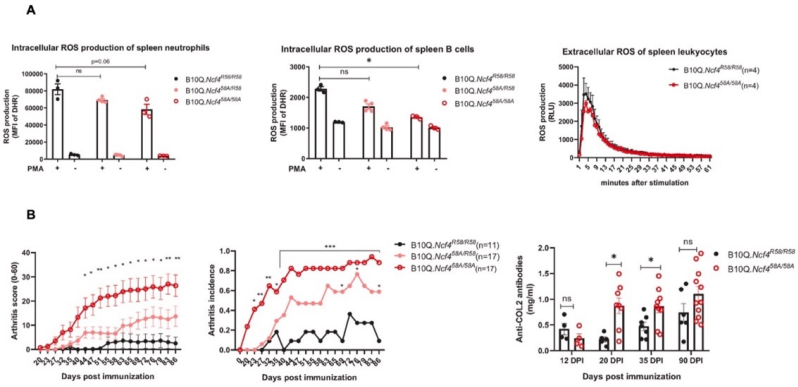

Figure 10. ROS measurement and increased disease severity in the *Ncf4*^{R58A} group. (A) Intra and extracellular ROS production in neutrophils and B cells. **(B)** Disease severity and incidence and anti-COL2 antibody production at different immunization time points. Figure reused and adapted from (112).

5.2.2 T cell responses are unaffected by *Ncf4*^{R58A} mutation while ASCs are increased

Given the increased anti-COL2 antibodies after immunization, we thought this was T-cell dependent. We observed that antigen processing and pro-inflammatory cytokine secretion in a T cell recall assay were not affected by the mutation, as well as the percentage of effector/naïve/memory T cells after immunization (**Figure 11A**). What we could observe instead was increased numbers of ASCs, anti-COL2 antibodies, plasma cells, and LLPCs formation (**Figure 11B**).

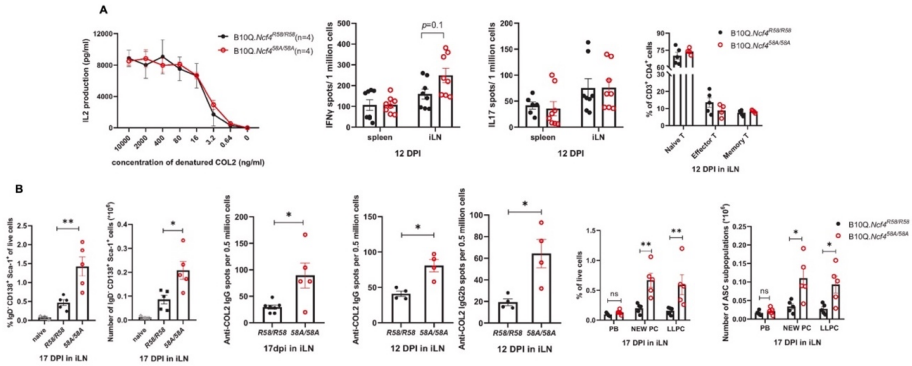

Figure 11. Unaffected T cell responses but increased ASCs and anti-COL2 antibodies. (A) Antigen-presentation, ELISpot of pro-inflammatory cytokines, T cell populations after COL2 immunization. **(B)** ASCs and anti-COL2 antibodies. Figure reused and adapted from (112).

5.2.3 NCF4 regulates ASC formation

Using the μMt mouse model that lacks B cells, we could still see increased anti-COL2 antibody secretion. Moreover, *in vitro*, differentiated B cells to plasmablasts with LPS were increased in the *Ncf4*^{R58A} group accompanied by increased antibody titers (IgM) (**Figure 12A**). CXCR4 was decreased in the absence of ROS and CXCR3 was increased (**Figure 12B**).

