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REACTIVE OXYGEN SPECIES IN INFLAMMATION

by

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“The whole of science is nothing more than a refinement of everyday thinking.”

Albert Einstein

“Desperation is a necessary ingredient to learning anything, or creating anything.
Period.
If you ain't desperate at some point, you ain't interesting.”

Jim Carrey

ABSTRACT

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Reactive oxygen species in inflammation

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Chronic inflammation is the underlying cause of many common disabling conditions such as rheumatoid arthritis (RA), multiple sclerosis, coeliac disease, type I diabetes and coronary artery disease. NOX2 complex derived reactive oxygen species (ROS) are known to regulate joint inflammation in rats and mice, and additionally, recent genetic evidence associates phagocyte ROS and the development RA in humans. *Ncf1* mutated mice have lost the functionality of their NOX2 complex and thus have no phagocyte ROS production. These mice suffer from exacerbated arthritis. The immune suppressive effect of the NOX2 complex derived ROS is mediated by monocytes/macrophages that downregulate the activation of autoreactive T cells.

The aim of this thesis was to study how ROS modulate immune responses in different arthritis models and in tumor development. Additionally, genome wide gene expression profiling was carried out to assess the global effects of NOX2 complex derived ROS.

Firstly, these results confirmed the potent anti-inflammatory nature of phagocyte ROS in arthritis models that were driven by the adaptive immune system. Secondly, arthritis models with predominantly innate immunity induced pathophysiology were moderately enhanced by phagocyte, more specifically, neutrophil derived ROS. Thirdly, the ROS induced immune suppression mediated by the adaptive immune system allowed development of bigger implanted tumors, while phagocyte ROS production did not affect the development of spontaneously growing tumors.

Lastly, genome wide gene expression analysis revealed that both humans and mice with abrogated phagocyte NOX2 complex ROS production had an enhanced type I interferon signature in blood, reflecting their hyperinflammatory immune status.

Keywords: Reactive oxygen species, inflammation, arthritis, NOX2 complex

TIIVISTELMÄ

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Happiradikaalit tulehdusreaktion säätelijöinä

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Nivelreuma, multippeli skleroosi, keliakia, tyypin I diabetes ja sepelvaltimotauti ovat vakavia pitkittyneen tulehdusreaktion aikaansaamia sairauksia. Syöjäsolujen tuottamat happiradikaalit vaimentavat tulehdusreaktiota niveltulehduksen eläinmalleissa ja lisäksi, genetiikkaan pohjautuva tieteellinen näyttö tukee happiradikaalien roolia myös ihmisen nivelreumassa. Syöjäsolujen (erityisesti monosyyttien) tuottamat happiradikaalit säätelevät isäntäänsä vastaan reagoivien (autoreaktiivisten) T-solujen aktivoitumista ja siten suojaavat isäntää tämän omien solujen hyökkäyksiltä.

Tämän väitöskirjan tavoitteena oli eritellä happiradikaalien vaikutuksia erilaisissa niveltulehdusmalleissa, sekä selvittää niiden vaikutusta syöpäkasvainten kasvuun. Lisäksi tavoitteena oli genominlaajuisen geeni-ilmentymisanalyysin keinoin tutkia, miten happiradikaalit säätelevät ihmisen ja hiiren geenien ilmentymistä.

Happiradikaalien tulehdusta rauhoittava vaikutus toistui niissä hiimalleissa, jotka pohjautuvat adaptiiviseen, soluvälitteiseen immuniteettiin. Tämän lisäksi happiradikaalien havaittiin maltillisesti lisäävän tulehdusaktiviteettia malleissa, jotka pohjautuvat luonnolliseen immuniteettiin. Yksi immuunijärjestelmän tärkeistä tehtävistä on rajoittaa pahanlaatuisten solujen lisääntymistä. Syöpäkasvainten kehitys oli hitaampaa hiirissä, joiden syöjäsolut eivät tuottaneet happiradikaaleja. Tuloksista voidaan päätellä, että happiradikaalit vaimentavat immuunivasteita ja samalla heikkenee kyky tuhota syöpäsoluja.

Genominlaajuinen geeni-ilmentymisanalyysi paljasti voimakkaamman tyypin I interferonivasteen sekä hiirissä että ihmisissä, joiden syöjäsolut ovat menettäneet happiradikaalientuottokykynsä. Samalla validoimme *Ncf1*-mutantin hiiren hyväksi malliksi tutkia kroonista granulomatoosia ja siihen liittyvää voimistunutta, kroonista tulehdusta.

Avainsanat: Happiradikaalit, tulehdus, artriitti, NOX2 kompleksi

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ABBREVIATIONS

7-AAD	7'amino-actinomycin D
ACPA	Anti-citrullinated protein antibody
ACR	American college of rheumatology
ADP	Adenosine diphosphate
APC	Antigen presenting cell
ATP	Adenosine triphosphate
B16-luc	B16-F10-luc-G5 melanoma cells
BSA	Bovine serum albumin
CAIA	Collagen antibody-induced arthritis
CCD	Charge-coupled device
CD	Cluster of differentation
CFA	Complete Freund's adjuvant
CGD	Chronic granulomatous disease
CIA	Collagen-induced arthritis
CII	Type II collagen
CRP	C-reactive protein
CTLA	Cytotoxic T-lymphocyte antigen
DHR	Dihydrorhodamine
DMARD	Disease-modifying antirheumatic drug
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
DUOX	Dual oxidase
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
EULAR	The European League Against Rheumatism
fMLF	N-formyl-methionyl-leucyl –phenylalanine
GFP	Green fluorescent protein
GPI	Glucose-6-phosphate isomerase
GSH	Glutathione (reduced)
GSSG	Glutathione disulfide (oxidized)
HE	Hematoxyoin and eosin
HLA	Human leukocyte antigen
IBD	Inflammatory bovel disease
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunogolbulin

INFL	Inflammatory transcript (Paper IV)
IPA	Ingenuity pathway analysis
IRG	Interferon regulated gene (Paper IV)
LAT	Linker of activated T cells
LC	Lymphocyte related gene (Paper IV)
LLC	Lewis lung carcinoma
LM	Lipomannan
LPS	Lipopolysacharide
MHC	Major histocompatibility complex
Misc.	Unclassified gene (Paper IV)
MPL	Monophosphoryl lipid A
MS	Multiple sclerosis
NAD	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NOX	NADPH oxidase
NSAID	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PMA	phorbol myristate acetate
Q-RT-PCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROS	Reactive oxygen species
RT	Room temperature
SH2	Src Homology 2
SLE	Systemic lupus erythematosus
SNP	Single-nucleotide polymorphism
SOD	Superoxide dismutase
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF-alpha	Tumor necrosis factor-alpha
TRAMP	The transgenic adenocarcinoma of the mouse prostate
VLA-4	Very late antigen
YFP	Yellow fluorescent protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by Roman numerals (I-V). The original communications are reproduced with the permission of the copyright holders.

I **Superoxide dismutase 3 limits collagen-induced arthritis in the absence of phagocyte oxidative burst.** Kelkka T, Laurila JP, Sareila O, Olofsson P, Laukkanen MO, Holmdahl R. Mediators of Inflammation. 2012;2012:730469.

II **Reactive oxygen species produced by the NADPH oxidase 2 complex in monocytes protect mice from bacterial infections.** Pizzolla A, Hultqvist M, Nilson B, Grimm MJ, Eneljung T, Jonsson IM, Verdrengh M, Kelkka T, Gjertsson I, Segal BH, Holmdahl R. The Journal of Immunology. 2012 May 15;188(10):5003-11. *Copyright 2012. The American association of Immunologists, Inc*.

III **Enhancement of antibody-induced arthritis via Toll-like receptor 2 stimulation is regulated by granulocyte reactive oxygen species.** Kelkka T, Hultqvist M, Nandakumar KS, Holmdahl R. The American Journal of Pathology. 2012 Jul;181(1):141-50. *Reprinted with permission from Elsevier.*

IV **Comparative analysis of chronic granulomatous disease and its mouse model reveals upregulated interferon signature and modified lymphocyte activation pathways.** Tiina Kelkka, Marjo Linja Outi Sareila, Malin Hultqvist, Peter Olofsson, Paulo Rodriques-Santos, Júlia Vasconcelos, Margarida Guedes, Laura Marques, Merja Helminen, Leena Kainulainen, Sirpa Jalkanen, Riitta Lahesmaa, Margarida Carneiro, Rikard Holmdahl. Manuscript.

V **NADPH Oxidase 2 Complex Derived Reactive Oxygen Species Suppress the Development of Implanted Tumors in Mouse.** Tiina Kelkka, Angela Pizzolla, Juha Petteri Laurila, Tomas Friman, Renata Gustafsson, Eva Källberg, Olof Olsson, Tomas Leandersson, Kristofer Rubin, Marko Salmi, Sirpa Jalkanen, Rikard Holmdahl. Manuscript.

1. INTRODUCTION

When the immune system recognizes a pathogen, it immediately launches an immune reaction to eradicate the impending threat. This is achieved by using a wide array of mechanisms and the process is characterized by the commonly known signs of inflammation including redness, swelling and pain. Just a quick search in scientific data bases will reveal that inflammation is regulated by a vast array of different mechanisms. The battle against foreign intrudes is initiated by special recognition molecules that recognize the danger. Recognition is followed by orchestration of an array of defense mechanisms needed for neutralization and elimination of the particular pathogen. When the pathogen is cleared, the immune reaction is switched off and the battleground with dead immune cells and destroyed tissue environment is cleaned. The last phase of a healthy immune response is characterized by tissue recovery and results in restoration of normal tissue function.

Chronic inflammation is a condition where inflammation is not regulated or switched off properly and it is the underlying cause of many common diseases such as rheumatoid arthritis (RA), other autoimmune diseases, allergies and atherosclerosis.

Reactive oxygen species (ROS) are highly reactive oxygen containing molecules that can oxidize and thus damage biologically important molecules such as fatty acids and genomic DNA. In addition to their potential to cause harm, ROS are instrumental regulators of immune responses and cell signaling. The last decade has revolutionized the way we understand the ROS' role in inflammation. Against the longstanding dogma describing the harmfulness of ROS (Chiurchiu, Maccarrone 2011), we have learned that reduced superoxide production by phagocytes (immune cells that "eat" other cells, pathogens and cell debris) actually enhances inflammation as reviewed in (Holmdahl et al. 2012). However, despite the novel findings in the field, we should not simply ignore the historical body of evidence describing ROS as inflammation propagating molecules, but rather try to explain the historical findings with novel understanding and techniques. In this thesis I have sought answers to build a bridge between the conflicting views of the role of ROS in inflammation.

As the immune system is too complicated to be imitated in cell culture models, mouse models are used as tools to experimentally dissect immunological questions. Today's research is aided by a numerous selection of genetically modified and well-characterized mouse strains. We also have access to different disease models to experimentally address specific questions. The ultimate goal of biomedical research is to better understand human diseases, and we need to acknowledge that there are differences between human diseases and their mouse counterparts. The last part of this thesis describes a project where genome-wide gene-expression analysis is used to validate the *Ncf1* mutated mouse as a good model to study human hyperinflammation.

2. REVIEW OF THE LITERATURE

2.1 The innate and adaptive immune systems

The purpose of the immune system is to protect the host against different dangers (Matzinger 1994) including pathogenic micro-organisms, tissue damage and arising malignancies. The first acting arm of the immune system is called the innate immune system. Innate immunity comprises all forms of defense that do not have immunological memory and it can be roughly divided into its cellular and non-cellular constituents. The cellular compartment contains phagocytes (granulocytes, macrophages, dendritic cells) mast cells and natural killer cells, while the non-cellular part comprises different pattern recognition receptors (Toll-like receptors, Nod-like receptors and many others) anti-microbial peptides, the complement system and various chemical mediators of inflammation such as leukotrienes, cytokines, and prostaglandins. The innate immune system recognizes a wide variety of danger signals, is quick to respond and it efficiently activates adaptive immune responses. Thus, due to the interplay between the innate and adaptive systems, there is only a thin line between innate and the adaptive immunity.

The most striking characteristic of the adaptive immune system is its memory i.e. its ability to remember previously met pathogens and quickly mount a targeted and specific reaction against these. When the innate immune system recognizes a pathogen, the pathogen is engulfed and digested in the phagocytes' phagosomes, where after the peptides of pathogen origin are loaded into MHC II molecules and presented to T cells. The T cells either direct a cytotoxic attack against the target (cytotoxic T cells) or stimulate either cellular (T helper type 1, Th1) or humoral i.e. antibody mediated immune responses by activating B cells (T helper type 2, Th2). More recently, 2005, Th17 cells were identified as the third major Th cell subset, with special tasks in mucosal immunity and inflammatory conditions (Park et al. 2005). Both arms of the adaptive immune system can produce so called memory cells and when these long lived lymphocytes re-encounter the same danger signal, they can trigger a potent inflammatory response without delay.

The hallmarks of inflammation include redness and swelling of the inflamed area together with pain and increased body temperature. If inflammation is caused by a micro-organism which can be isolated from the inflamed tissue, the condition is called infection. A healthy immune system can efficiently evade dangers, and it also dismounts the reaction when the danger has been resolved. Sterile chronic inflammation without pathogen involvement can either develop spontaneously upon an autoinflammatory processes or it can be a consequence of unsuccessful resolution of infection. In most inflammatory disease it remains unclear, how the chronic inflammation is originally triggered.

2.2 Rheumatoid arthritis is a chronic inflammatory disease

Conditions that are characterized by chronic inflammation include allergies, atherosclerosis and autoimmune diseases such as multiple sclerosis (MS), RA and inflammatory bowel disease. Autoimmune diseases, in which an adaptive and specific immune reaction is targeted against one's own tissues, constitute a unique group of inflammatory diseases. Autoimmune diseases can be roughly divided into systemic and organ specific disorders. Sjögren's syndrome, systemic lupus erythemasus (SLE) and RA are examples of systemic autoimmune disorders with occurrence of autoantibodies without evident tissue association. Type I diabetes (pancreatic beta-cells), coeliac disease (gliadin, small intestine), pernicious anemia (gastric parietal cells), Hashimoto's thyroiditis (thyroid gland) and autoimmune hemolytic anemia (red blood cells) serve as examples of tissue specific autoimmunity.

2.2.1 RA is incurable, but nowadays well treatable

RA is a common chronic inflammatory disease affecting 0.5-1% of the population in industrialized countries (Scott, Wolfe & Huizinga 2010). The risk of developing RA is attributable to both genetic and environmental factors. HLA region (Stastny 1976) is the most important genetic region to determinate the susceptibility to RA. *PAD14* (Suzuki et al. 2003) and *PTPN22* (Begovich et al. 2004) are other genes consistently and repeatedly associated with RA (Plenge et al. 2005, Barton et al. 2008, Daha et al. 2009, Wellcome Trust Case Control Consortium 2007). While many possible environmental factors have been suggested (Liao, Alfredsson & Karlson 2009), smoking is the only replicated risk factor for the development of RA (Heliövaara et al. 1993, Stolt et al. 2003, Carlens et al. 2010). The mechanism how cigarette smoke supports developing autoimmunity remains elusive.

