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Comprehensive Invited Review

Rheumatoid Arthritis: The Role of Reactive Oxygen Species in Disease Development and Therapeutic Strategies

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ABSTRACT

Autoimmune diseases such as rheumatoid arthritis (RA) are chronic diseases that cannot be prevented or cured. If the pathologic basis of such diseases would be known, it might be easier to develop new drugs interfering with critical pathways. Genetic analysis of animal models for autoimmune diseases can result in discovery of proteins and pathways that play a key function in pathogenesis, which may provide rationales for new therapeutic strategies. Currently, only the MHC class II is clearly associated with human RA and animal models for RA. However, recent data from rats and mice with a polymorphism in *Ncf1*, a member of the NADPH oxidase complex, indicate a role for oxidative burst in protection from arthritis. Oxidative burst-activating substances can treat and prevent arthritis in rats, as efficiently as clinically applied drugs, suggesting a novel pathway to a therapeutic target in human RA. Here, the authors discuss the role of oxygen radicals in regulating the immune system and autoimmune disease. It is proposed that reactive oxygen species set the threshold for T cell activation and thereby regulate chronic autoimmune inflammatory diseases like RA. In the light of this new hypothesis, new possibilities for preventive and therapeutic treatment of chronic inflammatory diseases are discussed. *Antioxid. Redox Signal.* 9, 1541–1567.

I. INTRODUCTION

To increase understanding of the pathologic basis of disease, the genes that underlie the involved pathologic pathways should be identified (84). With the developments of the last decades regarding genetic technologies and knowledge about the genome of several species, this should in theory be easy. In practice, however, this only turned out to be true for monogenic diseases, where a change in one gene leads to an obvious change in the highly penetrant phenotype. However, many diseases do not follow simple Mendelian patterns of inheritance, but are complex and dependent on many genes and the interacting environment. Identification of genes underlying such complex diseases therefore requires a different approach. Our group has a long history of trying to identify genes, conferring susceptibility to arthritis and encephalomyelitis in rodents, as models for human diseases. A significant number of quantitative trait loci (QTLs) have been discovered throughout the genome of both rats and mice, linking specific genetic regions to the disease phenotype. From the PIA4 QTL in rats, linked to arthritis severity, a gene was cloned that determines production of reactive oxygen species (ROS) and arthritis severity. This review on the role of oxidative burst in regulating arthritis and the immune system is based on the positional cloning of this gene. The identified arthritis-regulating gene is *Ncf1* (*p47phox*) that is translated to the Ncf1 (p47phox) protein. Ncf1 is part of the phagocytic NADPH oxidase complex and regulates the levels of oxidative burst in phagocytes. Our finding surprisingly showed that a low oxidative burst rather enhanced arthritis, inflammation, and immune responses, which was opposite to the general dogma that ROS, as produced by the NADPH oxidase complex, are harmful. We therefore had to challenge this dogma without any detailed or historic knowledge of the field and we discovered that this field is very complex with many diverging and contrasting reports. This might be due to the fact that many published experiments have only been done *in vitro*, which may not reflect the *in vivo* situation. Moreover, many *in vivo* experiments have been done with non-genetically controlled set-ups (*i.e.*, genetically manipulated strains, most likely with linked genetic fragments, are compared to non-littermate controls, making interpretation of the results difficult). Therefore, we here emphasize our opinion that an unbiased but solid finding of a naturally selected genetic polymorphism isolated on a pure genetic background, will help to clarify the role of redox regulation as determined by the NADPH oxidase complex in the immune response. We here review our findings concerning the discovery of polymorphisms in *Ncf1* and their role in autoimmune disease. We will discuss mechanisms and pathways that might be involved in immune regulation and put them in context to known literature.

II. RHEUMATOID ARTHRITIS

A. The disease

Rheumatoid arthritis (RA) is a rather common autoimmune disease with an incidence of ~1% in the Western world (44). Prevalence, however, varies amongst different populations and depends on the genetic background, as reflected by, for example, the observation that Caucasians are more susceptible to develop RA than Asians and Africans (150). Women are about three times more often affected by RA as compared to men, but the cause of this difference is unknown, although it is likely to be related to both environmental and genetic factors. Probably, sex hormones modulate the disease course and contribute to the observed sex difference. However, their influence is complex; estrogens potently suppress T cell-dependent autoimmune diseases like arthritis and encephalomyelitis in animal models, and it is likely that this is also the case in humans. This is, however, in contrast to effects of sex hormones in systemic lupus erythematosus, where estrogen rather enhances production of antibodies and formation of immune complexes (171). Genetically, not only sex chromosomes are likely to play a role but some autosomal chromosomes as well, since all genes interact with the different context shaped by gender. This is underscored by the regulatory effects of sex steroids on various autosomal genes.