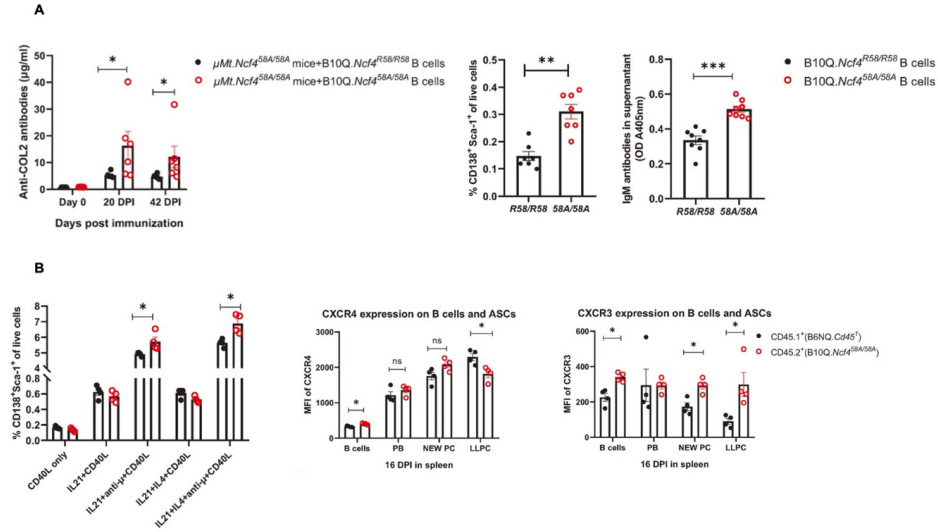

Figure 12. Antibodies and plasma cells. (A) Anti-COL2 antibodies in μMt mice transferred with *Ncf4* wild-type or mutant B cells. **(B)** *In vitro* differentiation of B cells in plasmablasts and CXCR3/4 expression. Figure reused and adapted from (112).

5.3 The role of NCF1-ROS in B cells

From study II we learned how a mutation in the NCF4 protein, part of the NOX2 complex could affect arthritis, but also shape B cell responses, which could in turn explain the disease phenotype. In **paper III** (unpublished data) we investigated the role of ROS restricted to B cells by using conditional mice models harboring a low or a high ROS NCF1 allele.

5.3.1 Expression of the high ROS NCF1^{T153} allele in B cells

To restore the ROS production capacity in B cells only, we used two mice models: one harboring a systemic *Ncf1*$^{*/*}$ mutation and the *Cre* recombinase under the *Mb1* promoter; another one harboring the low ROS NCF1^{153M} and the high ROS NCF1^{T153} alleles (**Figure 13A**) but the latter in reverted orientation, therefore unfunctional. Crossing these two mouse models, resulted in a progeny harboring the high ROS NCF1^{T153} allele restricted to B cells, which could be confirmed by a DHR123 assay (**Figure 13B**) as well as by proteomics (**Figure 13C**).

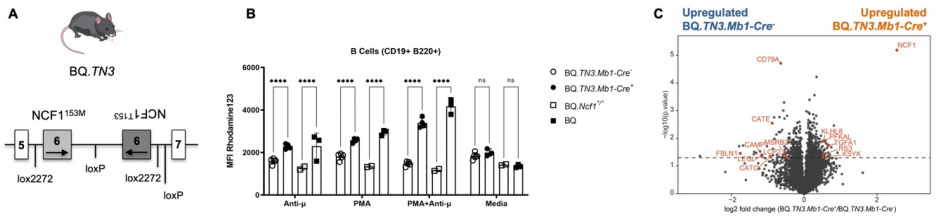

Figure 13. Mice models and their characteristics. (A) Mouse model harboring low and high NCF1 alleles. **(B)** Intracellular ROS measurement of stimulated B cells by DHR123 assay. **(C)** The proteomic landscape of B cells from immunized mice.

5.3.2 B cell-derived NCF1-ROS decreases arthritis susceptibility and antibody production

After confirming ROS restoration in B cells specifically, we wanted to address how this would impact murine models of arthritis. CIA model showed lower arthritis scores and lower anti-COL2 antibodies in mice expressing the high ROS NCF1^{T153} allele (**Figure 14A**). To see if this effect could be transferred, we injected B cells with the different ROS NCF1 alleles into susceptible animals. B cells harboring the high ROS NCF1^{T153} allele protected against arthritis, both 10 and 35 days post-COL2 immunization (**Figure 14B**). Taking advantage of the mouse model described in paper V, which is protected against arthritis but has the protection wrecked when the *Ncf1*$^{*/*}$ mutation is present, we introduced the BQ.*TN3.Mb1-Cre* mutation and could observe that B cell-derived ROS improved disease outcomes compared to littermates (**Figure 14C**).