The diagnostic criteria for RA have been recently updated by rheumatologists from the European League against Rheumatism (EULAR) together with rheumatologists from The American College of Rheumatology (ACR). The main problem with the previously widely used criteria defined by ACR in 1987 (Arnett et al. 1988) was the difficulty to identify patients with early disease. The updated criteria (presented in Table 1) now take into account the advances made in diagnostics (Neogi et al. 2010) and hopefully will allow earlier and accurate diagnosis of RA.

Table 1 ACR/EULAR 2010 criteria for RA

1. Joint involvement (0–5)
    - One medium-to-large joint (0)
    - Two to ten medium-to-large joints (1)
    - One to three small joints (large joints not counted) (2)
    - Four to ten small joints (large joints not counted) (3)
    - More than ten joints (at least one small joint) (5)
2. Serology (0–3)
    - Negative RF and negative ACPA (0)
    - Low positive RF or low positive ACPA (2)
    - High positive RF or high positive ACPA (3)
3. Acute-phase reactants (0–1)
    - Normal CRP and normal ESR (0)
    - Abnormal CRP or abnormal ESR (1)
4. Duration of symptoms (0–1)
    - Less than 6 weeks (0)
    - 6 weeks or more (1)

- Points are shown in parentheses.
- Cut-point for RA is 6 points or more.
- Patients can also be classified as having RA if they have: (a) typical erosions; (b) long-standing disease previously satisfying the classification criteria.

Adapted from (Scott, Wolfe & Huizinga 2010).

Despite the lack of curative treatment, good treatment modalities are available for RA patients. Methotrexate is the widest used disease-modifying antirheumatic drug (DMARDs) and it is prescribed as the first-line treatment to most patients. Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate pain, but their use in RA is limited due to concerns about their gastrointestinal and cardiac side-effects and their limited effect on disease progression. The third group of classical RA drugs are glucocorticoids that, due to their side-effects (osteoporosis and infections), are mainly used to stop highly active disease as short oral courses or as local intra-articular injections (Scott, Wolfe & Huizinga 2010).

During the last decade, the treatment of RA has been revolutionized by the use of novel biological agents. Encouraged by promising results from mouse models (Williams, Feldmann & Maini 1992, Keffer et al. 1991) a high-affinity chimeric anti-TNF-alpha antibody (Knight et al. 1993) was shown to reduce pain and swelling as well as suppress inflammation activity (CRP and/or erythrocyte sedimentation rate) in patients with RA (Elliott et al. 1993). Today, in addition to the original chimeric antibody (Infliximab) there are a number of other TNF-alpha binding biological agents on the market available for treating RA (Tak, Kalden 2011).

Other novel tools in RA therapy include Rituximab, a chimeric B-cell depleting anti-CD20 monoclonal antibody (Edwards et al. 2004, Cohen et al. 2006), Abatacept, costimulation blocking CTLA-4Ig fusion protein (Moreland et al. 2002) and Tocilizumab, and Anakinra monoclonal antibodies that block interleukin-6 and interleukin-1 receptors respectively (Choy et al. 2002, Bresnihan et al. 1998).

2.2.2 Animal models of arthritis

As visualized in Figure 1, the pathologic process in RA is initiated several years before the diagnosis (Aho et al. 1985, Rantapää-Dahlqvist et al. 2003). This highlights the necessity for early diagnosis and for the development of novel advanced treatments that interfere with the etiological causes and early preclinical phases of the disease.

Animal models can provide us with knowledge of the early events of arthritis development and progression. Animal models can also be used as tools to develop and validate novel therapeutic and even prophylactic approaches to cure RA (Kollias et al. 2011). In addition, RA patient material is restricted to blood and synovial samples and by using animal models, research can be extended into otherwise unobtainable immunologically relevant tissues (spleen, draining lymph nodes and whole joints). Furthermore, animal models enable the study and experimental intervention of untreated arthritis, which, due to obvious ethical reasons, cannot be done in RA.

Different animal models mimic various aspects of RA. Even though no single rodent model replicates all features of human RA (van den Berg 2009), a lot of clinically relevant information can be gained by combining the use of different animal models. Mouse and rat are the most commonly used species in arthritis research. The most commonly used mouse models together with models with special importance for the work presented in this thesis are discussed in the next chapters. Also spontaneously arthritis developing mouse strains, with special importance in RA research are included.

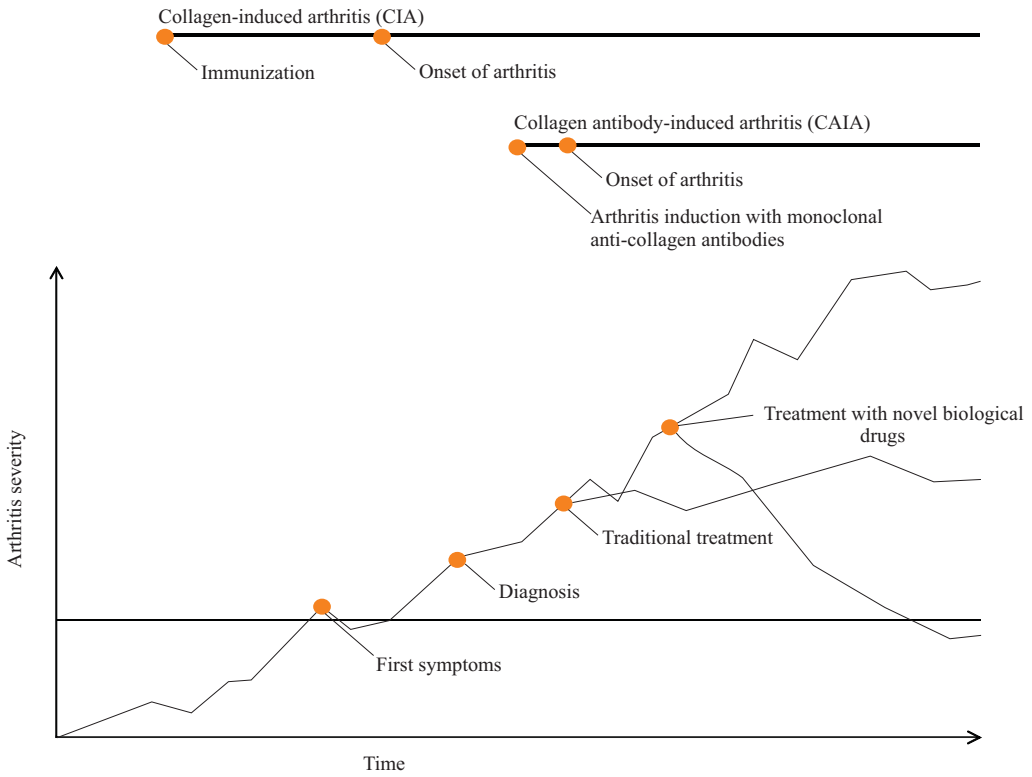


Figure 1. The relationship between RA and its animal models.
The pathological process leading to rheumatoid arthritis (RA) is initiated several years before the onset of the first symptoms. According to the diagnostic criteria, the first symptoms must be accompanied by other symptoms before establishing clinical diagnosis and initiation of the treatment. Novel biological drugs combined with classical treatment with antirheumatics often give good response and alleviate the symptoms. Collagen-induced arthritis can be used to study even the pre-clinical phase of RA, while collagen antibody-induced arthritis is a good tool to address the local inflammation in the joints (Idea for the figure adapted from lecture notes R. Holmdahl).

2.2.2.1 Arthritis models induced by immunization

Collagen-induced arthritis is the most commonly used arthritis model and can be induced in both mice and rats (Trentham, Townes & Kang 1977, Courtenay et al. 1980). In mice, CIA is usually induced by an intra-dermal injection of type II collagen (CII) emulsified in complete Freund's adjuvant (CFA), which contains highly immunogenic mycobacteria (Wooley et al. 1981, Brand, Latham & Rosloniec 2007). CIA is initiated with a phase of immune priming and in two to three weeks' time, the initial immune activation leads to initiation of arthritis in arthritis susceptible strains. The symptoms include redness and swelling of the affected joints as depicted in Figure 2. The external signs of inflammation are followed by the destruction of the synovial joint with formation of erosive pannus tissue and bone remodeling. In most cases,

inflammation subsides leaving the paw free from inflammation but deformed (Holmdahl et al. 2002). Arthritis severity is commonly assessed by macroscopic analysis of the paws and complemented with histology. Cross-laboratory comparisons are challenging as different laboratories use different scoring protocols.

The induction of CIA is dependent on and actively regulated by T cells and arthritis severity is typically reflected in the antibody response against CII (Holmdahl et al. 1986b). The most commonly applied variant of CIA is induced in DBA/1 mice, which is a highly CIA susceptible mouse strain. This strain is unfortunately also susceptible to environmental stress and prone to spontaneous arthropathy due to grouping stress (Holmdahl et al. 1992). Another susceptible and well characterized strain that develops CIA, is the black (C57BL/10) strain that carries the MHC II q haplotype (Holmdahl et al. 2002, Holmdahl et al. 1986a). Also C57BL/6 mice are shown to develop CIA, although this strain requires repeated immunization with chicken collagen emulsified in CFA and the use of larger volume of emulsion (Campbell, Hamilton & Wicks 2000). Importantly, CIA in C57BL/6 mice is not recommended as the first line model of RA. The MHC II b haplotype expressed by C57BL/6 mice does not allow the development of arthritis that is dependent on autoreactive T cells, but rather favors T cell responses against impurities (pepsin) in the collagen preparation (Backlund et al. 2012).

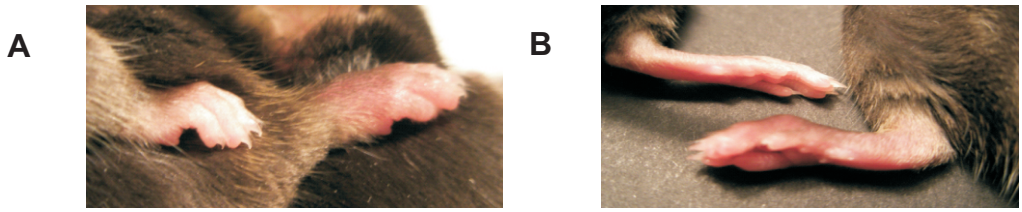


Figure 2. CIA in mice.
Ncf1 mutated (*Ncf1*/**) mice develop severe arthritis with red and swollen front (A) and hind (B) paws. Similar immunization with CFA does not result in inflammation in the wild type (*Ncf1*+/+) mice.

Proteoglycan-induced arthritis is closely related to CIA as it is induced by immunization with cartilage derived proteoglycan emulsified in adjuvant (Glant et al. 1987, Stoop et al. 2013). These chronic arthritis models are both dependent on T cells and share the associated B cell mediated autoantibody phenotype (Glant et al. 2011).

2.2.2.2 Antibody-induced arthritis models

CAIA

RA patients are shown to produce autoantibodies against CII (Cook et al. 2004, Cook et al. 1994, Rönnelid et al. 1994) and other cartilage collagens (Jaalinoja et al. 2008). These antibodies induce arthritis when injected in recipient mice (Wooley et al. 1984).

Similarly, pathogenic auto-antibodies are formed in CIA (Holmdahl et al. 1986b) and these antibodies can also transfer the disease (Stuart, Dixon 1983). Anti-CII antibodies have been extensively characterized and the main antigenic epitopes have been identified along the triplehelical CII molecule.

This knowledge has been exploited to develop collagen antibody-induced arthritis (CAIA), a B and T cell independent mouse model of the effector phase of arthritis. The model used in this thesis is induced by injection of a cocktail containing four monoclonal anti-collagen antibodies in mice and within two to three days first signs of arthritis appear (Nandakumar, Svensson & Holmdahl 2003). The antibody cocktail, which is also utilized in this thesis, recognizes the following CII epitopes: the C1 epitope (aa 359–369), recognized by mAb CIIC1, J1 (aa 551–564) recognized by mAb M2139, U1 (aa 494–504), recognized by mAb UL1, and the F4 epitope (aa 932–936) recognized by mAb CIIF4 (Kraetsch et al. 2001, Holmdahl et al. 1989, Schulte et al. 1998).

Antibodies alone induce mild and transient arthritis, symptoms gradually disappearing in approximately a week. Thus, for most experimental needs, the antibody-induced arthritis needs to be enhanced by using some additional immunological stimulant. The standard protocol describes LPS as the routinely used stimulant (Nandakumar, Svensson & Holmdahl 2003). Other agents so far described to enhance CAIA are monophosphoryl lipid A (MPL) (Kelkka et al. 2012a) and TLR2 agonists lipomannan (LM) (Hultqvist et al. 2010) and Pam-3-Cys (Cook et al. 2010).

KRN

Another important antibody-induced arthritis model is the so called KRN model (reviewed in (Monach, Mathis & Benoist 2008)). KRN arthritis develops in transgenic mice that express a self-reactive T cell receptor together with MHC class II molecule A(g7) (Korganow et al. 1999). This K/BxN mouse mounts an immune reaction against the ubiquitous self-antigen glucose-6-phosphate isomerase (G6PI) and by means of intense B cell differentiation and proliferation develops high levels of anti-G6PI antibodies. These antibodies induce arthritis with varying penetrance when transferred to new recipient mice from different inbred strains (eg. Balb/c, B6, B10, DBA/1) (Ji et al. 2001). The transferred arthritogenic antibodies appear to bind to endogenous cationic G6PI, which is preferentially associated with cartilage surfaces (Maccioni et al. 2002).

Furthermore, immunization with G6PI and even with the immunodominant self-peptide derived from G6PI, induce chronic, T lymphocyte dependent arthritis in DBA/1 mice (Schubert et al. 2004, Bruns et al. 2009). These models, where the immune response is directed against a ubiquitous antigen, but that precipitate in the synovial joints reflect the systemic aspects of RA.

2.2.2.3 Genetic models with spontaneous arthritis

The expansion of different genetically modified mouse strains has provided arthritis research with an increased number of mouse models that address specific pathways in arthritis development. In 1991, Kollias and coworkers provided insight into the possible role of TNF-alpha in arthritis by introducing a mouse model overexpressing human TNF-alpha that developed chronic polyarthritis with 100 % incidence (Keffer et al. 1991). Today, this model is widely used to study the downstream effects of TNF-alpha in arthritis.

The role of T cells as mediators of autoimmunity is highlighted in the so called SKG model, where a mutation in Src homology 2 (SH2) domain of ζ-associated protein of 70 kDa (*ZAP-70*) gives rise to autoimmune inflammation and arthritis (Sakaguchi et al. 2003). ZAP-70 is a key mediator of TCR signal transduction and the SKG mutated variant of *ZAP-70* impairs positive and negative selection of T cells in the thymus, thus leading to production of arthritogenic T cells. SKG mice develop spontaneous arthritis in conventional animal houses, but in specific pathogen free conditions, the disease must be triggered with an additional immunostimulant (Yoshitomi et al. 2005) such as the fungal products zymosan, beta-glucans (Yoshitomi et al. 2005) and mannan (Hashimoto et al. 2010, Ruutu et al. 2012).