The development of RA is likely to start relatively early in life, at least several years before the clinical onset. The time of

clinical onset seems to be spread along the life span but has a peak around 40–50 years of age. It is believed that genetic factors play a role (~60%) as well as the environment (118). Several genes have been identified and linked to development of RA, as will be discussed below. Also different environmental factors influence both onset and disease course. It has been shown that some infectious agents play a role in causation of the disease, although they do probably not account for a large percentage of cases (112). Dietary factors have also been suggested to influence RA. It is hypothesized that a diet containing high amounts of antioxidants (like vitamins C and E) might protect from the development of disease. However, no strong linkages between RA and serum levels of antioxidants have been described (72). Consumption of certain fatty acids has also been investigated in relation to RA. Omega-3 fatty acids are believed to diminish formation of omega-6 fatty acids that are precursors for arachidonic acid, which is necessary for formation of pro-inflammatory eicosanoids, such as leukotrienes and prostaglandins (99). However, no strong correlations between dietary omega-3 fatty acid intake and reduced risk to develop RA have been found either (141). A factor that has been proven to increase the risk to develop RA is smoking (103). Smoking is associated with the concentration of rheumatoid factor (RF), antibodies to citrullinated proteins, pulmonary involvement, and radiographic damage. Several pathways are likely to be affected by smoking, such as nitric oxide pathways and the redox balance and might thereby disturb immune functions (176).

Although the hallmark of RA is bilateral symmetric polyarthritis, not all patients present with these complaints. Many suffer from fatigue, malaise, weight loss or depression, which have been shown to be related to the disease. This often goes together with some less obvious joint complaints. Most commonly, peripheral joints in hands and feet are involved, whereas involvement of larger joints like shoulders or knees, are often involved later and in more severe diseases. A different pattern of affected joints might indicate another type of arthritis, such as infectious arthritis or osteoarthritis. To help standardize the classification of RA world-wide, the American Rheumatism Association has made a list of seven criteria, of which at least four should be fulfilled by the patient to be diagnosed with classical RA (Table 1) (6). It is important to acknowledge that the classification of RA is a classification of a syndrome with a shared clinical picture, but that several different underlying diseases may lead to this same clinical result (82). Thus, RA should be referred to as a syndrome rather than a single disease.

B. Current therapeutic strategies for arthritis

Since untreated RA has an aggressive disease course, an early and effective treatment is necessary. Today, there is no direct cure for RA available; the main goals of treatment are therefore to ameliorate the symptoms of the disease (*i.e.,* diminish pain and decrease inflammation and joint destruction). Different types of treatments are currently used as summarized below.

1. NSAIDs and glucocorticoids. Nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are often used to decrease the inflammation and pain. Both these drugs act by inhibiting the generation of prostaglandin E2 (PGE2) that is formed from arachidonic acid via the enzymes cycloxoygenase 1 (COX-1) and COX-2. NSAIDs specifically inhibit COX activity and can be either selective (*i.e.,* inhibitors of COX-2) or nonselective (*i.e*., inhibits both COX-1 and COX-2). Both these groups of NSAIDs are commonly used to decrease the symptoms of RA even though there are limitations in their efficacy. Even if these drugs decrease the symptoms, the treatment does not change the long-term fate of the disease. The inhibitory effect of NSAIDs on COX-1 and COX-2 and thereby on prostaglandin synthesis, explains most of their actions (192). COX-1 is constitutively expressed and is involved in production of physiological levels of prostaglandins, while COX-2 is mostly an inducible form with increased expression in inflammatory sites. Overexpression of the COX-2 protein thus plays

Table 1. Diagnostic Criteria of Rheumatoid Arthritis in Humans and Animal Models

Diagnostic criteria	*RA (human)*	*PIA (rat)*	*CIA (mouse)*
Morning stiffness	+	ND	ND
Swelling of hand joints	+	+	+
Swelling of three or more joints	+	+	+
Symmetric swelling of joints	+	+	+
Rheumatoid nodules	+	ND	ND
Abnormal serum rheumatoid factor	+	+	+
Erosions or decalcifications, visible o X-ray	+	+	+
Disease triggers	Unknown	Pristane	Heterologous collagen II in CFA
Clinical onset	40–50 years	Day 12	After boost
Progression	Chronic	Chronic	Chronic

ND, not done.

Points 1–4 should be met for at least 6 weeks. In the right columns it is shown that pristine-induced arthritis (PIA) in rats and collagen-induced arthritis (CIA) in mice resembles human arthritis to a large extent.

an important role in several inflammatory diseases. Also other working mechanisms that are not related to prostaglandin synthesis have been suggested for NSAIDs. For example, NSAIDs were shown to inhibit NF-kB and AP-1. Both these transcription factors are important and could play a role in disease severity (183). In the light of our findings, another interesting proposed mechanism for NSAIDs is that they could inhibit neutrophil ROS production (50).