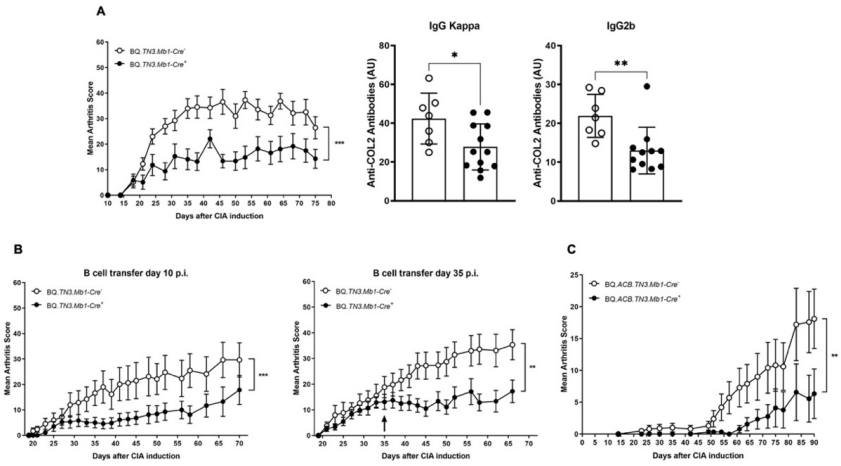

Figure 14. Impact of B cell-derived ROS in CIA mouse models. (A) CIA and anti-COL2 antibodies. **(B)** CIA in BQ.Ncf1*/* susceptible mice transferred with B cells on day 10 or day 35. **(C)** CIA in BQ.*ACB* mice harboring the BQ.*TN3.Mb1-Cre* construct.

5.3.3 T cell responses are profoundly affected by B cell-derived NCF1-ROS

B cells are important antibody-producing cells, but they are also professional APCs, presenting peptides to the T cells via MHCII. We could observe a profound effect in T cell responses, with increased secretion of pro-inflammatory cytokines (**Figure 15A**), but also in COL2 tetramer T cells, however, these cells displayed a regulatory phenotype (**Figure 15B**). Moreover, the proteomic landscape of T cells from immunized animals was also affected, with the SEM4D protein (CD100) being upregulated in the low ROS NCF1^{153M} allele (**Figure 15C**).

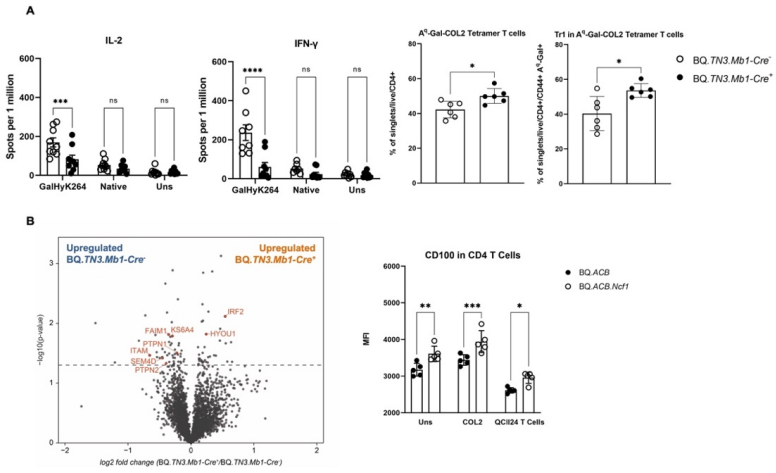

Figure 15. Affected T cell responses from B cell-derived ROS. (A) Pro-inflammatory cytokine production and COL2 tetramer T cells. **(B)** Proteomic analysis of CD4 T cells from immunized mice.