The lack of ROS production from phagocytes has also been reported to induce spontaneous arthritis (Hultqvist et al. 2004, Lee et al. 2011). The *Ncf1* mutated mouse, however, develops arthritis late in life and with too low penetrance to be used as a spontaneous model as such.

2.2.2.4 Local and bacterial irritation models

Local irritation models comprise the last group of arthritis models discussed here. These models can be divided into models of septic arthritis induced with live bacteria and models that are induced by injecting only the arthritogenic component of the micro-organism.

Borrelia burgdorferi (Campfield et al. 2012) *Salmonella enterica* (Noto Llana et al. 2012) and *Yersinia enterocolitica* (Heesemann, Gaede & Autenrieth 1993) are examples of bacteria known to induce arthritis. Intra-articular injection of zymosan induces chronic inflammatory arthritis with mononuclear cell infiltration, synovial hypertrophy and pannus formation (Keystone et al. 1977). Peptidoglycan is another substance that, when injected directly in the joint, induces joint inflammation (Rosenzweig et al. 2011). In addition, systemic injection of peptidoglycan has the potential to induce arthritis in selected mouse strains, Balb/c and DBA/1 mice being particularly susceptible (Koga et al. 1985). Other substances used to induce local arthritis via intra-articular injections include curdlan, mannan (Rosenzweig et al. 2011) and LPS (Abu-Ghefreh, Masocha 2010).

2.3 ROS

Redox (reduction-oxidation) reactions include all chemical reactions where atoms' oxidation state is altered. Oxidation is the loss of electrons and reduction is the gain of electrons. The reductant is oxidized when it reduces its target. Similarly, when the oxidized product is again reduced, the reductant is oxidized. This kind of cyclic repetition of redox reactions is called redox cycling. ROS are a heterogeneous group of oxygen radicals and other strongly oxidizing molecules and share common features with closely related reactive nitrogen species. After generation, ROS are further converted into other oxidative species or neutralized by carefully regulated enzymatic and non-enzymatic antioxidative reactions as depicted in Figure 3.

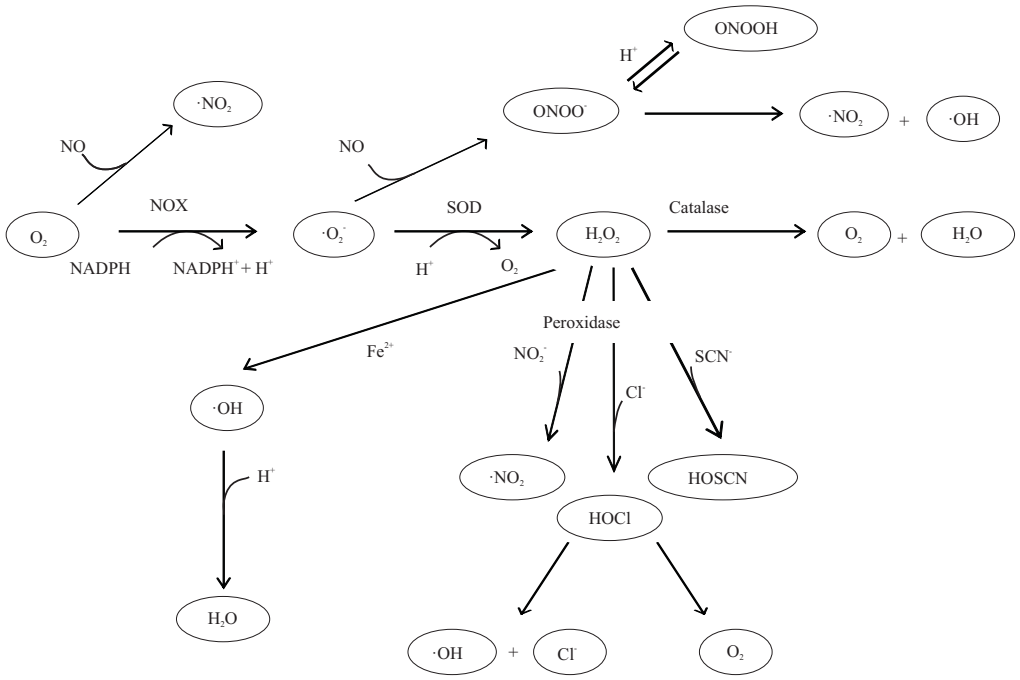


Figure 3. Reactive oxygen species and their reactions.
O_2, molecular oxygen; NO, nitric oxide; $\bullet NO_2$; nitrogen dioxide; $\bullet O_2^-$, superoxide anion; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; H_2O_2, hydrogen peroxide; •OH, hydroxyl radical; H_2O, water; HOCl, hypochlorous acid; HOSCN, hypothiocyanous acid; NOX, NADPH oxidase; SCN, thiocyanate; SOD, superoxide dismutase.

Molecular oxygen (O_2) is converted into superoxide anion (O_2^-) by the action of specialized enzymes, in mitochondria during cellular respiration, by ionizing and UV radiation and during the metabolism of a wide range of xenobiotic substances and drugs (Winterbourn 2008). Superoxide can either spontaneously or by the action of one of the three superoxide dismutases (SOD1-3) be further converted into hydrogen peroxide (H_2O_2). The reaction between superoxide and nitric oxide produces peroxynitrite ($ONOO^-$), converging reactive nitrogen and oxygen species metabolism.

Peroxynitrate can further react to produce hydroxyl radicals ($HO^{\bullet}$) and nitrogen dioxide radicals ($NO2^{\bullet}$) (Merenyi et al. 1998). When hydrogen peroxide meets ferrous iron (Fe^{2+}), the initiated so called Fenton reaction leads to the production hydroxyl radicals ($HO^{\bullet}$) (Fenton 1894). Catalase catalyzes the neutralization of hydrogen peroxide onto molecular oxygen and water (Loew 1900). Highly reactive radicals are also produced when myeloperoxidase turns hydrogen peroxide into hypochlorous acid (HOCl) (Klebanoff 1968) and when eosinophil peroxidase consumes hydrogen peroxide to form hypobromous acid (HOBr) (Ten et al. 1989, Sakamaki et al. 1989). In addition to hypochlorous and hypobromous acid, hypothiocyanous acid (HOSCN) which is produced by lactoperoxidase (Polis, Shmukler 1953), make an important radical involved in antimicrobial defense.

2.3.1 Cellular sources of ROS

Mitochondrial respiration is one of the major sources or ROS in biological systems. Mitochondria are the power plants of the cell and produce ATP to satisfy the cell's energy demands in a process called cellular respiration. Electron transfer to molecular oxygen takes place via the four complexes of the electron transfer chain in a process that generates water and during which protons (H^{+}) are pumped into the intermembrane space at complex I (NADH dehydrogenase), complex III (cytochrome c reductase), and complex IV (cytochrome c oxidase). The formed H^{+} gradient is the major contributor to the mitochondrial inner membrane potential. When the protons flow back into the matrix through the ATP synthase complex, ATP is synthesized from ADP and inorganic phosphate. During respiration, some electrons escape form the transfer chain to generate superoxide (Rigoulet, Yoboue & Devin 2011). The estimates of how much of the consumed oxygen is released as superoxide vary, latest and probably most realistic estimates suggesting 0.1 % of the respiratory rate during steady state metabolism (Tahara, Navarete & Kowaltowski 2009).

The leakage of electrons from the respiratory chain was long referred to as a byproduct, but more recent findings have assigned important regulatory functions for these escaped electrons. Most proteins harbor disulfide bridges that are maintained by the cellular redox balance and inhibition of mitochondrial ROS production can lead to errors in protein folding and transport ultimately affecting cell proliferation related signaling (Yang, Song & Loscalzo 2007). Mitochondrial ROS have been implicated as enhancers of inflammation by many researchers (West et al. 2011, West, Shadel & Ghosh 2011, Mitchell et al. 2010, Ostrakhovitch, Afanas'ev 2001). In the light of today's litterature, the proinflammatory effects of mitochondrial ROS are mainly mediated by the innate immune system (West et al. 2011)

Xanthine oxidase is a molybdenium containing flavoenzyme (Ichida et al. 1993) that catalyzes the oxidation of hypozanthine to zanthine and further to uric acid and generate both superoxide and hydroxyl radicals (Kuppusamy, Zweier 1989, Romao et

al. 1995, Huber et al. 1996). Human zanthine oxidase is expressed in most tissues (Xu et al. 1994).

Detoxification of lipophilic xenobiotics such as many drugs and environmental pollutants by the catalytic activity of the cytochrome p450 enzyme produces superoxide and hydrogen peroxide (Zangar, Davydov & Verma 2004). There is genetic evidence suggesting that polymorphisms in cytochrome p450 may play a role in the development of RA (Yen et al. 2003a) and ankylosing spondylitis (Yen et al. 2003b).

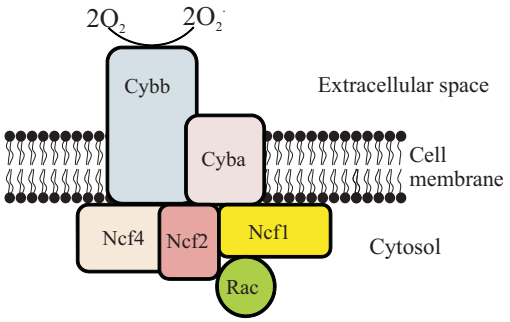
2.3.2 NOX2 belongs to the NOX family

NADPH oxidases (NOX) are a family of transmembrane oxidases that reduce molecular oxygen to superoxide using energy derived from the oxidation of NADPH/NADH to NADP/NAD. NOX enzyme family consists of five NOX enzymes (NOX1-5) and two Dual oxidases (DUOX1-2). NOX isoforms differ in their inducibility, expression pattern and they also require different sets of accessory proteins for their enzymatic activity as excellently reviewed in (Bedard, Krause 2007)).

NOX2 complex is the firstly identified and best studied member of the NOX family. It is expressed by phagocytes (granulocytes, monocytes, dendritic cells) and its expression has also been reported in other cells of the immune system such as natural killer cells, B cells and mast cells. Lower levels of NOX2 expression has also been detected in T cells (Jackson et al. 2004, van Reyk et al. 2001). Interestingly, recent published data report that neuronal expression of NOX2 regulates stress-response behavior in rats and mice (Schiavone et al. 2012, Sorce et al. 2010).

The NOX2 complex encompasses CYBB (also known as gp91phox) and accessory molecules that enable and regulate its function. The transmembrane catalytic core of the NOX2 complex associates with the other transmembrane protein CYBA (also known as p22phox) and upon activation this heterodimer complex is directed from granule membranes to the lipid rafts of the plasma membrane to form the catalytically active NOX2 complex as presented in Figure 4. These lipid rafts are subsequently, upon phagocytosis directed to form endosomes and phagosomes. Efficient superoxide production requires the concerted action of the CYBB-CYBA heterodimer and a set of cytosolic proteins. In resting state the cytosolic subunits of the NOX2 complex NCF1, NCF2 and NCF4 (also known as p47phox, p67phox and p40phox) reside in the cytosol associated together (Lapouge et al. 2002). Similarly, in resting state, the GDP-bound small GTPase RAC is in complex with the inhibitory GDI, and is located in the cytosol separated from the other cytosolic subunits (El-Benna, Dang & Gougerot-Pocidalo 2008). Upon activation, all cytosolic components together with RAC move to the cell membrane and build complex with the membrane bound enzymatic core to initiate superoxide production (El-Benna, Dang & Gougerot-Pocidalo 2008). Interestingly,

there are two RAC isoforms and they differ in their cellular distribution; RAC1 predominates in monocytes and RAC2 in neutrophils (Zhao, Carnevale & Cathcart 2003).



Gene	Protein
Cybb	CYBB (gp91phox, NOX2)
Cyba	CYBA (p22phox)
Ncf1	NCF1 (p47phox)
Ncf2	NCF2 (p67phox)
Ncf4	NCF4 (p40phox)
Rac1	RAC1
Rac2	RAC2

Figure 4. The NOX2 complex.
The schematic structure and organization of the supreoxide producing activated NOX2 complex (Modified from (Hultqvist et al. 2009)). Genes coding for NOX2 complex proteins are listed in the left panel.

During priming the location, conformation and phosphorylation status of the subunits are altered to lower the threshold for activation. Priming can be achieved by e.g. inflammatory cytokines, TLR agonists, chemoattractants, chemicals and other factors such as peroxynitrite and adhesion as reviewed in (El-Benna, Dang & Gougerot-Pocidalo 2008). Priming facilitates the following full activation and superoxide production, which is triggered by a much more selected repertoire of substances including N-formyl-methionyl-leucyl –phenylalanine (fMLF), complement component C5a, and phorbol myristate acetate (PMA) (McPhail, Clayton & Snyderman 1984). The NOX2 complex is also activated during phagocytosis (Sbarra, Karnovsky 1959).

NOX1 was the first homologue that was described for NOX2 (Suh et al. 1999). NOX organizer 1 (NOXO1, NCF1 homolog), the organizer subunit of NOX1 and NOX activator 1 (NOXA1, NCF2 homolog) are the cytosolic accessory molecules needed for superoxide production by NOX1 complex (Banfi et al. 2003, Geiszt et al. 2003a, Takeya et al. 2003). High expression of NOX1 has been detected on colon epithelium (Szanto et al. 2005). Additionally, lymphocytes (Szanto et al. 2005), vascular smooth muscle (Lassegue et al. 2001), endothelial cells (Kobayashi et al. 2004a), prostate, uterus (Suh et al. 1999), placenta (Cui et al. 2006), osteoclasts (Lee et al. 2005) and retina (Manea, Raicu & Simionescu 2005) are reported to express NOX1. NOX1 deficient mouse has decreased blood pressure (Matsuno et al. 2005, Matsuno et al. 2005) and develop enhanced hyperoxia induced acute lung injury (Carnesecchi et al. 2009), suggesting an inflammation regulating role for NOX1 derived ROS.

NOX3 (Cheng et al. 2001) is expressed in the inner ear and can produce ROS using both NCF1 and NOXO1 organizer subunits (Banfi et al. 2004, Cheng, Ritsick & Lambeth 2004). In addition, NOX3 expression has been reported to take place on hepatocytes (Li et al. 2010). NOX4 (Cheng et al. 2001, Geiszt et al. 2000, Shiose et al. 2001) was originally identified from kidney, but it is also expressed in many other tissues including endothelium (Ago et al. 2004), fibroblasts (Colston et al. 2005) and hematopoietic stem cells (Piccoli et al. 2005). Interestingly, upon heterologous expression NOX4 is active without cell stimulation and does not require cytosolic subunits for its activity (Martyn et al. 2006).

NOX5 was identified as a calcium activated (Bánfi et al. 2004) NADPH oxidase in various fetal tissues, lymphocyte rich areas in spleen and lymph nodes and in uterus and in testis (Cheng et al. 2001, Banfi et al. 2001, Bedard, Jaquet & Krause 2012). The last two enzymes belonging to the NOX family are the thyroid dual oxidase 1 and 2 (DUOX 1 and 2) (De Deken et al. 2000). Contrary to other NOX enzymes and despite of some disagreement in the field, it is likely that dual oxidases convert the produced superoxide directly into hydrogen peroxide (Bedard, Krause 2007). Inactivating mutations in DUOX2 constitute the causative reason for congenital hypothyroidism (Moreno et al. 2002), highlighting the importance of these enzymes to thyroid function. In addition, there is also evidence that mucosal surface host defense is supported by hydrogen peroxide derived from the DUOX enzymes (Geiszt et al. 2003b).