Glucocorticoids (GC) are effective in controlling joint inflammation and are considered to be one of the most effective types of anti-inflammatory and immune-suppressive substances known (16). GCs play an important role in RA treatment and are used in different doses depending on the indication. GCs not only have a rapid effect but have also been shown to have disease-modifying properties by reducing progression of joint damage (102). However, long-term high-dose treatment leads to adverse effects. This dual effect of GCs has lead to development of improved drugs, such as GCs with lower toxicity or increase in the effective dosage. One such example of designed glucocorticoids is the group of selective glucocorticoid receptor agonists (SEGRAs). Since it is believed that many of the adverse effects are mediated via transcription activating mechanisms while the immuno-modulatory effects are regulated via suppressive pathways, these selective GCs should have less side effects but still be as effective as conventional GCs (16, 160).

2. DMARDs. Disease-modifying antirheumatic drugs (DMARDs) are agents that change immune responses and thereby the disease course in a long-term fashion and more dramatically than NSAIDs. The DMARDs form a group of substances that is frequently used in the treatment of RA and include synthetic compounds such as methotrexate (MTX). Nowadays, DMARDs are the main therapeutic regimen for RA, together with biological agents including receptors, receptor antagonists, or antibodies to immunological factors. MTX was originally developed as a therapeutic for malignancies (43) but low dose MTX is now a standard treatment for RA (195, 199). The cytotoxic effect of MTX is believed to be due to its properties as a powerful antimetabolite for folate, necessary for the synthesis of pyrimidines and purines. The anti-inflammatory effect of MTX however is suggested not to be dependent on this pathway, since folate supplementation in RA patients on MTX does not compromise the clinical efficiency (127). Some of the effects of MTX have been stated to be due to increased release of adenosine. It has been shown that mice treated with low dose MTX show an adenosine-dependent inhibition of inflammation mediated via occupancy of adenosine A2 receptors (29). Recently, *in vitro* studies on human blood lymphocytes has shown that low dose concentrations of MTX induces apoptosis in activated but not resting lymphocytes (55), which might lead to clonal deletion of T cells that are activated by an antigen at the time of MTX treatment. In line with these results, it has been shown *in vivo* that CD4+ T cell populations from active RA patients are reduced after MTX treatment (74).

Another way in which MTX has been proposed to affect the inflammatory response is via influencing the redox balance. Since ROS are important mediators of numerous inflammatory responses, attempts have been made to connect MTX treatment with alterations in ROS production. It has been shown that MTX treatment of peripheral blood neutrophils induces an increase in peroxide levels (63). *In vivo*, ROS are generated in response to MTX in a human T cell line, resulting in apoptosis and reduced adhesion capacity of a monocytic cell line (138). The same group also showed that ROS scavengers inhibit the MTX-induced cell cycle arrest. A more recent study also suggests that the apoptosis inducing effect of MTX is dependent on ROS formation, linking these two theories together. T cell lines have been shown to be more affected than monocytic cell lines by MTX treatment (73). Taken together, this evidence supports an involvement of ROS in the mechanism of action of MTX treatment in RA and implies an important role for ROS in diminishing arthritis pathology.

3. Biological agents. As a result of progress in understanding immune and inflammatory mediators, specific therapies specifically interfering with inflammatory pathways have been developed or are under development. Potential therapeutics derived from such an approach are biological agents that target individual pro-inflammatory cytokines, chemokines, adhesion molecules, proteolytic enzymes, and angiogenic factors. An example of such a target molecule is tumor necrosis factor alpha (TNF-α), considered to be a major pro-inflammatory cytokine involved in many inflammatory disorders. Blockade of TNF-α was first shown to have a profound protective effect on arthritis severity in the CIA model in the mouse (198) and could later be shown to have a significant therapeutic effect on RA (45). Since then, strong evidence supports the beneficial effects of TNF-α inhibition in RA (162). Although the exact mechanism of action is not known, the treatment leads to a partial neutralization of TNF-α, followed by a decrease in lymphocyte migration into the joints, reduction of angiogenesis in the joint, and protection against inflammatory destruction. TNF-α is a central component in the cascade of cytokines release during RA and mediates its effect through binding to either TNF receptor type 1 or 2, found on immune cells and endothelial cells. There are several ways in which TNF-α action can be blocked: anti-TNF-α antibodies (*i.e.*, infliximab and adalimumab) or soluble type 2 TNF-α receptors (etanercept). However, there is a large heterogeneity in the response to these agents and many adverse effects are also recorded. More recently, several other immunological structures are also targeted with biological agents including CD20 on B cells (40), IL-6 (158, 174), and CTLA-4 (107).