5.4 The role of NCF1-ROS in GC B cells

Given the results from paper III and considering that GC formation is T cell-dependent, in **paper IV** (unpublished data) we sought to investigate the role of NCF1 in GC-B cells.

5.4.1 Expression of high ROS NCF1 allele in GC-B cells

We took advantage of the mouse model harboring the low and high ROS NCF1 alleles but using an *AID-Cre* mouse model instead. We confirmed the hCD2 expression under the AID promoter and (**Figure 16A**) and ROS production by B cells from the BQ.*TN3.AID-Cre*$^{+}$ after stimulation (**Figure 16B**).

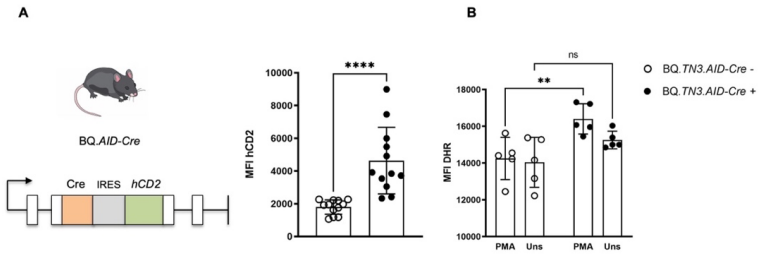

Figure 16. Mouse model and its characteristics. (A) Genetics of the BQ.*AID-Cre* mouse, hCD2 expression in GC-B cells. **(B)** ROS production by B cells after stimulation.

5.4.2 Impact of GC-B cell-derived ROS in CIA and anti-COL2 antibody production

As we observed a dramatic effect in CIA scores in paper III, we were curious to see if restoration of NCF1 in GC-B cells would lead to the same effect. We could observe decreased arthritis signs in mice harboring the high ROS NCF1^{T153} allele in GC-B cells, accompanied by lower anti-COL2 antibodies when compared to mice harboring the low ROS NCF1^{153M} allele (**Figure 17A**). Moreover, in vitro different B cells from this mouse secreted less antibodies (**Figure 17B**).

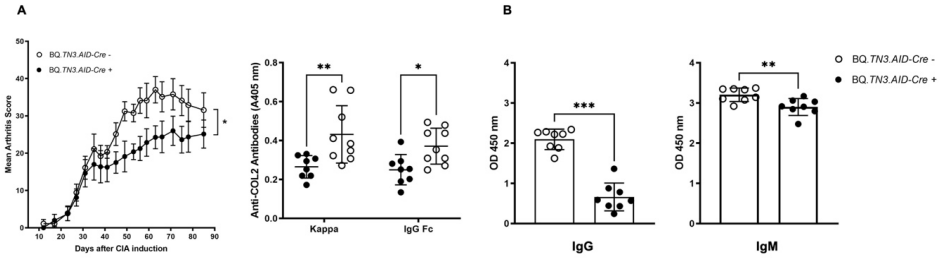

Figure 17. CIA, anti-COL2 antibodies, and general IgG/IgM antibodies. (A) CIA in littermate controls. **(B)** Anti-COL2 antibodies from sera of mice in (A) 15 days post-immunization and *in vitro* IgG and IgM antibody production by B cells stimulated with LPS.

5.4.3 Profound effect in GC formation and T cell responses

Since we observed decreased arthritis signs, with less anti-COL2 antibody production, we thought to evaluate the GC formation as well as plasma cells, as these are the antibody-secreting cells (**Figure 18A**). We could see that GC-B cells are expanded in mice harboring the low ROS NCF1^{153M} allele, but the plasma cell formation remains unaffected. However, like what we observed in paper III, T cell responses were affected. In this case, these were Tfh cells, responsible to provide signals and help to B cells in a GC reaction. These cells had higher expression of the inducible T cell costimulatory molecule (ICOS) as well as phosphorylated signal transducer and activator of transcription 3 (pSTAT3) (**Figure 18B**).