2.3.3 The antioxidant systems

ROS production and clearance are important parts of healthy metabolism. The tissue redox-balance is maintained by concerted actions of oxidant producing and oxidant consuming systems. ROS play an indispensable role in pathogen clearance and act as important signaling molecules regulating many physiological processes. To avoid excessive oxidant load and damage in cellular and tissue structures it is important that these highly oxidative metabolites are promptly removed when not needed. Uncontrolled ROS production or failure in antioxidant defense can lead to oxidative stress and result in DNA strand breakage, protein damage and lipid peroxidation (Kohen, Nyska 2002).

Antioxidants can be dived into enzymatic and non-enzymatic ROS neutralizing mechanisms. The most important enzymatic antioxidant systems include SOD enzymes, catalase, the thioredoxin, glutathione peroxidase, and peroxiredoxin systems. Dietary tocopherols (vitamin E) and ascorbic acid (vitamin C) are low-molecular-mass antioxidants that can neutralize ROS by directly reducing them.

The firstly identified superoxide dismutase (SOD) enzyme was the cytoplasmic copper/zink SOD (SOD1) (McCord, Fridovich 1969). The next SOD enzyme to be found was the mitochondrial iron/manganese SOD (SOD2) (Weisiger, Fridovich 1973)

and almost ten years later, the third SOD enzyme, the extracellular SOD (SOD3) was discovered (Marklund 1982). All three SOD enzymes catalyze a reaction where superoxide is dismutated into hydrogen peroxide, but structurally these enzymes are unrelated. The correct balance between superoxide and hydrogen peroxide is instrumental for the vitality of any biological organism as demonstrated in infertility of female SOD1 knockout mice (Matzuk et al. 1998) and in the early death of the SOD2 knockouts at three weeks of age (Lebovitz et al. 1996).

SOD enzymes are known to regulate inflammation. A low molecular weight mimetic of SOD has been reported to ameliorate collagen-induced arthritis in rats (Salvemini et al. 2001), similarly as SOD1 has been shown to limit ischemia reperfusion injury in rats (Burton 1985). The expression of mitochondrial SOD2 is upregulated in psoriasis skin lesions (Lontz et al. 1995) and in addition, its expression can be induced by inflammatory cytokines and LPS (Masuda et al. 1988, Akashi et al. 1995, Visner et al. 1990). The third, the extracellular ROS, has mostly been studied as a suppressor of lung inflammation (Folz, Abushamaa & Suliman 1999, Yao et al. 2010, Auten et al. 2006), while it has also been shown to limit ischemia reperfusion injury (Sjoquist et al. 1991) and experimental arthritis (Yu et al. 2008, Dai et al. 2003, Iyama et al. 2001a).

Catalase, a peroxisomal enzyme that is expressed by nearly all aerobic cells, catalyzes the decomposition of hydrogen peroxide to water and oxygen (Loew 1900, Purdue, Lazarow 1996) and thus, unlike SOD enzymes, it is a true antioxidant enzyme. Local, joint specific expression of catalase is shown to have an inflammation limiting effect in antigen-induced arthritis in rats (Dai et al. 2003).

The thioredoxin system consists of two antioxidant oxidoreductase enzyme families: thioredoxins and thioredoxin reductases. Thioredoxins (Wollman et al. 1988) are kept in reduced state by thioredoxin reductases in a NADPH-dependent reaction and serve as a good example of redox cycling as presented in Figure 5. Reduced thioredoxins are efficient reducers of oxidized disulfides in proteins and peptides (reviewed in (Nordberg, Arner 2001). Thioredoxin reductases possess a selenocysteine residue in the conserved C-terminal sequence, which is required for the catalytic activity (Zhong, Holmgren 2000). Increased expression of thioredoxin has been reported in patients with Sjögren's syndrome and RA (Saito et al. 1996, Yoshida et al. 1999).

The family of glutathione peroxidases consists of eight isoforms in humans. The active site of all glutathione peroxidases contains a selenocysteine (Forstrom, Zakowski & Tappel 1978, Aumann et al. 1997) and mediates a reaction where glutathione is oxidized and hydrogen peroxide is reduced to form water (Rotruck et al. 1973). Glutathione reductase completes the redox cycle and reduces the glutathione back to its reduced state using NADPH as energy source (Fujii et al. 2011)

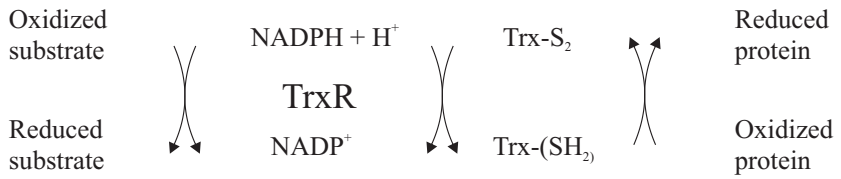


Figure 5. The thioredoxin system as an example of redox cycling
Thioredoxin reductase (TrxR) reduces the active site in thioredoxin (Trx) using NADPH as energy source. Reduced thioredoxin (Trx-SH2) can reduce disulfides in proteins and peptides. This continous circle of oxidation and reduction reactions is called redox cycling and is the basis of most antioxidant systems. Adapted from (Nordberg, Arner 2001).

Mammals express six different peroxiredoxins which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) based on the number and position of cystein residues in the active site (Rhee, Chae & Kim 2005). These selenoenzymes mediate similar type of redox cycling as the thioredoxins and, in fact, five of these can use thioredoxin as the electron donor for their catalytic cycle. Peroxiredoxins reduce and thus detoxify hydrogen peroxide, aliphatic and aromatic hydroperoxides and peroxynitrate (Bryk, Griffin & Nathan 2000, Dubuisson et al. 2004, Poole, Hall & Nelson 2011).

2.4 ROS regulate physiological events

2.4.1 Two levels of regulation

There are two levels of redox regulation in biological systems (Winterbourn, Hampton 2008). The first level of regulation is relatively slow and is maintained by the constant ROS production from the mitochondria and non-inducible ROS producing enzymes and is regulated by slow oscillations of the glutathione buffer system. Protein thiols vary in their redox potential, which means that in a given redox environment some are more reduced than others. When the cell becomes more oxidized, the glutathione buffer's GSH:GSSG ratio decreases shifting the disulfide content of the thiol proteins thus altering the protein properties (Schafer, Buettner 2001).

The second level of redox regulation is more specific and much quicker to respond. The mechanisms of this second level of regulation are based on the kinetic properties of some redox sensitive target proteins. This regulation can take place without obvious changes in the total redox balance of the cell (Go et al. 2004). Protein tyrosine phosphatases with a critical active site cysteine are sensitive to reversible oxidation and inactivation directly affecting cellular signaling (Tonks 2005). Additionally, certain protein kinases and GTPases have key cysteines in their regulatory or nucleotide

binding domains rendering them susceptible to redox-regulation (Cross, Templeton 2004, Heo 2004). Examples of immunologically important proteins subject to redox regulation include PTPN22 (Tsai et al. 2009), T cell receptor and its signaling molecule lck (Cemerski, van Meerwijk & Romagnoli 2003) and the co-stimulatory molecule VLA-4 which is also found in the immunological synapse (Kim et al. 2010, Laragione et al. 2003). Increase in cellular oxidation status has also been reported to support activation of the pleiotropic cytokine TGF-beta, which is involved in maintaining the suppressor function and Foxp3 expression of CD4+Cd25+ regulatory T cells, Th-17 differentiation, wound healing and immune suppression (Jobling et al. 2006). In addition, oxidative stress in inflamed joints has been shown to induce the physical translocation of linker of activation of T cells from the plasma membrane to cytosol, thus impairing T cell receptor signaling (Gringhuis et al. 2002, Gringhuis et al. 2000).

2.4.2 ROS in pathogen defense

Patients suffering from chronic granulomatous disease (CGD) (Bridges, Berendes & Good 1959) lack functional NOX2 complex. This rare (birth rate from 1/200 000 to 1/250 000) (Winkelstein et al. 2000) congenital disease is caused by mutations in any of the subunits of the ROS producing complex and is characterized by recurrent life threatening bacterial and fungal infections (van den Berg et al. 2009, Holland 2010). The most commonly affected subunit is the catalytic CYBB, giving rise to the x-linked form of CGD and accounts for approximately 70 % of the cases (Roos et al. 2010b). The autosomal forms of CGD are divided into cases with mutations in *CYBA* (Roos et al. 2010a) (6 %), *NCF2* (Roos et al. 2010a) (6 %) and in *NCF1* (Roos et al. 2010a) (20 %). Only one patient with mutated *NCF4* (Matute et al. 2009) has been described so far. Additionally, mutation in *RAC2* (Ambruso et al. 2000, Williams et al. 2000) also results in human neutrophil immunodeficiency with severe bacterial infections and poor wound healing.

Staphylococcus aureus is the most common finding in bacterial cultures from CGD patients (van den Berg et al. 2009), while *Aspergillus fumigatus* and *Aspergillus nidulans* infections are the most common findings of fungal origin (Winkelstein et al. 2000, Segal et al. 1998). The most important bacterial infections in addition to *Staphylococcus aureus* are *Burkholderia cepacia*, *Serratia marcescens* and *Salmonella* spp (van den Berg et al. 2009, Holland 2010). Importantly, a recent study reported that patients with residual ROS production have improved long term survival (Kuhns et al. 2010).

ROS exert their pathogen killing functions both by directly being toxic to the intruders and more importantly by activating neutrophil primary granule proteases (Reeves et al. 2002, Segal 2005). More recent evidence has introduced the concept of neutrophil

extracellular traps that enable capturing of yeast conidia and hyphae in a NOX2 complex dependent mechanism (Bianchi et al. 2009).

The increased occurrence of hyperinflammatory and autoimmune manifestations among CGD patients and heterozygous carriers has received increased attention. Inflammatory bowel disease (IBD) and other gastrointestinal manifestations are the most common non-infectious inflammatory manifestations among CGD patients (Marciano et al. 2004, Marks et al. 2009, Kobayashi et al. 2008). Anti-phospholipid syndrome, IgA neurophathy (De Ravin et al. 2008) Kawasaki disease (Yamazaki-Nakashimada, Ramirez-Vargas & De Rubens-Figueroa 2008) and polyarthritis (Lee, Yap 1994) are other autoimmune manifestations reported to occur in CGD patients. In addition, female CGD carriers are reported to exhibit an increased susceptibility to SLE and lupus erythematosus -like symptoms (De Ravin et al. 2008, Gomez-Moyano et al. 2010, Badolato et al. 2003, Ananworanich, Shearer & Abramson 2000, Cobeta-Garcia et al. 1998, Dohil et al. 1997, Schmitt et al. 1995, Manzi et al. 1991, Smitt et al. 1990, Strate, Brandrup & Wang 1986, Stalder et al. 1986).

Most CGD patients are prophylactically treated with mold-active antifungal and antibacterial compounds, while the only curative treatment for CGD is stem cell transplantation. CGD is a good candidate disease for gene therapy, as expression of the functional variant of the mutated gene can rescue the NOX2 complex function and restore phagocyte ROS production. Researchers are actively developing improved gene delivery techniques to achieve long standing and high enough expression rate of the delivered gene (Segal et al. 2011).

As discussed earlier, CGD can be caused by several different mutations affecting any of the genes coding for the NOX2 complex subunits. Similarly, several translational mouse models are used to experimentally address the CGD phenotype. The most commonly used models include knock out strains of *CYBB* and *Ncf1,* both strains replicating the defects in pathogen defense and associated hyperinflammation (George-Chandy et al. 2008, Jackson, Gallin & Holland 1995). These models, together with other NOX2 complex related mouse models are discussed in more detail in chapter 2.4.4.2.

2.4.3 ROS as immune regulators

As discussed in earlier, the immune system and ROS are linked together in numerous different ways. However, modern science is only starting to understand the cascade starting from ROS sensitive molecular sensors that induce the cascade leading to regulation of local and systemic inflammation.

Genetic association studies confirm the functional data and support the connection between NOX2 complex and autoimmunity. *NCF4* (Olsson et al. 2007) as well as *NCF1* are associated with RA (Olsson et al. 2011), while *NCF2* is associated with SLE

(Jacob et al. 2012, Cunninghame Graham et al. 2011) and Chron's disease (Muise et al. 2011). Similarly, *NCF4* and *RAC2* are associated with Chron's disease (Muise et al. 2011, Rioux et al. 2007, Somasundaram et al. 2012, Roberts et al. 2008), while *RAC2* is the only NOX2 complex component so far with association with MS (Sironi et al. 2011).

Based on today's knowledge, we can separate three different ways, how ROS affect inflammation (summarized in Figure 6). Firstly, metabolic ROS and ROS from inflammatory neutrophils may promote inflammation. Secondly, T lymphocyte activity is dramatically downregulated by monocytes in a ROS dependent manner and thirdly, also inflammation without T cell involvement can be downregulated by NOX2 derived ROS.

2.4.3.1 The traditional view

Oxidative stress i.e. overload of oxidant production is a hall mark of neutrophilic inflammation and suggested as a marker of disease activity in RA (Kundu et al. 2012) and SLE (Shah et al. 2011). The concept of ROS as proinflammatory mediators has been largely justified by experiments assessing the effect of antioxidant administration on inflammation severity (discussed in 2.3.1). In addition, experimental evidence suggests that ROS deficiency leads to less severe influenza virus inflammation (Snelgrove et al. 2006a, Snelgrove et al. 2006b). Administration of SOD3, catalase (Ostrakhovitch, Afanas'ev 2001, Dai et al. 2003), vitamin E (De Bandt et al. 2002) as well as the natural bioflavonoid rutin (Ostrakhovitch, Afanas'ev 2001) suppress joint destruction and inflammation in animal models of arthritis. Experimental data collected by using inflammation models in mice and rats supports the hypothesis that presents antioxidants as anti-inflammatory. However, these results are in sharp conflict with evidence from large clinical antioxidant studies that have not found any inflammation suppressive (Rosenbaum et al. 2010) or other beneficial effects for the daily intake of large amounts of dietary antioxidant supplements (vitamins A, C and E). As revealed by a recent Cochrane review analyzing clinical trials with the total of almost 300 000 subjects, the intake of antioxidant preparations may even increase general mortality (Bjelakovic et al. 2012).

Similarly as in mouse models of arthritis, ROS that is produced in the inflamed foci during the active inflammatory phase of SLE, give rise to local increase in oxidant load (Avalos et al. 2007). Excessive ROS production as a result of pathological immune activation may, in fact, prove as a clinically useful biomarker for the disease activity (Shah et al. 2011, Minhas, Das & Bhatnagar 2011).