III. SUSCEPTIBILITY GENES IN RA

RA is partly heritable, suggesting that upon identification of susceptibility genes, new pathways might be discovered that can lead to new and more effective therapeutic strategies. By means of twin analyses, it is estimated that the heritability of RA is up to 60%. The published concordance rates vary between studies, most likely because of differences in disease severity of the cases studied. The monozygotic (MZ) twin concordance rate for RA is four times higher than the dizygotic (DZ) twin concordance rate, indicating a heritability of 40%–60%. The overall MZ twin concordance rate is 12%–15% (1, 130, 172). Many studies undertaken to identify genes that are responsible for this 60% heritability level showed that the

genetic factors underlying RA are far more complex and variable as anticipated before. The most obvious region that has consistently been shown to be associated with RA is the major histocompatibility complex (MHC) region, located at chromosome 6. However, the 3.6 Mb MHC region is quite dense and contains ~220 genes, many of which are involved in immune regulation. First suggested in 1976 by Stastny *et al*., it is now widely appreciated that DRB1 alleles in the MHC class II region confer a higher significant risk to develop RA as compared to any other single gene or allele as identified so far (178). That different specificities of DRB1 are related with RA has been explained by their sharing of a conserved sequence within the third hypervariable region of the DRβ-1 chain. This sequence, from residue 70–74 and either being QRRAA, RRRAA, or QKRAA, is also referred to as the 'shared epitope'. Although this shared epitope hypothesis implies that the DR molecules with this sequence are directly related to RA, the exact mechanisms are unknown (62). In fact, various mechanisms as well as several different genes have been suggested to account for this MHC effect (81, 151, 182). The putative responsible MHC class II molecule with a specific shared epitope binding pocket, could bind a peptide from a joint-related antigen but might also present peptides from other antigens. As a consequence, effector as well as regulatory T cells might be influenced. It has even been suggested that the shared epitope in fact is the source of the peptide and binds to other MHC molecules itself (182, 206). The presence of the shared epitope is clearly not sufficient or necessary to develop RA. So taken together, despite the well-known genetic influence of MHC class II on RA accounting for maybe >30% of the inheritance, we still do neither know the responsible genes nor the mechanisms that account for the remainder.

Recently some non-MHC genes have been suggested to contribute to RA susceptibility, as found by association studies: PTPN22 (13, 61), CTLA-4 (203), and PADI4 (180). These candidate genes probably represent only the tip of an iceberg of numerous genes influencing this complex disease. It is important to confirm the relevance of each candidate gene and to understand their functional importance. As both linkage analysis and association studies require very large cohorts, it will remain a significant challenge for a long time to discover and confirm new genes, despite the improving technical possibilities. Animal models provide an opportunity to decrease the statistical thresholds through fixation of the environment and breeding of large genetically controlled cohorts, which makes linkage analysis possible. After identification of the major QTLs, it is possible to isolate the contributing genes by breeding them into congenic strains. Although this is time consuming and laborious, it is possible to conclusively identify susceptibility genes for complex disease using such a hypothesis free approach. One such gene is modified *Ncf1* that we isolated via this congenic method. Modified functionality of *Ncf1* due to genetic polymorphisms, indeed, had effects that would have been difficult to predict beforehand (132).

IV. EXPERIMENTAL MODELS FOR RA

To identify susceptibility genes for complex diseases like RA, huge populations of patients and controls are required, whereas black and white answers by genetic analysis are seldom observed. Animal models can provide a complementary alternative. Rodents are kept in standardized environmental conditions and genetic backgrounds can be fixed by breeding, to diminish influences of these factors on susceptibility to disease. Rats and mice are available as inbred strains and several useful models are available that mimic various aspects of RA. Obviously, animals are not humans and RA does not occur in inbred rodents (96). As in animals, however, RA in humans is likely to be an induced disease, although we do not know the responsible environmental factors. In addition, human RA is very heterogeneous. Experimental models for RA should resemble human RA in several ways, such as chronicity, tissue specificity, the association with MHC class II, and the production of autoantibodies. Animal models mimicking the classification aspects of RA (Table 1) are thus most optimal and there are a number of such models available. Infectious agents, non-bacterial adjuvants, or cartilage proteins can induce arthritis in animals. In some strains arthritis may develop spontaneously, usually due to a specific genetic deletion or overexpression. Commonly used models are collagen-induced arthritis (CIA) in mice and rats and various forms of adjuvant-induced arthritis in rats (83). Genetic modification through transgenic expression of T cell receptors or TNF-α, or mutations in *ZAP70* or *Ncf1* may lead to spontaneous development of arthritis (88, 120, 155, 201). Some of the inbred mouse strains may also spontaneously develop arthropathy with various degrees of inflammation (28, 98) especially when combined with specific environmental conditions, including hormones, stress, or infections. Since human RA presents in such a diverse range, the different animal models will represent different pathological pathways as found in humans and allow discovery of different genes regulating these pathways. Fixing the MHC class II locus in such animal experiments, allows discovery of other genes, that are less strongly associated with disease (204). These pathways can subsequently be investigated in the human situation.