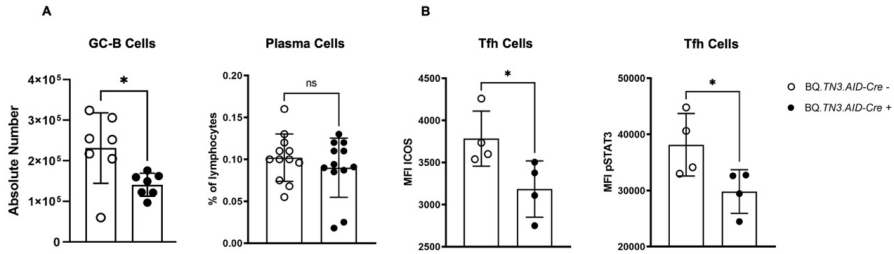

Figure 18. Affected GC formation and Tfh cells. (A) GC-B number after COL2 immunization with no difference in plasma cells. **(B)** Differential expression of ICOS and pSTAT3 in Tfh cells.

5.4.4 The proteomic landscape of GC-B cells is affected by ROS

Since we saw an expansion in GC formation in mice harboring the low ROS NCF1^{153M} allele, we thought to sort GC-B cells after COL2 immunization and analyze their proteome. Proteomic analysis of GC-B cells showed differentially expressed proteins in the absence of functional NCF1, both in the BQ.*TN3.AID-Cre*$^{+/-}$ mice models (**Figure 19A**) as well as in the BQ and BQ.*Ncf1*$^{*/*}$ (**Figure 19B**). Importantly, the NCF1 protein was upregulated in the BQ.*TN3.AID-Cre*$^{+}$, showing proper construction of the animal model.

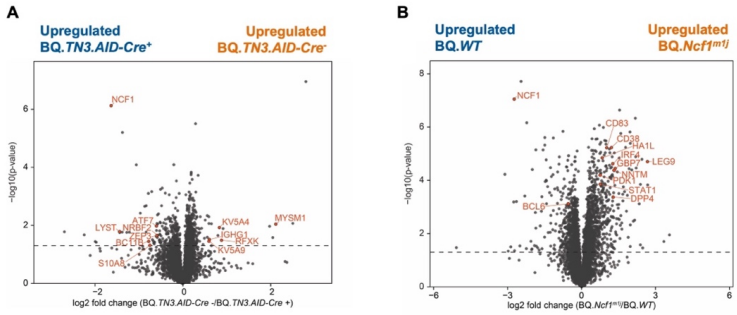

Figure 19. Affected proteomic landscape in GC-B cells after COL2 immunization. (A) GC-B cells from BQ.*TN3.AID-Cre*$^{+/-}$. **(B)** GC-B cells from BQ and BQ.*Ncf1*$^{*/*}$.

5.5 Positively selected autoreactive B cells

According to the immunology textbooks, autoreactive B or T cells undergo different negative selection mechanisms to be deleted. In **paper V** (unpublished data) we describe a new subset of autoreactive B cells, that are positively selected and have suppressive properties in a COL2-antigen-specific fashion.

5.5.1 Ignorance of negative selection and arthritis disease suppression

We created a knock-in mouse model of the heavy chain of the CB20 hybridoma, specific for the C1 epitope, denoted ACB (anti-C1 B cells). We did not observe clonal deletion, receptor editing, or anergy, mechanism of negative selection for autoreactive B cells. B cells from the ACB mouse responded to COL2 in a dose-dependent manner, primed ACB B cells with COL2 could activate transgenic HCQ3 T cells, these cells need to be alive to process the antigen, and C1 B cells have higher expression of MHCII, pointing for superior antigen presentation capability (**Figure 20A**). We then investigated mouse models of autoimmunity, where we found that ACB B cells transferred to susceptible BQ.*Ncf1*$^{*/*}$ animals protected them against arthritis, and this happens in an antigen-restricted manner as in an EAE model the animals get equally sick. This protection is independent of IL-10 and this effect can be transferred by C1-B cells (**Figure 20B**).