Furthermore, ROS modified neo-epitopes and following epitope spreading to autoantigens have been hypothesized to play a role in the initiation of autoimmune inflammation (Kurien, Scofield 2008). Natural polyreactive antibodies also acquire

improved binding to autoantigens upon modification by physiological levels of ROS (Dimitrov et al. 2008). Both these hypothesis support the idea of autoimmunity being originally triggered by infection and associated ROS response. Alternatively, these suggested mechanisms can be understood as means of autoimmunity progression as it is known that the anti-citrullinated protein antibody repertoire expands during early disease progression in RA (van der Woude et al. 2010). It should be noted, however, that the source of antigen/antibody modifying ROS has not been conclusively experimentally addressed.

Taken together, these results suggest that ROS modulates local inflammation and may facilitate the development of autoimmunity. However, systemic administration of antioxidants has repeatedly failed to moderate autoimmune inflammation.

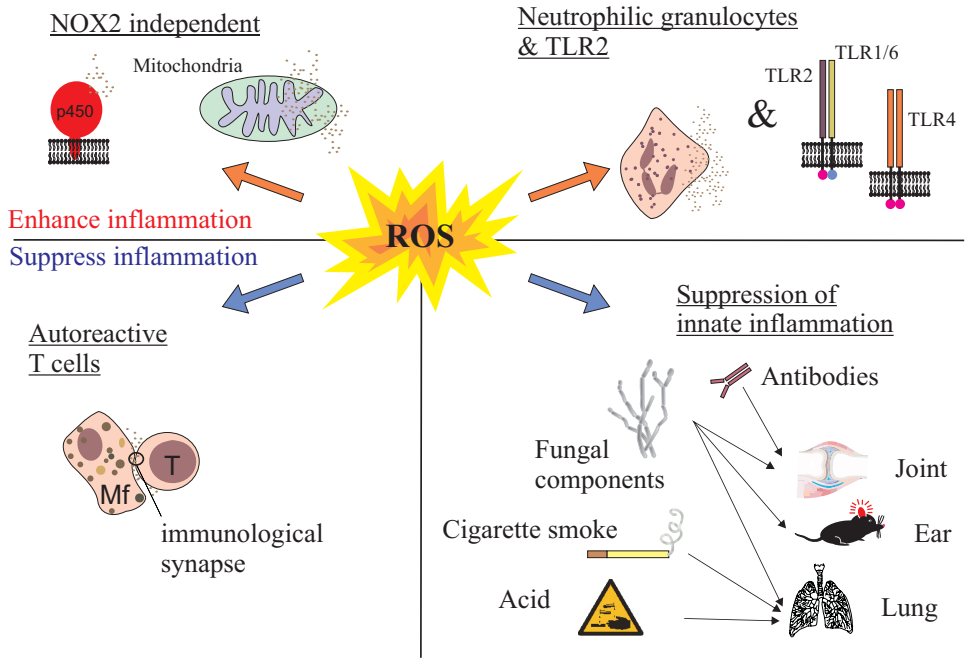


Figure 6. ROS can both suppress and enhance inflammation
Mitochondrial ROS and neutrophil secreted NOX2 complex derived ROS locally enhance inflammation. Monocyte derived NOX2 complex ROS suppress both adaptive and innate immune responses.

2.4.3.2 ROS suppress T cell activation

Ncf1, the gene coding for the activator subunit of the NOX2 complex was identified as an important regulator of pristane-induced arthritis in rats using positional cloning approach (Olofsson et al. 2003). For the authors' surprise, rats with lowered ROS production were more susceptible to arthritis than their littermates with intact

superoxide production. Similar results were obtained from mouse (Hultqvist et al. 2004) and some years later it was revealed that it is monocyte/macrophage derived ROS that suppress T cell autoreactivity (Gelderman et al. 2007). In addition, the lack of functional NCF1 gives rise to sporadic cases of spontaneous arthritis in post-partum female and elderly mice (Hultqvist et al. 2004, Lee et al. 2011).

Furthermore, the severity of experimental autoimmune encephalomyelitis (EAE), the animal model of MS, was also suppressed by phagocyte ROS (Hultqvist et al. 2004) and similarly, MS patients with more severe disease are reported to produce lower levels of ROS (Mossberg et al. 2009). Further support for the immune suppressive character of ROS is derived from an inflammatory disorder called the SAPHO syndrome, where the disease phenotype was shown to be associated with reduced oxidant production (Ferguson et al. 2008). And similarly also a mouse model of lupus was recently shown to be suppressed by NOX2 complex derived ROS (Campbell, Kashgarian & Shlomchik 2012). Remarkably, all this data is generated *in vivo* and comparable results arise from experiments using both human patient material and animal models.

Although a closer analysis of the effects of ROS on adaptive immune system is underway, there is already enough evidence to suggest that the activity of all major Th cells is regulated by phagocyte ROS. Firstly, ROS was reported to suppress Th1 activity (Gelderman et al. 2007). These results were followed by a report showing downregulation of IL-17 production by NOX2 complex derived ROS (George-Chandy et al. 2008) and the following year, NOX2 complex derived ROS were reported to down regulate Th2 type of immunity (Hagenow et al. 2009). NOX2 derived ROS suppress T cell autoreactivity in an antigen dependent fashion, suggesting that the critical ROS are secreted during the process of antigen presentation. In addition, a recent report describing the ROS dependency of regulatory T cell induction by monocytes, support the concept of the anti-inflammatory nature of monocyte derived ROS (Kraaij et al. 2010).

Also structural aspects support the idea of ROS dependent immune suppression to take place at the sites of antigen presentation i.e. in immunological synapses. MHC class II-peptide complexes and lipid rafts accumulate in immunological synapses (Hiltbold, Poloso & Roche 2003) and similarly, NOX2 complex components accumulate in lipid rafts upon activation (Shao, Segal & Dekker 2003). This allows the concreted action of ROS production and antigen presentation close to the target T cell. The exact molecular targets that mediate the immune suppression are not known and undeniably, the secreted ROS affect in addition to the T cell also the ROS producer (monocyte) itself.

2.4.3.3 ROS suppress innate immunity

The third pathway how ROS regulates immunity, is the innate suppression pathway. This pathway has been studied using thioglycollate peritonitis (Segal et al. 2002), acid aspiration-induced lung inflammation (Segal et al. 2007), intrasynovially induced zymosan-induced arthritis (van de Loo et al. 2003) and cigarette smoke-induced lung inflammation (Yao et al. 2008). Furthermore, sterile ear inflammation induced by a number of bacterial and fungal components such as (β(1–3)(1–6)-glucan (from *Saccharomyces cerevisiae)*, mannan (from *Saccharomyces cerevisiae)*, and lipoteichoic acid (from *Staphylococcus aureus*) is enhanced in mice deficient in phagocyte ROS production (Schappi et al. 2008). Similarly to the T cell dependent immune suppression, the ROS mediated suppression of innate inflammation is governed by ROS producing monocytes as demonstrated recently by Deffert et al. using sterile ear inflammation model induced by intradermal β1,3-glucan from *Alcaligenes faecalis* (Deffert et al. 2012).

The mechanism how ROS suppress innate immunity has received increased interest during the last years. Already ten years ago, it was noted that dying neutrophils and phagocytizing macrophages from CGD patients secreted less anti-inflammatory mediators than cells from healthy controls (Brown et al. 2003). There are only sporadic observations how the innate immune suppression is induced by ROS. By using zymosan-induced lung inflammation, researchers have found that ROS induce an increase in the level of the anti-inflammatory transcription factor Nrf2 (Sussan et al. 2009) in the cell nucleus (Segal et al. 2010). In addition, a specialized subset of immunosuppressive macrophages, namely myeloid derived suppressor cells, are suggested to mediate immune suppression at least in part, by secreting ROS (Corzo et al. 2009). All these observations are very recent and more research is needed to understand how ROS suppress inflammation. With increasing knowledge, it will be intriguing to see how similar or dissimilar are the cellular and molecular mechanisms mediating the ROS dependent adaptive and innate pathways of immunosuppression.

2.4.4 Experimental aspects

Reactive oxygen species are short-lived oxidants with limited diffusion rates. Hydrogen peroxide can diffuse up to 1.5 mm from the production site and can readily pass cell membranes. Superoxide is shorter-lived, membrane impermeable and can easily dismutate to hydrogen peroxide (Winterbourn 2008). Other ROS are much less studied in biological context. When the diverse ROS are combined with all the dynamic antioxidant systems present in the biological system, the result is a delicate and carefully regulated balance that is challenging to study. There are different *in vitro* and *in vivo* strategies to dissect the role of ROS in biology and all approaches have their strengths and weaknesses as outlined below.

2.4.4.1 Measuring ROS

There is a long-standing tradition in measuring oxidative stress-induced changes in cells. These methods do not directly measure ROS, but are ways to indirectly assess the extent of ROS-induced damage in the target tissue or cell population. DNA damage can be measured by using the comet assay (Karlsson 2010) or by staining for the DNA double strand breaks (Monks et al. 2006), while mass spectrometry is the recommended approach to study lipid peroxidation (Yin, Xu & Porter 2011). It should be noted, that these assays detect cellular damage and are of limited or no use when studying ROS as physiological messengers.

ROS can be measured in numerous different ways as reviewed by (Maghzal et al. 2012). Luminol and isoluminol are luminescent probes, with closely related chemical structures, that produce light upon contact with radicals. Luminol is cell permeable, while isoluminol cannot pass the cell membrane, thus making it a good probe to analyze extracellular oxidant production (Lundqvist, Dahlgren 1996). These probes are not toxic to cells and can be used for *in vitro* and *ex vivo* cell assays and the luminescence can be recorded over time after the addition of the desired stimulant. However as a cell suspension based assay, this technology is of limited use, when the identity of the ROS producing cell type needs to be resolved.

Dihydrorhodamin-123 (DHR-123) overcomes the problem of cell type identification in phagocyte ROS production analysis as it is used in flow cytometry and can be combined with cell surface markers (Emmendorffer et al. 1990). However, as the fluorescence emission spectrum of DHR-123 (excitation with the blue laser at 488 nm) is wide, it can only be combined with conjugates with different excitation wavelengths. Thus, a modern flow cytometer is preferred when the ROS production of any population defined with more than one surface marker is desired. DHR-123 is fast, cost effective and easy to use. The limitation of this, and other flow cytometry based techniques is demonstrated in dense cell populations, as ROS from other cells may diffuse and give rise to false positive ROS signal in neighboring cells (Van Pelt et al. 1996, Bylund et al. 2010).

As already mentioned all ROS are extremely reactive and resemble each other in their chemical properties. In addition, reactive nitrogen species may also react with ROS sensitive dyes and further complicate the interpretation of the results. In *in vitro* assays, the issue of reactive species cross-reactivity can at least in part be overcome by using enzymes that enzymatically remove specific ROS from the assay. For example adding SOD or a SOD mimetic will quickly convert superoxide into hydrogen peroxide and the use of catalase removes hydrogen peroxide. These enzymatic approaches are suitable for exclusion the effects of certain ROS, but are not enough to collect evidence to point out any specific ROS. Some ROS probes are reported to be selective for only one type of ROS as reviewed in (Kalyanaraman et al. 2012), but these reports should

be interpreted with some precaution as the probes are often validated for only a limited set of reactive species, most often for superoxide and hydrogen peroxide, thus ignoring the potential cross-reactivity with other reactive species. Due to the technical limitations of ROS and their related nitrogen species measurements *in vivo*, the following *in vivo* discussion does to not even attempt further specify the roles of different reactive species in inflammation.

2.4.4.2 Studying ROS biology

The cheapest and quickest way to study ROS in *in vitro* cultures or *in vivo* is to use blockers of ROS production. Unfortunately, the use of blockers easily introduces technical artifacts that may severely compromise the reliability of the obtained results. Diphenylene iodonium (DPI) and apocynin are commonly used NOX inhibitors, which in addition to blocking the NOX enzymes, also fundamentally alter cell metabolism. To illustrate this, genetic and shRNA mediated inhibition of NOX2 complex function (van Bruggen et al. 2010) could not reproduce findings reported earlier in a highly cited journal using DPI and apocynin induced blockage of the NOX2 complex (Dostert et al. 2008). Thus, when scientific reliability is pursued, the use of chemical and pharmacological blockers of NOX enzymes should be avoided or complemented with more reliable techniques.

CGD patients are deficient in their NOX2 complex function and make an excellent reservoir of natural knockout cells of human origin. These samples are not, however, accessible to all research as the disease is (fortunately!) extremely rare and the accessibility of CGD samples is further complicated by the fact that most affected individuals are young people and children.

A much more expensive and time consuming alternative to chemical inhibitors are the genetically modified animals. Knockout mice of many ROS related genes have been developed during the last years and the most commonly used strain to study NOX2 complex function is the *Cyba* knockout (Pollock et al. 1995). Targeted knockout strains lacking functional *Ncf1* (Jackson, Gallin & Holland 1995), *Ncf2* (international mouse strain resource (IMSR) and *Ncf4* (Ellson et al. 2006b) enable the assessment of the physiological roles of the different NOX complex subunits *in vivo*. In addition to the targeted knock out strain, *Ncf4* has also been subjected to targeted germline mutagenesis to yield a more physiological mouse model to work with (Ellson et al. 2006a).

Most knockout mice are created on 129 genetic background, while most research is performed on black background. Thus, in addition to the inserted gene disruption cassette, in most knockout mice, there is a contaminating gene fragment around the gene of interest compromising the reliability of the littermate controls (Ahlqvist, Hultqvist & Holmdahl 2009, Wolfer, Crusio & Lipp 2002). Furthermore, the complete

removal of a specific protein may have severe and unpredicted developmental consequences that complicate the interpretation of the resulting phenotype.

The *Ncf1* mutated mouse used in this thesis (Hultqvist et al. 2004, Huang et al. 2000) is free from these problems. The *Ncf1*^{m1J} mutation is a spontaneous point mutation and in addition, an extensive SNP analysis has confirmed that the *BQ.Ncf1*.^{m1} strain widely used in this thesis does not carry a contaminating gene fragment from the original C57BL/6J genetic background. Furthermore, this mouse completely and globally lacks the functionality of the NOX2 complex, which results in total lack of inflammatory NOX2 complex derived superoxide. In rats, the same *Ncf1* gene exists in different naturally occurring functional variants (Olofsson et al. 2003). In contrast to the *Ncf1* mutated mouse, the congenic ROS deficient rat model has some remaining NOX2 complex activity. Lastly, ethylnitrosourea induced mutagenesis has produced a mutant strain lacking functional *Cyba* (Nakano et al. 2008).

The emerging knowledge in manipulation of the rat genome (Geurts et al. 2009) will, most likely provide us with an increasing number of genetically modified rats, thus expanding the possibilities and challenges of mouse research into rat research.

The role of different cell populations *in vivo* can be studied by using transgenic mice expressing the desired protein using cell specific promoters. The use of human CD68 promoter results in monocyte specific / predominant expression of the transgene (Gelderman et al. 2007, Gough, Gordon & Greaves 2001). The transgene may be present in several copies in the genome and it integrates in random places, thus potentially endangering the normal gene expression regulation of the host. The emergence of different conditional knockout and knockin mice combined with different tissue specificities will most likely provide the redox field with new tools to study physiological pathways affected by ROS.