V. POSITIONAL IDENTIFICATION OF *Ncf1* AS AN ARTHRITIS REGULATING GENE IN RATS

Pristane-induced arthritis (PIA) in rats provides a model that resembles human RA in many aspects, such as symmetrical disease, serum rheumatoid factors, radiographic changes, and swelling of joints for at least 6 weeks (Table 1) (193). In addition, PIA also is a chronic disease. In this model, arthritis is induced by injecting the mineral oil pristane intradermally, neither bacterial products nor exogenous antigens are involved in the disease induction. Arthritis develops 2 weeks after injection of pristane in susceptible rats. Because no joint-derived proteins are used for immunization, no specific immune responses are elicited. However, PIA has been shown to be T cell mediated and dependent on MHC class II (80). For these reasons, PIA is a good model to use, when investigating genes responsible for specific disease characteristics that might play a similar role in the human situation.

Genetically segregating crosses can be used when inducing disease and quantitative disease traits (*e.g.*, paw swelling) can

be linked to specific chromosomal regions containing genes interfering with the disease phenotype. To determine loci responsible for different disease traits in PIA, a rat strain susceptible to disease, the Dark Agouti (DA) rat and a resistant strain, the E3 rat, were crossed and segregation analyses were performed. About 20 QTLs were identified, affecting onset, severity, or chronicity, and they were often shared between different types autoimmune models, including pristine-induced arthritis, collagen-induced arthritis, and experimental autoimmune encephalomyelitis (132, 194). One of the QTLs identified in this way was the *Pia4* locus on chromosome 12. This locus did not only determine susceptibility for arthritis, but also for experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS) (14). By backcrossing to the susceptible DA strain and selecting on resistance for arthritis, this locus was isolated in a congenic strain containing the *Pia4* fragment, as originating from the E3 strain (*i.e*., DA.*Pia4*). By additional backcrossing and identifying new recombinants, the congenic fragment could be minimized and tested for association with arthritis. When the E3-derived fragment was reduced to 300 kb, a physical map was made using PAC/BAC screening techniques, and through sequencing additional markers were selected. Using these new markers, other recombinants were found and a congenic fragment containing only two genes, *Ncf1* and *Gtf2i*, was identified in a small congenic fragment (Fig. 1). Ncf1 (also denoted p47phox—for simplicity we will use Ncf1 for both the gene and the protein throughout) is a subunit of the NADPH oxidase complex that is mainly expressed in phagocytes but also in some other cell types. This complex produces reactive oxygen species (ROS) in phagosomes and the extracellular space upon activation. Gtf2i is thought to be a substrate of Bruton's tyrosine kinase involved in the B cell receptor signaling pathway (205). To investigate which of these two genes

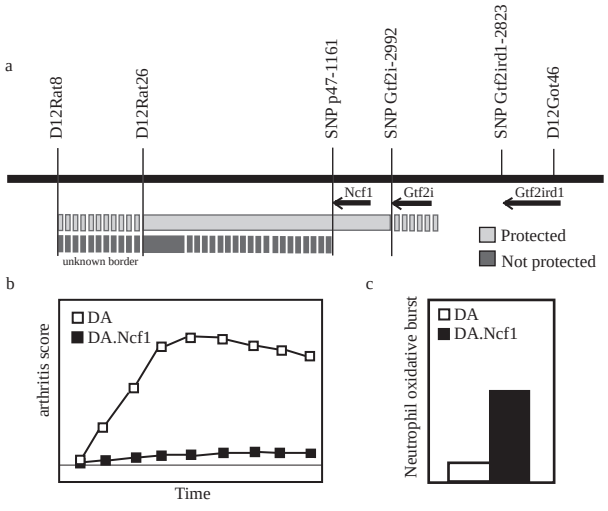


FIG. 1. Positional cloning of Ncf1. (**a, b**) After reducing the size of the QTL Pia4 responsible for the difference in arthritis severity in DA rats and congenic DA.*Pia4* rats, two genes were left in this small fragment that turned out to be *Ncf1* and *Gtf2i*. (**c**) Functional assays revealed a difference in oxidative burst between the two rat strains, indicating that Ncf1 was responsible for the difference in arthritis severity. Based on Olofsson *et al*. (132).