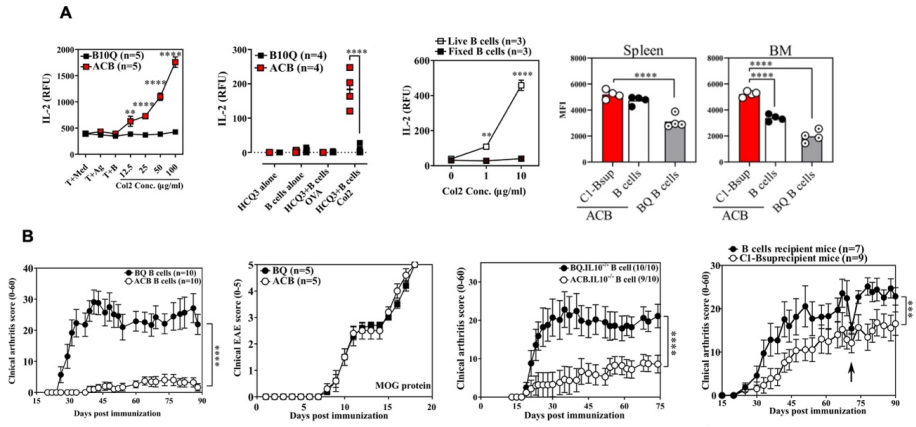

Figure 20. C1-B Cells neglect negative selection and protect against arthritis. (A) COL2-antigen processing and MHCII expression in C1-B cells. **(B)** Autoimmune models of arthritis and EAE.

5.5.2 Regulatory T cells are induced by C1-B cells

COL2 immunized animals recurrently displayed an increased frequency, activation, and proliferation of regulatory T cells, both conventional Foxp3+ Tregs as well as unconventional Tr1 (Lag3+ CD49b+) (**Figure 21A**). C1-B cells transferred to susceptible BQ.*Ncf1*$^{*/*}$ animals displayed the same phenotype, with increased activated Tregs (CD44+

Foxp3+), and expansion when stimulated with COL2 and the survival cytokine IL-2. This effect was observed in a contact-dependent manner (**Figure 21B**).

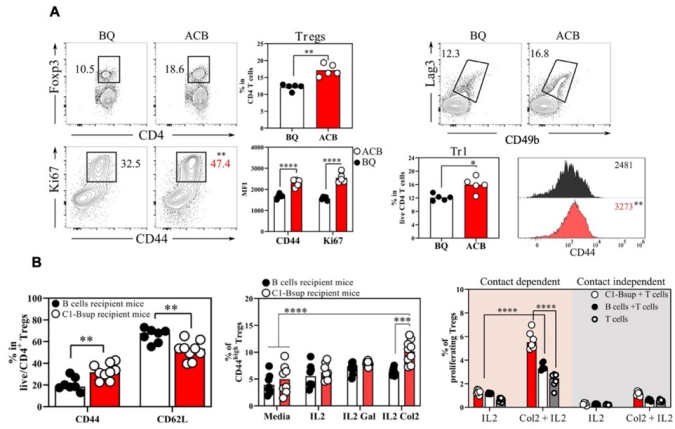

Figure 21. Treg induction by C1-B cells. (A) Frequency of Tregs (Foxp3+; CD49+ Lag3+) in the inguinal lymph nodes of ACB or BQ 10 days after COL2-immunization. **(B)** Tregs in mice transferred with C1-B cells and Tregs induction in a contact-dependent manner.

5.5.3 Upregulation of CCR7 and CD72

RNA-seq analysis of human C1-B cells showed upregulation of CCR7 and CD72, which could be observed in animal models after COL2-immunization, increasing in the first 10 days post-immunization, and only C1-B cells depicted this signature. To dissect the role of CD72, we co-cultured B and T cells from ACB and BQ with a CD72 blocker. Overall, we observed no changes in activation markers, however, we did observe a reduction in Treg induction (**Figure 22A**). Injection of an anti-CD72 antibody broke the tolerance of the ACB mice, with non-proliferating Tregs expanded, and more B cells that could explain more anti-COL2 and anti-C1 antibodies (**Figure 22B**).