2.4.4.3 Imaging ROS

The distribution of large biomolecules such as proteins in the tissues of interest can be studied using histology and special staining methods. The use of conventional imaging approaches in redox biology is of limited use as ROS are short-lived and thus not well suited for *ex vivo* analysis. The use of different *in vivo* imaging techniques enables us to visualize the locations of ROS production. There are two main approaches to quantitate ROS production *in vivo*: genetically encoded fluorescent ROS indicators and injected, mostly luminescent ROS sensitive probes.

The genetically encoded indicators such as HyPer (fusion protein of YFP and OxyR) (Belousov et al. 2006) allow the expression of the desired probe in the cells of interest and can be used to dissect the compartmentalization of the ROS response even within a single cell (Malinouski et al. 2011). HyPer was successfully integrated in C. elegans genome (Back et al. 2012), suggesting that it may be possible to integrate this probe

also in the mouse genome. Another genetically encoded redox sensitive probe, that holds future potential as a ROS marker *in vivo*, is a GFP derivative (roGFP) that has been expressed as a transgene in erythrocytes (Xu et al. 2011). Stable expression of Peredox, which also is a GFP based probe, has allowed the analysis of cytosol redox balance in cultured and primary cells (Hung et al. 2011).

These genetically encoded ROS indicators are reported to tolerate even high doses of ROS. However, when fluorescence is used as a read-out in experiments comparing ROS sufficient and deficient cells or animals, it should be remembered that ROS may quench the fluorescent signal, thus potentially compromising quantitative analysis. In addition, these genetically encoded probes are subjected to the same limitations as other transgenic mice. Crossing the reporter mouse together with the gene of interest is a time and money consuming process that can be further complicated if the desired genes only exist on different genetic backgrounds.

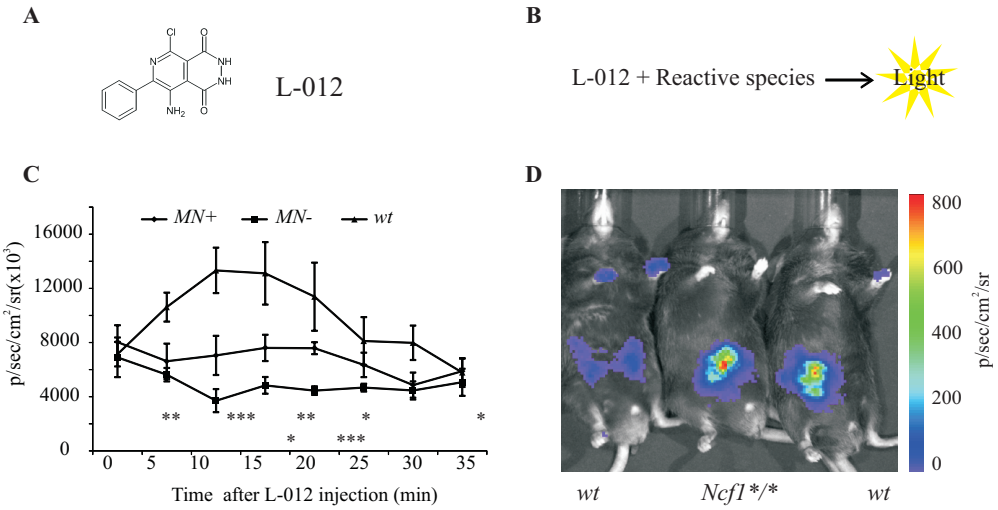


Figure 7. L-012 as a tool to image ROS *in vivo*

A) The chemical formula of L-012. B) L-012 is a chemiluminescent probe that produces light when in contact with reactive species. C) Naïve mice were injected with 20 mg/kg L-012 and the luminescent signal in their front paws was followed for 35 minutes. Wild type (wt) mice gave significantly stronger luminescence signal than the *Ncf1* mutated mice (*MN*-). Also mice with corrected ROS production on monocytes (*MN*+) produced significantly more ROS than their completely mutated littermates. Upper row of asterisks indicate significant differences between wild type and *Ncf1* mutated mice, while the lower row stands for the differences between *MN*+ and *MN*-. D) Mice with ongoing collagen antibody-induced arthritis were injected with 20 mg/kg L-012 and luminescence intensity was visualized. In wild type mice all paws with even mild clinically apparent arthritis emitted ROS signal, while no ROS signal could be detected from the *Ncf1* mutated mice that lack functionality of the NOX2 complex. Modified from (Holmdahl et al. 2012).

L-012 is an isoluminol derivative that holds improved sensitivity when compared to isoluminol and is a promising tool for *in vivo* imaging of ROS (Kielland et al. 2009). L-012 reacts with superoxide and peroxynitrite to emit light that can be detected (Daiber et al. 2004). This method is sensitive enough to detect the base line difference in ROS production between naïve NOX2 sufficient and deficient mice as visualized in Figure 7 and reproduced in (Han et al. 2012). This technique can visualize both very early and more advanced joint inflammation independently of the used arthritis model. Additionally, as an injected probe, it does not require mouse breeding and it is shown to work in nude, white and even black mice (Holmdahl et al. 2012, Kelkka et al. 2012a, Kielland et al. 2009, Pizzolla et al. 2012, Kelkka et al. 2012b). The weakness of this method is its low resolution. The resolution can be, however, improved and even three dimensional images can be obtained with a modern instrument.

3. AIMS

The goal of this thesis was to dissect role of ROS as immune regulators. Previous work in the research group headed by professor Rikard Holmdahl has established ROS as potent suppressors of adaptive immunity (Hultqvist et al. 2004, Olofsson et al. 2003, Gelderman et al. 2007, Hagenow et al. 2009, Gelderman et al. 2006), while the historical long standing scientific tradition describes ROS as enhancers of inflammation. The aim of this thesis was to analyze the role of ROS in T cell independent mouse models of arthritis and to extend the analysis to other immunologically relevant disease models such as cancer and peritonitis. The detailed aims of the sub-projects were as following.

I: To analyze whether SOD3 can regulate inflammation severity in the absence of NOX2 complex derived superoxide.

II: To study the role of NOX2 derived ROS in a T cell independent arthritis model and assess the effect of different immune stimulants on arthritis in mice with and without phagocyte ROS production.

III: To study the role of macrophage ROS in septic arthritis and bacterial infection.

IV: To analyze gene expression profiles of *Ncf1* mutated mice and CGD patients. Different stages of developing experimental murine autoimmune arthritis were included.

V: To examine the role of NOX2 derived ROS in tumor development.

4. MATERIALS AND METHODS

For more detailed technical information the reader is referred to the materials and methods sections of the constituent original publications and manuscripts.

4.1 Mice

All mice were backcrossed onto the C57BL/10.Q.rhd genetic background, a strain with the DBA/1 H-2^q fragment on a C57BL/10 background, shortly referred to as B10.Q (Holmdahl et al. 1986a). The *Ncf1* mutated mouse carried the m1J mutation and the *B10.Q.Ncf1*$^{m1J/m1j}$ (abbreviated *Ncf1*/*)* (Hultqvist et al. 2004). The transgenic *B10.Q.Ncf1*$^{m1J/m1j}$*-Tg(Ncf1)1Rhd* (briefly *MN+*) strain carried a transgene expressing functional *Ncf1* on macrophages using the human CD68 promotor (Gelderman et al. 2007). The transgene was always bred heterozygous and mice denoted as *MN-* are *Ncf1*/** littermate controls.

4.2 Inflammation models

4.2.1 CIA

Collagen-induced arthritis (CIA) was induced under isoflurane anesthesia by injecting 100 µg rat collagen type II (CII) (purified from rat chondrosarcoma as described earlier) (Smith et al. 1975) emulsified in complete Freund's adjuvant (CFA) subcutaneously at the base of the tail.

4.2.2 CAIA

CAIA was induced by a single intravenous injection of the four monoclonal antibody cocktail as described earlier (Nandakumar, Holmdahl 2005). Arthritis was enhanced i.p. five or seven days later with the following immunostimulants: 25 µg LPS (from *E. Coli 055:B5*), 200 µg peptidoglycan (from *S. aureus*), 400 ng pertussis toxin (from *B. pertussis*), 2 mg zymosan A (from *S. cerevisiae*) and 200 µl aluminum hydroxide gel (alum). These were purchased from SigmaAldrich, St Louis, Missouri, USA. Synthetic MPL (25 µg/mouse), flagellin (From *B. subtilis,* 7 µg/mouse), gardiquimod (77 µg/mouse), poly(I:C) (300 µg/mouse) and LM (from *M. smegmatis*, 35 µg/mouse) were purchased from InvivoGen, San Diego, California, USA.

4.2.3 Supportive techniques for arthritis

Scoring

Arthritis development was monitored using a scoring system based on the number of inflamed joints in each paw, inflammation being defined by simultaneous swelling and redness of the affected joint (Holmdahl et al. 1998). Briefly, one point was given for

each swollen and red toe or knuckle and five points for a swollen and red ankle. Maximum score per paw was 15 points summing up to the maximum of 60 points per mouse.

Histology

Formalin fixed joints were decalcified in EDTA, tris and polyvinyl pyrridoline containing buffer (pH 6.9), dehydrated and embedded in paraffin blocks and 5µ sections were stained with either hematoxylin-eosin (HE) or safranin.

Anti CII antibody analysis

Serum anti-CII antibody titers were analyzed as described in (Hultqvist et al. 2004). Briefly, Maxisorb plates (Nunc) were coated over night with 50 µl of 10 mg/ml of rat CII. Plates were blocked with 1 % BSA in PBS for 1h at RT and diluted serum samples (50 µl) were incubated overnight +4ºC. Bound antibodies were detected with biotinylated antibody against immunoglobulin kappa chain and detected with streptavidin conjugated europium (Perkin Elmer) with Victor plate reader (Perkin Elmer, Turku, Finland). Serial dilutions from pooled serum from *B.10.Q.Ncf1*/** mice with CIA were used to create the standard curve.

4.2.4 Peritonitis

Peritonitis was induced with 5% proteose peptone (BD Difco, Sparks, MD, USA) and 10 ng IL-1β (R&D Systems, Minneapolis, MN, USA) in 1 ml PBS. After 18 hours peritoneal infiltrating cells were collected by peritoneal lavage, counted and subjected to differential counting.

4.3 Viral expression vectors

Replication deficient adenoviral E1-partially-E3-deleted AdBglII vectors (developed from serotype Ad5) expressing rabbit SOD3 (Ade-SOD3) or bacterial β-galactosidase lacZ (Ade-LacZ) (Laukkanen et al. 2001) were used in both *in vitro* and *in vivo* experiments.

4.4 Tumor models

The B16-F10-luc-G5 melanoma cells (briefly B16-luc) (Xenogen), which stably express luciferase, injected subcutaneously in flanks under general anesthesia. *In vivo* bioluminescence imaging was performed as described in (Marttila-Ichihara et al. 2008). Mice were injected s.c. in the left flank with 150,000 Lewis Lung Carcinoma cells (ATCC, Manassas, USA) in the volume of 50 µl. Female C57Bl/6 TRAMP mice (Greenberg et al. 1995) heterozygous for the Probasin SV-40 Tag transgene were bred to non-transgenic C57Bl/6 males (Taconic M&B, Ry, Denmark), as well as to *Ncf1* mutated mice on a C57BL/6 background.

4.5 ROS analysis

4.5.1 *In vitro* DHR and flow cytometry

Red blood cells were lysed from heparinized whole blood with hypotonic lysis buffer and leukocytes were surface stained with monoclonal antibodies (Table 2). Cells were incubated for 10 min at 37˚C with 3 µM dihydro-rhodamine 123 (DHR-123; Molecular Probes and Invitrogen Life Technologies) followed by 20 min activation at 37˚C with 200 ng/ml PMA (Sigma-Aldhrich). The cells were acquired using the LSR II flow cytometer equipped with FACS Diva software (BD Biosciences). The results were analyzed with Flowing Software (Cell Imaging Core, University of Turku). The membrane impermeable DNA-binding dye 7-amino actinomycin (7-AAD) was used stain dying cells with compromised cell membrane integrity.

Table 2 antibodies used in flow cytometry

Ab	Conjugate	Clone	Manufacturer
CD16/CD32	Fc-block	2.4G2	BD
CD3e	FITC	145-2C11	BD
CD4	A647	RM4-5	BD
CD8	PerCp-Cy5.5	53-6.7	BD
CD8	PE	53-6.7	BD
B220	Pacific Blue	RA3-6B2	BD
CD11b	eFluor450	M1/70	eBioscience
Gr-1	FITC	RB6-8C5	BD
Gr-1	PE	RB6-8C5	BD
Gr-1	APC	RB6-6C5	eBioscience
Ly6g	PE-Cy7	1A8	BD
Ly6c	FITC	AL21	BD
CD45	APC-Cy7	30-F11	BD
F4/80	APC	BM8	eBioscience

4.5.2 *In vivo* L-012

Isoflurane anesthetized mice were injected intraperitoneally with 20 mg/kg L-012 probe (Wako Chemicals, Neuss, Germany) dissolved in physiological saline (Kielland et al. 2009). The luminescent signal was detected with IVIS 50 bioluminescent system (Xenogen, Alameda, California, USA) after the probe injection. Image acquisition and analysis were performed with Living Image software (Xenogen).

4.6 Statistics

Mann-Whitney U (non-parametric data or data that was not normally distributed) and Student's T test (parametric and normally distributed data) were used for two-group

comparisons, while Kruskall-Wallis (non-parametric data or data that was not normally distributed) or ANOVA (parametric and normally distributed data) were used for comparisons of three of more groups. Intregroup comparisons of arthritis incidence were analyzed using Fisher's exact test and survival analysis in tumor experiments was performed using log rank test. Average ±SEM is presented in all figures and all p-values < 0.05 were considered as significant.

4.7 Genome wide gene-expression analysis

Mouse samples were hybridized on Sentrix BeadChip Array MouseRef-8 v2 (Illumina) arrays and HumanHT-12 v3 Espression BeadChip (Illumina) arrays were used for all human samples. The microarray data was quartile-normalized and log2-transformed. Differentially regulated genes were identified using linear modeling (LIMMA packages, Bioconductor). For all further analysis adjusted p-vales <0.05 are considered as significantly differentially regulated. The canonical pathways were generated through the use of Ingenuity pathway analysis (IPA, (Ingenuity Systems, www.ingenuity.com). Selected transcripts passing inclusion criteria (adj. p-value < 0.05 and FC > 1.5 or FC < -1.5, for mouse spleen samples FC > 2 or FC < -2) were manually classified into three functional categories: interferon regulated genes (IRG), lymphocyte related genes (LC), genes with clear inflammatory function (INFL) and miscellaneous (Misc.).