was responsible for the observed differences in PIA severity, both genes were sequenced and resulting sequences were compared between DA and E3 rats. It was found that *Ncf1* contained three polymorphisms (single nucleotide polymorphism, SNP), two of which led to an amino acid change. These SNPs were changed amino acids at position 106 and 153 and resulted in a Met/Val and Met/Thr alteration, respectively. In *Gtf2i* only one polymorphism was found. However, this polymorphism was located outside the translated region. Expression analysis of Gtf2i and Ncf1 did not reveal any differences in mRNA levels between the susceptible and resistant strain. Since only *Ncf1* had mutations in the coding region, this was most likely to be responsible for the protective effect seen in the DA.*Pia4* congenic rat. To clarify the effect of the identified polymorphism in *Ncf1*, functional assays were performed. When determining oxidative burst levels in DA and DA.*Pia4* congenic rats, it was shown that DA rats had significantly lower levels of oxidative burst. Agents that upregulate burst could restore the difference, indicating that *Ncf1* was the gene responsible for the difference in both oxidative burst and susceptibility to PIA. Another confirmation came from a different rat strain; the Brown Norway rat. This rat shares two of the three SNPs with the DA rat, but not the alteration at position 153 and showed normal levels of burst, comparable with those in the E3 rat. This indicated that this one amino acid difference in *Ncf1* between Brown Norway and DA is responsible for the decreased efficacy of Ncf1 and subsequent increased arthritis severity (132).

To determine whether the identified polymorphism was specific for the DA strain, as being an inbred strain, a series of rats was analyzed that derived from the wild. Importantly, the *Ncf1* gene was extensively polymorphic with many recombinants within the gene, including the disease-associated mutation that occurred in approximately half of the wild rats. Thus, a naturally occurring polymorphism affecting both oxidative burst and arthritis had been identified. To determine that the reduced oxidative burst was the causative mechanism in the pathway eventually leading to arthritis, it was necessary to identify another mutation in *Ncf1* that had the same effect. In mice, a QTL was identified on chromosome 5, containing the *Ncf1* gene but we found that *Ncf1* was not polymorphic. However, a spontaneous mutation in the *Ncf1* gene had earlier been discovered in a C57BL/6 mouse strain, also carrying a mutation in the leptin receptor gene (86). Peritoneal macrophages isolated from these *Ncf1*-mutated (C57BL/6J-mdb/db) mice were not able to exert a significant oxidative burst upon stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol 12-myristate 13-acetate (PMA). This mutation affected a splicing site upstream of exon 8 and analysis of Ncf1 expression levels revealed absence of the 47 kD band, whereas the other members of the NADPH oxidase complex were present in normal amounts. When mRNA was isolated and corresponding cDNA was synthesized by PCR, it was shown that different types of aberrant splicing products were formed. To confirm the effect on arthritis, we backcrossed this mutation to the B10.Q strain and ascertained that there was no fragment left from B6, not even closely linked to the mutation. Backcrossing to B10.Q was necessary to be able to test arthritis susceptibility, since this strain expresses the H2-Aq gene that allows development of CIA (21). This Ncf1-mutated B10.Q strain (B10.Q$^{Ncf1*/*}$) developed severe and chronic collagen- induced arthritis (CIA)

and an enhanced autoimmune response to type II collagen, thus confirming the results in the rat. In addition, some of these mice developed arthritis spontaneously in the period after pregnancy, when mice are very sensitive to the development of arthritis due to the drop of estrogen levels (121). We concluded that mutations in *Ncf1* cause a reduced oxidative burst capacity that lead to a higher autoimmune response and a dramatically more severe and chronic arthritis.

$$O2 + e- \rightarrow O2^{-\cdot}$$
$$2O2 + NADPH \rightarrow 2O^{-\cdot} + NADP^{+} + H^{+}$$
$$2O2^{-\cdot} + 2H^{+} \rightarrow H2O2 + O2$$

FIG. 3. Production of ROS. The electron as donated by NADPH reacts with oxygen and forms the oxygen radical. This quickly reacts with water to form hydrogen peroxide (H2O2), and therefore has a very short half-life.