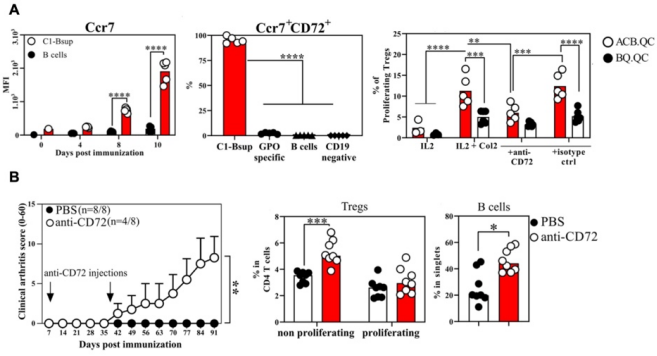

Figure 22. Upregulation of CCR7 and CD72. (A) CD72 and CCR7 expression after COL2 immunization. **(B)** Effect of anti-CD72 injection on arthritis, Tregs, and B cells.

6 Conclusions

Overall, a general conclusion that can be drawn from the studies that compose this thesis is that B cells are fundamental for the control and development of animal models of rheumatoid arthritis (RA), and reactive oxygen species (ROS) can shape B and T cells responses and act as immune modulators.

From **study I**, we clinched that co-administration of the glycolipid αGalCer with the inflammatory cytokine IL-18 leads to an expansion of autoreactive B cells. αGalCer has been utilized as an adjuvant for vaccines, antitumor treatment, and even for the regulation of autoimmune diseases. These results shed light on the dual facet of αGalCer in the context of inflammation.

From studies **II, III**, and **IV** we learned that NOX2-derived ROS in B cells plays a crucial role in murine arthritis models, shaping B and T cell responses and increasing autoimmunity if ROS levels are reduced.

From **study V** we conclude that B cells have important regulatory properties, not relying on IL-10 production, a subset of cells that should be better explored and can potentially improve RA disease treatment.

A more detailed conclusion for each study is:

Study I – iNKTs cells regulate B cell responses and control their autoreactivity. However, in the context of inflammation, these cells shift their phenotype, leaving autoreactive B cells unchecked and promoting their expansion.

Study II – A mutation in the NCF4 protein, part of the NOX2 complex, leads to decreased ROS production of various cell types (neutrophils but mostly B cells) which increases autoimmunity, plasma cell formation, and autoantibodies.

Study III – Restoration of the NCF1-ROS in B cells specifically leads to decreased arthritis scores and incidence, accompanied by lower anti-COL2 antibodies, and a profound effect in T cells, especially regulatory T cells.

Study IV – Similar to study III, in this study we found that NCF1-ROS is important in GC-B cells, restoration of NCF1 in this cell type leads to decreased GC formation, arthritis scores, and anti-COL2 antibodies, with an effect on Tfh cells.

Study V – We identified a subset of B cells that are autoreactive and positively selected, shaping T cell responses mainly regulatory T cells, and suppressing arthritis.

7 Points of Perspective

In this thesis, we extensively investigated B cells, them being the focus or sub-focus of every study. In the three middle stories, we focused greatly on the role of reactive oxygen species (ROS) in B cells and its consequences in murine models of rheumatoid arthritis (RA).

ROS have long been regarded as detrimental for a wide variety of comorbidities, including autoimmune diseases and cancers. Back in 2003, the Medical Inflammation Research group discovered the Neutrophil cytosolic factor 1 (*Ncf1*) gene as responsible for arthritis severity in rats, a finding that has been until today the target of study and exploitation, for shifting the old view that ROS are bad guys.

With the work and results presented in this thesis, I believe that we made some further advances and contributed to the dissection of the role of ROS in non-phagocytic cells, such as B cells, which might have an impact on how future studies will be thought of and designed. As with everything, it is a matter of balance, and so are the levels of ROS and antioxidants. Excessive ROS will also not be a good thing. With these studies, we might have to consider if all the hype around the oral administration of antioxidants is correct.

These results will provide support and open doors to future research in the redox regulation of B cells, which could also be targeted in other models of autoimmunity.

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