4.8 Data presentation and validation

Novel, previously unpublished findings were always confirmed in two or more independent experiments with identical setup. Whenever possible, pooled data from all experiments is shown to avoid selection bias and normalization was carried out to adjust for baseline differences when appropriate. Experiments that were performed in different animal facilities were not pooled, but a representative experiment is selected. Similarly, ROS production assays (*in vitro* and *in vivo*) were not pooled as the results were successfully reproduced several times and representative results are presented in several of the constituent papers. To meet the three Rs principles for humane and ethical conduct of animal work (http://www.nc3rs.org.uk), negative results that were without scientific novelty were not repeated.

5. RESULTS

5.1 NOX2 complex derived ROS suppress arthritis (I, II, IV)

The arthritis suppressing capacity of phagocyte derived ROS was replicated in all CIA experiments presented in this thesis. In paper I, *Ncf1* mutated and wild type mice were analyzed in parallel in the same experiment and significantly more severe arthritis was observed the *Ncf1* mutated mice. Similarly, both CIA experiments that were performed to collect samples for genome wide gene expression analysis (paper IV) clearly demonstrated the arthritis suppressing capacity of phagocyte ROS. This is well in line with the previous findings (Hultqvist et al. 2004).

In paper II, *Ncf1* mutated mice that were over 100 days of age, developed more severe collagen antibody induced-arthritis than the wild type mice during the early phase of CAIA. No ROS induced difference could be observed in younger mice (< 100 days old).

5.2 NOX2 complex derived superoxide enhances arthritis that is induced by agents of bacterial origin (II & III)

Neutrophil derived ROS are traditionally considered as inflammation promoting and also this study revealed settings where ROS promotes inflammation. In paper II, *S. aureus*-induced septic arthritis was more severe in *Ncf1* wild type mice than in the ROS deficient *Ncf1* mutant mice. Similarly, in paper III, the wild type mice developed more severe LPS stimulated CAIA than the *Ncf1* mutated mice. In both cases, arthritis is induced or stimulated by bacteria or bacterial components and to dissect further the pathologic process driving the ROS dependent enhancement of inflammation, we continued by assessing the impact of different innate immunity stimuli on the severity of antibody-induced arthritis.

CAIA was stimulated with synthetic MPL and LM (from *M. Smegmatis*), a TLR4 and TLR2 agonist (Doz et al. 2007) respectively. Both substances enhanced arthritis, LM more potently than MPL, but only LM-stimulated arthritis was enhanced by phagocyte ROS. By using TLR4 deficient mice (Poltorak et al. 1998), we confirmed, that the ROS dependent arthritis enhancement was not affected by TLR4 signaling. Thus the results strongly suggest that the observed arthritis enhancement by ROS is mediated via a TLR2 regulated pathway.

Another question we wanted to elucidate in paper III, was the identity of the inflammation promoting immune cells in the ROS sufficient wild type mice. Macrophage predominant transgenic expression of NCF1 did not enhance LM stimulated arthritis and thus, we concluded that the ROS stimulated arthritis

enhancement is induced by neutrophils. Histological analysis of haematoxylin and eosin and safranin stained tissue sections confirmed the presence of massive neutrophil infiltration in the arthritic joints, further supporting the role of neutrophils as mediators of ROS driven arthritis enhancement. These results are in line with previous results from different antibody-induced arthritis models that have been reported to be dependent on neutrophilic granulocytes (Nandakumar, Svensson & Holmdahl 2003, Tanaka et al. 2006, Monach et al. 2010).

5.3 Superoxide dismutase 3 limits collagen-induced arthritis and peritonitis in the absence of phagocyte oxidative burst (I)

SOD3 has been repeatedly shown to suppress arthritis and other inflammatory diseases (Folz, Abushamaa & Suliman 1999, Yao et al. 2010, Auten et al. 2006, Sjoquist et al. 1991, Yu et al. 2008, Dai et al. 2003, Iyama et al. 2001a, Laurila et al. 2009a). The aim of this part of the project was to analyze whether SOD3 can regulate inflammation in the absence of NOX2 complex derived superoxide.

We used *Ncf1* mutated mice that lack NOX2 complex function together with wild type mice to assess the effect of virally expressed SOD3 on arthritis and peritonitis. As expected, CIA was significantly more severe in the *Ncf1* mutated than in the wild type mice (Hultqvist et al. 2004). As reported previously in the literature, the wild type mice with intact ROS production responded well to the SOD3 treatment (Yu et al. 2008, Dai et al. 2003, Iyama et al. 2001b) and the SOD3 treated paws exhibited significantly lower arthritis severity than the control virus treated. In wild type mice, arthritis severity was significantly reduced in the treated paw five days after the treatment injection, which coincided well with high expression of virally produced SOD3. Rather surprisingly, similar results were obtained from the *Ncf1* mutated mice; SOD3 expression suppressed arthritis severity also in the absence of phagocyte ROS production. The observed transient reduction in arthritis severity in both genotypes was restricted to the treated paw as the sum score of the non-treated paws was not affected. This suggests that SOD3 locally restricts inflammation by modulating the innate effector mechanisms, and does not modulate the underlying adaptive arthritogenic process.

The NOX2 complex independent, but SOD3 mediated reduction in inflammation severity was confirmed in peritonitis experiments. Peritonitis was terminated and peritoneal exudate was collected three days after peritonitis induction, at a time point that corresponded macrophage entrance into the inflamed peritoneal cavity. As reported in the literature, the SOD3 treated wild type mice had less infiltrating macrophages than the control virus treated (Laurila et al. 2009b). The reduction in peritoneal inflammation was reproduced in the NOX2 complex deficient mouse, although the difference war smaller. In peritonitis, there was no difference in peritoneal

cellularity between the *Ncf1* mutated and wild type mice, suggesting that NOX2 complex does not regulate proteose-peptone induced peritonitis.

Lastly, both the treatment virus and the control virus induced significant increase in inflammation severity in both inflammation models and in both genotypes investigated. A less immunogenic approach would be needed to perform a more detailed analysis of SOD3 mediated NOX2 complex independent suppression of inflammation.

Taken together, these results suggest that SOD3 can suppress inflammation in the absence of NOX2 derived superoxide. Supporting results, suggesting an anti-inflammatory role for SOD3 even in the absence of NOX2 superoxide have been obtained by using a SOD mimetic (Seleme et al. 2012). The question of the actual origin of the inflammatory radicals remains elusive, and would make an interesting future project.

5.4 NOX2 complex function can be monitored in naïve and arthritic animals with L-012 imaging (I, II, III)

L-012 is an isoluminol derived luminescent probe that produces light upon contact with ROS (Daiber et al. 2004). Previously, this probe has been used to image inflammation *in vivo* (Kielland et al. 2009). Here, the method was further characterized and applied in an extended array of inflammation models. In paper I, L-012 is shown to indicate arthritic paws of wild type mice with CIA and in paper II, the possibilities of the probe were extended to CAIA. In addition to visualization of the late and severe phases of arthritis, the use of L-012 allowed quantitative analysis of the very mild early phase of antibody-induced arthritis that is challenging to evaluate by visual scoring. L-012 imaging corresponded well with inflammation severity and confirmed the visual scoring results. In paper III, the technique was successfully applied in naïve mice. A significantly lower luminescent signal was recorded from the *Ncf1* mutant mice than from the wild type and the transgenic *MN*+ mice.

5.5 The loss of NOX2 complex function results in type I interferon response in mouse and man (IV)

The aim of the project reported in paper IV was to perform genome wide gene expression analysis in ROS deficient *Ncf1* mutated mice and CGD patients. Hypothesis free data-analysis by means of Ingenuity pathway analysis software was complemented with more detailed manual examination of the differentially regulated genes. Type I interferon signature was identified as the major common feature in ROS deficient subjects in both man and mouse. In the next step, the mice were subjected to CIA and upon immune priming, the type I interferon response was further enhanced in the *Ncf1* mutated mice and associated with signs of type II interferon response.

The second transcriptional signature identified in CGD patients was the lymphocyte signature; genes mostly belonging to the B lymphocyte pathway were upregulated in the CGD patient cohort. In CGD patients the B cell compartment was expanded, which at least in part, explained the identified B cell signature. Interestingly, CGD patients had a decreased population of memory B cells as previously reported in (Bleesing et al. 2006).

In mouse, the arthritis development was associated with downregulation of both B and T lymphocyte transcripts in the *Ncf1* mutated mice. This could not be explained by alterations in cell populations, but highlights the role of adaptive lymphocyte dependent immune responses in arthritis initiation and progression.

The third major ROS regulated transcriptional pathway operating in mouse was the inflammatory pathway that mainly consisted of transcripts of granulocyte origin. An emerging expression of granulocyte related transcripts was observed in the *Ncf1* mutated mice during the development of arthritis. This third pathway, however, could at least in part be explained by the slightly increased percentage of granulocytes in the analyzed blood and spleen samples.

5.6 The hyperinflammatory phenotype in the *Ncf1* mutated mice suppress the growth of implanted tumors (V)

The NOX2 complex is known as a potent modulator of immune responses and immune responses are known to regulate tumor growth. The exact role of ROS in the progression of solid tumors has not been addressed.

In this project we analyzed the growth development of four different tumor models in *Ncf1* mutated and wild type mice. *Ncf1* mutated mice developed smaller B16-FLT3L, B16-luc and LLC tumors than the wild type mice, while tumor development in the transgenic TRAMP-model was not affected by the *Ncf1* mutation. The results suggest that NOX2 complex derived ROS only supports tumor growth when the tumor is induced by injection of *in vitro* propagated cancer cells, but cannot affect the growth of endogenous tumors.

There was no difference in the vascularity of the B16-luc tumors collected from *Ncf1* and wild type mice, excluding the probability of NOX2 complex as regulator of tumor angiogenesis. Similarly, neither the expression of extracellular matrix components nor angiogenic factors was affected by phagocyte ROS in the LLC carcinoma model. Closer analysis of the cytokine profiles of the tumors revealed elevated expression of IL-4 in the LLC tumors collected from the *Ncf1* mutated mice, suggesting an immune-mediated, Th2 type of suppression of tumor growth in the *Ncf1* mutated mouse. This finding is in line with previous results showing Th2 mediated immunity as the enhancer of adjuvant-free arthritis in the absence of NOX2 complex derived ROS (Hagenow et al. 2009).

6. DISCUSSION

The five papers presented in this thesis have shed light on very different aspects of immunity ranging from different arthritis models to tumor biology and hyperinflammation observed in CGD patients. The broadness of the scientific focus has enabled a holistic approach to define of the role of ROS in immunity and inflammation.

6.1 Excessive ROS production enhances innate inflammation

The classical concept of ROS as mediators and enhancers of inflammation was further defined in this thesis. Firstly, septic arthritis (paper III) was less severe in the ROS deficient *Ncf1* mutated mice and secondly, in paper II, CAIA was enhanced by NOX2 complex derived ROS. The proinflammatory nature of superoxide was further underlined in paper I, where SOD3, an enzyme converting superoxide into hydrogen peroxide suppressed arthritis and peritonitis.

The results presented in the paper II showed that TLR2 stimulated arthritis is enhanced by NOX2 complex derived ROS. Previous results suggest that arthritis is controlled by many TLRs including TLR2, TLR4 (Abdollahi-Roodsaz et al. 2008a, Abdollahi-Roodsaz et al. 2008b, Abdollahi-Roodsaz et al. 2007), TLR7, TLR9 (Hoffmann et al. 2011) TLR5 (Chamberlain et al. 2012) and TLR3 (Roelofs et al. 2009, Meng et al. 2010, Ospelt et al. 2008). NOX2 derived ROS have previously been reported to enhance TLR4 mediated acute lung injury (Imai et al. 2008), while no other TLR has been linked to arthritis enhancement by ROS. Thus, the potential role of TLR4 as a ROS regulated arthritis enhancer in CAIA needed to be addressed. Our results show that the TLR4 agonist MPL enhanced arthritis, but the enhancement was not regulated by NOX2 complex derived ROS. TLR2 agonist LM, however, enhanced arthritis more potently in the presence of functional NOX2 complex. TLR4 deficiency was not found to affect arthritis severity in LM stimulated CAIA, further supporting the role of TLR2 in ROS mediated enhancement of inflammation.

Optimally, the results could have been further confirmed by using a TLR2 deficient mouse strain. This was not, however, done because both TLR2 deficient mouse strains that are available as alive repositories are knock-out mice (Takeuchi et al. 1999, Wooten et al. 2002) and even extensive backcrossing (at least 3 years of mouse breeding) would not have resulted in a genetically uncompromised *Ncf1* mutated TLR2 deficient mouse. Thus, further *in vivo* verification of the obtained results was not feasible with the tools available today. LM most likely modulates immune responses by binding to other innate receptors than the TLRs (e.g. NOD-like and RIG-I-like receptors and lectins). The work to define the exact receptor binding properties of LM, however, fell outside the scope of this thesis.

The interplay between NOX2 complex derived ROS and TLR2 has been suggested even earlier, as TLR2 is shown to induce ROS production via direct physical contact with NOX2 (Yang et al. 2009). In addition, it has been reported that a TLR2 blocking antibody can impair ROS production in human eosinophils thus suppressing inflammation (Driss et al. 2009).

Transgenic expression of NCF1 restores the functionality of the NOX2 complex on macrophages, but not on granulocytes in *MN*+ mice (Pizzolla et al. 2012). In TLR2 stimulated CAIA, this was not enough to enhance arthritis which enabled us to conclude that the ROS dependent enhancement of TLR2 stimulated CAIA was not mediated by macrophages.

Earlier studies addressing the role of neutrophils in antibody-induced arthritis have largely relied on cell depletion experiments using the monoclonal anti-Gr-1 (antibody clone RB6) antibody. These experiments have shown that Gr-1 positive cells are essential for arthritis development (Tanaka et al. 2006, Nandakumar et al. 2004, Wipke, Allen 2001), but as showed in (Daley et al. 2008) the use of Gr-1 antibody leads to co-depletion of a marked number of monocyte lineage cells. Monach et al. 2010 (Monach et al. 2010) used a knockout mouse (Gfi1-/-) lacking functional neutrophils to conclusively show the neutrophil dependency of serum-induced arthritis. The results presented in this thesis take advantage of a novel approach to study the role of different myeloid cell's role in antibody-induced arthritis. Instead of disrupting the physiological balance by depleting cells, we expressed functional NOX2 complex on monocyte lineage. The results show that monocyte derived ROS do not regulate CAIA and the essential role of neutrophils as regulators of CAIA severity is further supported by the neutrophilic infiltrate in the inflamed joints revealed by histopathology.

The experimental approach in septic arthritis and antibody-induced arthritis did not allow more detailed dissection of the specific radical that induced the enhancement of inflammation. Results from SOD3 experiments completed the picture by suggesting that superoxide mediates the enhancement of inflammation while locally produced hydrogen peroxide is significantly less inflammatory or even anti-inflammatory.

The anti-inflammatory effect of SOD3 has previously only been studied in ROS sufficient *in vivo* models. Here we showed that it counteracts inflammation even in the absence of functional NOX2 complex. Our results do not, however, address the question about the actual source of the superoxide that SOD3 converts into hydrogen peroxide in the absence of the NOX2 complex. Similar results as obtained in this study, were recently reported by Seleme et al. (Seleme et al. 2012), who reported that SOD3 downregulate the expression of proinflammatory mediators in both wild type and NOX2 complex deficient cells. Similarly to our data, the extent of immune suppression was less pronounced in the *Ncf1* mutated mice than in the wild type controls. Taken together, this data suggest that in NOX2 complex sufficient systems SOD3 removes

proinflammatory superoxide that is produced by the NOX2 complex and by some other ROS generating mechanism.