VI. THE NADPH OXIDASE COMPLEX AND OXIDATIVE BURST

The (leukocyte) NADPH oxidase complex is an enzymatic complex consisting of several subunits (Fig. 2). The enzymatic part of the complex is found in the membrane and consists of two proteins, Cybb (gp91phox) and Cyba (p22phox), that together form flavocytochrome b558. The other three subunits are located in the cytosol and play a regulatory role, these are Ncf1 (p47phox), Ncf2 (p67phox), and Ncf4 (p40phox). The three cytosolic phox-proteins form a complex, which remains inactive in the resting state, due to the fact that Ncf1 is in an auto-inhibited conformation. Upon activation, a series of phosphorylations at specific sites, especially on Ncf1, lead to a conformational change of the cytosolic complex and subsequent translocation to the plasma or phagosomal membrane where it, together with Rac and the enzymatic complex, forms the active NADPH oxidase (NOX) complex (66) (Fig. 2). Biochemical experiments have shown that Ncf2 and Rac are absolutely required for the complex to be functional, in contrast to Ncf1, although the level of radical production is lower in complexes without Ncf1, as shown in cell-free systems (48, 105). These data indicate that Ncf2 and Rac are activatory molecules, to induce the electron flow via Cybb, whereas Ncf1 and Ncf4 have a more organizing function in the interaction between Cybb and Ncf2. The complex catalyzes the production of superoxide from oxygen, using NADPH as donor of electrons (Figs. 2 and 3). The produced superoxygen (O2-; with a halflife of 1 μs) quickly reacts with other molecules to form different kinds of ROS, such as free radicals, hydrogen peroxide, and oxidized halogens (*e.g.*, hypochlorous acid), from which hydrogen peroxide is a more stable form with a 1000 times longer half-life (106), allowing it to be operative on biological relevant distances (144). These oxidants are, when produced by phagocyte NADPH oxidases, used to kill phagocytosed pathogens. Other NOX enzymes expressed in other cell types mainly produce ROS to be used in signal transduction or remodeling of the extracellular matrix (110). Nox1 is a homolog of Cybb that was identified first and is abundantly expressed in colon epithelial cells and in various other types of cells, including vascular smooth muscle cells, but at lower levels (10). Other nonphagocytic NADPH oxidases include three Nox enzymes (Nox3–5) and two dual oxidases (Duox1 and Duox2). These Duoxes contain two oxidase modules each: an N-terminal extracellular peroxidase-like domain and a C-terminal Cybb-homologous oxidase part. Nox3, was first identified as an oxidase expressed in the human fetal kidney and most closely resembles Cybb among the Nox family of oxidases (26). Nox4 is highly expressed in the adult and fetal kidney and in cardiovascular tissue and produces a small but significant amount of superoxide in a constitutive fashion (53, 64). Nox5, is abundantly expressed in the testis and spleen (11). Duox1 and Duox2 are highly expressed in the thyroid gland (33). Duox2 is essential for thyroid hormone synthesis; mutations in Duox2 lead to congenital hypothyroidism, even in heterozygous persons (125). Heterologous expression of Duox enzymes in several mammalian cells fails to reconstitute ROS production; suggesting that unknown oxidase components are required for Duox activity. These data indicate that different forms of NADPH oxidase complexes can be formed in different cell types.

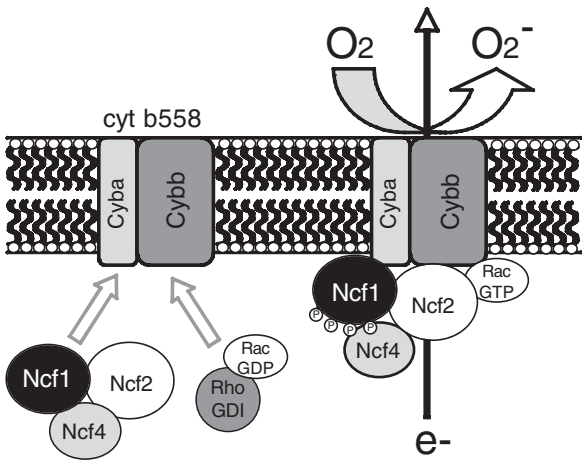


FIG. 2. The NADPH oxidase complex. The NADPH oxidase complex catalyzes the production of superoxide from oxygen, using NADPH as donor of electrons. The (leukocyte) NADPH oxidase complex is an enzymatic complex consisting of several subunits. The enzymatic part of the complex is found in the membrane and consists of two proteins, Cybb (gp91phox) and Cyba (p22phox), that together form flavocytochrome b558. The other three subunits are located in the cytosol and play a regulatory role, these are Ncf1 (p47phox), Ncf4 (p40phox), and Ncf2 (p67phox). The three cytosolic phox-proteins form a complex, which remains inactive in the resting state, due to the fact that Ncf1 is in an auto-inhibited conformation. Upon activation, a series of phosphorylations at specific sites, especially on Ncf1, lead to translocation to the plasma or phagosomal membrane where it, together with Rac and the enzymatic complex, forms the active NADPH oxidase complex

In addition, mitochondria also produce ROS. During normal mitochondrial respiration, ROS are produced and mitochondria can respond to exogenous or endogenous elevated ROS concentrations by increasing their own ROS production. This ROS-induced increase of ROS release (RIRR) can either be depen-

dent or independent on so-called mitochondrial permeability transitions pores (18). Unstable mitochondrial membrane potential and redox transitions can occur after insults like ischemia/reperfusion injury and toxin exposure, with negative consequences for mitochondrial integrity and cellular survival. Exposure to oxidative stress results in higher levels of ROS and when reaching a threshold level, opening of one of the mitochondrial channels is triggered. This subsequently leads to the collapse of the mitochondrial membrane potential and a transient increased ROS production by the electron transfer chain. This ROS can be released into the cytosol and trigger RIRR in other close-by mitochondria. This mitochondrion-to-mitochondrion ROS signaling provides a positive feedback mechanism for enhanced ROS production, leading to significant mitochondrial and cellular injury and potentially to induction of apoptosis (208). However, in the remainder of this review, we will focus on ROS produced by the phagocytic NADPH oxidase.