It should be noted that we did not directly address the question, whether the enzymatic activity of SOD3 is required for its anti-inflammatory function. In our study, the arthritis ameliorating effect of SOD3 was strictly limited to the injection site and could not downregulate arthritis in other, untreated paws. Thus, the results support the view of SOD3 as a local modulator of the innate immune system that does not modulate the underlying systemic adaptive immune response.

6.2 NOX2 complex derived ROS suppress T cell mediated inflammation

NOX2 complex derived ROS are shown to downregulate inflammation in many T cell dependent arthritis models as discussed in the literature review above. The arthritis suppressing capability of phagocyte ROS was replicated in several arthritis experiments reported in papers I, II and IV and additionally, an enhanced immune reaction against implanted tumors was observed in paper V.

NOX2 derived superoxide has been reported to suppress the collagen-induced arthritis mediating Th1 (Gelderman et al. 2007) and Th17 (George-Chandy et al. 2008) responses. Downregulation of both Th1 and Th17 responses was discovered in CIA models that were induced by using CFA. In addition, NOX2 complex derived ROS has also been shown to suppress the Th2 polarized adjuvant-free arthritis model induced by an injection of purified CII alone (Hagenow et al. 2009). In paper V, we found elevated expression levels of the typical Th2 cytokine IL-4 in implanted tumors that were collected from the *Ncf1* mutated mice. Our finding is in line with the previous findings as the anti-tumoral response in the ROS deficient mice developed without the use of adjuvants.

The antibody induced phase of CAIA was more severe in mice without functional phagocyte ROS production. This difference was only seen in mice that were over 100 days of age at the time of arthritis induction. An earlier report has shown that serum-induced arthritis is not controlled by NOX2 complex derived ROS. However, it should be noted that the experimental work was performed using young mice and remarkably, the NOX2 complex deficient mice showed a clear trend to develop more severe arthritis than the wild types (Wipke, Allen 2001).

The involvement of the adaptive immune system in regulation of the CAIA phenotype seen in the old mice cannot be conclusively demonstrated without using e.g. TCR knockout mice. The hypothesis suggesting adaptive immunity as the regulator of the early phase of CAIA is supported by the findings demonstrating autoantibody formation and spontaneous arthritis in elderly *Ncf1* knockout (Lee et al. 2011)and post-partum *Ncf1* mutated mice (Hultqvist et al. 2004).

The longer the mouse lives, the longer time its immune system has to interact with the surrounding flora. Results from Diane Mathis' group highlight the role of environmental flora in arthritis induction via the Th17 pathway (Wu et al. 2010). In addition, also the so called SKG mouse with a T cell mediated hyperinflammatory phenotype is shown to develop spontaneous arthritis in conventional animal facility, while clean specific pathogen free housing environment blocks the development of spontaneous arthritis (Yoshitomi et al. 2005). It has also been reported, that *Ncf1* mutated mice exhibit altered responses to bacterial and viral infections (Jackson, Gallin & Holland 1995, Imai et al. 2008) and thus the intestinal normal flora may be different in the genotypes. In the light of current literature it is tempting to speculate that environmental priming may differentially affect the activation status of the adaptive immune system in ROS sufficient and deficient mice and thus alter susceptibility to antibody-induced arthritis.

6.3 Type I interferon signature in the absence of phagocyte ROS in both mouse and man

Paper V describes a pronounced type I IFN response in both CGD patients and in its *Ncf1* mutated mouse model. Type I IFN response has not previously been associated with CGD, but is known to underlie autoimmune inflammation in SLE. Already as early as in the 70's two female heterozygous CGD carriers were reported to exhibit discoid lupus (autoimmune disease affecting the skin) (Schaller 1972). This association of CGD and SLE has been extensively confirmed and interestingly, Humbert et al. even used this information to identify CGD carriers among SLE patients (Humbert et al. 1976). Furthermore, a very recent report described exacerbated lupus in NOX2 complex deficient mouse (Campbell, Kashgarian & Shlomchik 2012). Thus, a lowered ROS response in humans, as well as in mice predisposes to type I IFN associated autoimmunity and SLE associated pathology.

SLE is the prototype autoimmune disease with an upregulated type I IFN signature (Bennett et al. 2003, Blanco et al. 2001). Other related autoimmune syndromes with Type I IFN signature include Sjögren's syndrome and inflammatory myopathies (dermatomyositis, polymyositis and juvenile dermatomyositis) as reviewed in (Pascual, Chaussabel & Banchereau 2010). Despite the increased interest, the role of type IFN I signaling in autoimmune arthritis remains controversial.

In paper V we report simultaneous upregulation of type I and II IFN responses in arthritic mice. Many reports have identified a type I IFN signature in samples collected from RA patients (Thurlings et al. 2010, Kraan et al. 2007, Mavragani et al. 2010, van der Pouw Kraan et al. 2008), while others have identified coexisting type I and II IFN signatures (van Baarsen et al. 2010, Karonitsch et al. 2012) or no type I signature at all (Smiljanovic et al. 2012). Most likely the variation in observations reflects the

heterogeneity of RA patients, and highlights the need for improved classification criteria for different patient groups.

Interestingly, local administration of IFN-beta has been reported to ameliorate adjuvant induced arthritis in rats and similarly, pristane-induced arthritis in rats is milder in animals with an enhanced type I IFN signature (Adriaansen et al. 2006, Olofsson et al. 2007). However, IFN-beta administration could not ameliorate disease activity in RA (van Holten et al. 2005). The variation in results could be, however, explained by the differences between rat and human arthritis.

The mechanism how the observed type I IFN signature arises was not addressed in our study. The first obvious candidate to trigger the type I IFN response would be viral infections. Other possible explanations include bacterial infections (Tomita et al. 2003, Apperloo-Renkema et al. 1995) and similarly, also immunocomplexes containing amino acids released from necrotic /late apoptotic cells are shown to trigger type I IFN signaling in humans (Vallin et al. 1999) as reviewed in (Rönnblom, Alm & Eloranta 2011).

CGD patient's gene expression profiles have been analyzed twice in the litterature and both studies have identified an increased proinflammatory gene expression pattern in the CGD patients. The granulocyte signature was not associated with type I IFN signature in either of the studies. The difference in obtained results may be explained by the selection of analyzed sample material: We used peripheral whole blood, while the previous studies were performed using either stimulated neutrophilic granulocytes (Kobayashi et al. 2004b) or with selected monocytes (Brown et al. 2008). In our study, a pronounced granulocyte signature was observed in the arthritic mice, while naïve mice and CGD patients did not show signs of granulocyte signature. This verifies the good health status of the included CGD patients and our mouse colony.

The third main finding revealed by the gene expression analysis was the lymphocyte signature, which, similarly as the IFN response, was found in both mouse and man. CGD patients showed a pronounced upregulation of mainly B cell related transcripts, whereas in mouse, arthritis development was accompanied by an downregulation of both B and T cell associated genes. The B cell related lymphocyte signature detected in CGD patient samples could be explained by an expanded B cell compartment in the patient samples. Flow cytometry analysis of mouse samples, however, revealed an increase in B cell compartment but no marked differences could be detected in the T cell populations.

6.4 The (dis)similarities between mouse and man

As discussed in (Mestas, Hughes 2004) there are multiple differences between the murine and human immune systems. The two species mostly differ on the molecular

level, while the main immunological pathways share a remarkable degree of similarity. The TLR repertoire serves as a good example to illustrate this. Humans express TLR10, while due to a retrovirus insertion the expression of TLR10 is inhibited in mouse. However, the functionality of the pathogen sensing pathway, the expression of the related TLR1, TLR2 and TLR6 is conserved between the two species (Guan et al. 2010).

Ncf1 was identified as an arthritis regulator in rats, because it is polymorphic between different laboratory rat strains with different susceptibility to pristane-induced arthritis (Olofsson et al. 2003). In mouse, however, none of the NOX2 complex components shows an essential degree of polymorphism. Furthermore, in humans more than one hundred polymorphisms increase variance in phagocyte oxidant production capacity (Holmdahl et al. 2012). Despite the significant differences in NOX2 complex genetics, all three species with abrogated ROS production display highly similar hyperinflammatory phenotype. This functional similarity suggests that despite the gene level differences, the oxidation regulated immunological pathway is highly conserved between the three species.

The translational biomedical research is also challenged by the experimental disease models' capacity to imitate the human counterparts. Recent genome-wide expression analysis results published in PNAS show that acute inflammation models in mouse do not correlate well with corresponding human diseases (Seok et al. 2013). Furthermore, complex diseases that have large phenotypic variability even between different patients are not easy to mimic in experimental set-ups. This is well exemplified in MS, where the use of current animal models has produced important immunological understanding, but at least in part failed to respond to the needs of MS research (Croxford, Kurschus & Waisman 2011).

As discussed in the literature review, different arthritis models reflect different aspects of RA. Acute models display the characteristics of the fulminant inflammation taking place in the affected joints, while chronic models help to dissect the underlying adaptive immune reaction. Thus, one should always carefully interpret the experimental results keeping in mind the limitations of the used model. As exemplified by the TNF-alpha pathway blocking agents that are nowadays commonly used to treat RA (Feldmann, Maini 2001), animal models have instrumentally helped the scientific community to develop novel improved arthritis therapy modalities.

The results presented in the constituent paper IV validate the *Ncf1* mutated mouse model as an excellent tool to study ROS deficient hyperinflammation observed in CGD patients. These results also identify the ROS deficient mouse model a validated tool to study molecular pathways affected in RA patients with type I IFN signature (et al. 2007).

6.5 L-012 Imaging

As presented in three of the constituent papers, an *in vivo* imaging technique that is based on the use of a luminescent probe (L-012) was validated as an easy and reproducible tool to analyze ROS production and inflammation in live mice. The benefits of this method include its high sensitivity and harmlessness to the mouse, thus allowing repeated imaging sessions using the same animals. The effect of L-012 imaging to arthritis development was not directly assessed and would make an interesting future project. Thus, in CAIA experiments that were analyzed with L-012 imaging, all mice were subjected to L-012 injection and imaging associated exposure to anesthetics. In CIA, L-012 imaging was performed either when clinical data collection was terminated or alternatively using a satellite batch of mice. Naïve mice that were subjected for L-012 imaging were not included in arthritis experiments, but, when possible, these mice were reused to setup and optimize novel ex vivo methods after a recovery period.

L-012 imaging can be performed without special genetic inserts allowing its easy use on a broad range of mouse strains with low cost. L-012 cannot be used to detect any single reactive species. It produces light upon contact with superoxide and shows great sensitivity to peroxynitrite (Kielland et al. 2009, Daiber et al. 2004). It should be noted that as a chemical probe L-012 does not differentiate between reactive species originating from different sources. In addition to NOX2 complex activity (Kielland et al. 2009) and nitroglycerin administration (Minamiyama et al. 2006), xanthine oxidase activity (Westover, Harrison & Selemidis 2009) and mitochondrial respiration have been shown to give rise to L-012 luminescence (Daiber et al. 2004).

6.6 Clinical implications and future directions

From the translational point of view, the most important finding reported in this thesis is the type I IFN response that was shared between mice and man lacking phagocyte ROS production. We were the first to successfully explain the immunological pathway leading to the hyperinflammatory findings repeatedly observed in CGD patients. This finding does not directly affect the treatment of the CGD patients. The key problem in these patients is the recurrent severe infections and the curative stem cell transplantation performed for most patients is also likely to stop the autoimmune progression. However, it will be interesting to follow the field and see whether the stem cell transplanted CGD patients with restored phagocyte ROS production will also lose the autoimmunity associated type I IFN signature. Furthermore, this work did not address the origin of the type I IFN signature in the ROS deficient subjects. Hopefully future work will define the role of environmental microbial priming as well as the role of endogenous antigens in the development of type I IFN response in the ROS deficient hyperinflammatory phenotype.

There is an increasing body of evidence connecting ROS and chronic inflammation (reviewed in (Holmdahl et al. 2012)). The observed type I IFN response in the autoimmunity prone ROS deficient subjects offers novel insights into the development of autoimmune inflammation and thus opens new possibilities for therapeutic intervention.

As reported by Hultqvist et al. (Hultqvist et al. 2006), phytol, a pro-oxidant counteract arthritis development in rats. The use of oxidants to suppress chronic inflammation could directly suppress the disease driving adaptive immune reaction. The results presented here support the use of pro-oxidants to suppress T cell activity in chronic inflammation.

In addition to the potent anti-inflammatory properties of the NOX2 complex derived ROS, this thesis increased our awareness of their pro-inflammatory, innate immunity stimulating role in certain situations. Neutrophil derived ROS enhanced antibody-induced arthritis by stimulating TLR2 dependent signaling as presented in the paper II. Arthritis enhancement was, however, transient and, in difference to real autoimmunity, not operated by the adaptive immune system. Similarly, in paper I SOD3 mediated conversion of superoxide into hydrogen peroxide was shown to suppress inflammation locally. In conclusion, the pro-inflammatory effects of ROS operated independently of the adaptive immune system. Even though antioxidants do not hold much potential to treat autoimmune diseases, local administration of antioxidants e.g. SOD3 may prove useful in inflammatory conditions that are not chronic and are locally orchestrated by the innate immune system.

7. CONCLUSIONS

The results presented in this this thesis confirmed the anti-inflammatory nature of phagocyte derived ROS. However, these results also illuminated the proinflammatory properties of ROS that mainly operate in innate immunity induced inflammation. Specific conclusions based in papers I-V are presented below.

I: SOD3 was shown to suppress arthritis severity and peritoneal inflammation even in the absence of NOX2 complex derived superoxide.

II: NOX2 complex derived ROS enhanced antibody-induced arthritis that was enhanced by a TLR2 ligand. The results strongly suggest that this enhancement of innate joint inflammation was mediated by neutrophilic granulocytes.

III: Macrophage derived ROS gave protection in bacterial infections, but enhanced the associated septic arthritis. *In vivo* imaging of ROS production with L-012 revealed a significantly higher ROS signal in the naïve NOX2 complex sufficient mice than in the *Ncf1* mutated mice.

IV: Gene expression analysis revealed a shared type I IFN signature in CGD patients and in the *Ncf1* mutated mice, both lacking NOX2 complex derived ROS. Importantly, this work validated the *Ncf1* mutated mouse as a highly relevant model to examine the ROS deficiency associated hyperinflammation in CGD patients.

V: NOX2 derived ROS was shown to suppress the development of implanted tumors.

These results significantly increased our knowledge on the role of ROS as regulators of innate and adaptive immunity and in pathogenesis of different inflammatory diseases. This thesis described ROS as potent suppressors of adaptive immunity and inducers of type I IFN signature, but moderate enhancers of innate, neutrophilic granulocyte mediated inflammation. Thus, the previously conflicting wievs of ROS as suppressors or enhancers of inflammation could be merged and explained.

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