VII. REDOX REGULATION

It is becoming more and more clear that ROS play a role in (auto)immunity. However, which role they exactly play in the different phases of the immune response (priming, expansion, and effector phase) is still not known. During oxidative stress, ROS are important mediators of damage to cell structures (187). The hydroxyl radical is known to react with all components of DNA, damaging both the deoxyribose backbone and the purine and pyrimidine bases (186). In addition, the side chains of amino acids are susceptible to oxidation events, although some amino acids are more vulnerable than others, as outlined below. Phospholipids are also affected by ROS and their polyunsaturated fatty acids are extremely sensitive to oxidation (170). When ROS are excreted extracellularly, matrix molecules, like collagens and proteoglycans, can be damaged and structurally modified. This might result in increased inflammation and immune activation against neo-epitopes in the joint during arthritis in both experimental and human RA (77). So it is expected that ROS are extremely harmful during oxidative stress at the inflammatory site. However, ROS may act in a beneficial way, thus it is important to recognize the quantitative difference between oxidative stress and redox regulation and the exact location where ROS production takes place. In both extreme oxidative stress responses and during oxidative regulation, ROS are involved, but during oxidative stress massive amounts of ROS are formed that have the damaging effects on cellular contents as described above and the production of oxidants exceeds the capacity to neutralize them (166). In contrast, during redox regulation the amount of ROS produced is much lower and more localized and does not lead to cell death or malfunctioning. To function properly, cells need to maintain an adequate redox balance. This requires precise monitoring and controlling of the redox status within the cell. Organisms have developed different defense mechanisms to protect themselves from a variety of ROS from different sources. These defense mechanisms involve preventive mechanisms, repair mechanisms, physical defense, and antioxidant defense. Enzymatic antioxidants comprise superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Antioxidants that do not work in an enzymatic fashion are ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), and some others. A balance between the levels of these antioxidants and their activity is required for normal functioning of the cell.

The antioxidant defense in cells is often initiated by oxidative modification of an amino acid side chain. The amino acids that have a major role in cellular sensing and the antioxidant response are cysteine, methionine, phenyalanine, tyrosine, and tryptophan. Chemically seen, the sulfur and phenolic redox systems rely on the ability to undergo both reversible and irreversible redox transformations under different oxidative states, to exert their sensing and response properties. Different redox mechanisms drive these changes in oxidation states, like electron transfer, hydroxylation, and exchange reactions. Only when the appropriate intracellular redox potential is met and in the presence of particular oxidants, sensing of the redox balance is possible. Activation of these redox mechanisms often is a response to a change in the intracellular redox status. The changes in amino acids that subsequently occur, act as sensors for changes in the redox balance. There are two major sulfur-based antioxidant systems. One is centered around glutathione as a reducing substrate and the other one is focussed on thioredoxin (Trx). The glutathione system has been shown to be more effective in reducing small disulfide molecules and in directly reacting with ROS. The Trx system is more an antioxidant system used to reduce oxidized cysteine proteins (202). Reduced glutathione (GSH) is present in millimolar concentrations in cells and is one of the most important intracellular antioxidants. It acts as a radical scavenger by trapping ROS that otherwise would react with cellular thiols and disturb the redox equilibrium. GSH exerts its antioxidant function through enzyme-catalyzed reactions. GSH is equilibrized by oxidized glutathione (GSSG) and the ratio of these two can be used as an indicator of the redox status of the intracellular compartment. Normally the intracellular redox status is in a reduced status; accordingly there is a high concentration of GSH in the cytosol. The GSH/GSSG ratio is ~50/1 in the cytosol, dramatically different from the state outside the cytosol (endosomes, lysosomes, extracellularly), which has about a 1/1 ratio (56). Consequently, most cysteines of cytosolic proteins are present as free thiols (-SH) whereas these are oxidized and form S–S bridges extracellularly. It is common belief that the few disulfide bonds that do exist intracellularly are transient and depend on enzymatic reactions, although this view has been challenged recently (19). It has been shown that GSH forms disulfide bridges with many proteins, a process known as glutathionylation. In addition some studies showed, by using redox proteomics, that certain cell types have proteins that contain disulfide bridges even in the cytosol (19, 30). However, this type of disulfide bridges is likely to be regulatory rather than structural. They might exist in more reversible forms, to allow existence of both oxidized and reduced forms of the same protein (56, 57). This could be a way of controlling the function of specific proteins.

In contrast to the intracellular compartment, the plasma has a much more oxidized status, with a GSH/GSSG ratio around 1. This means that plasma has a relatively poor antioxidant potential mediated by thiols, which is compensated for by other mechanisms (36). In contrast, the extracellular environment has an oxidizing nature and theoretically excludes presence of –SH

groups (37). Thiol groups are therefore thought to exist as disulfide bonds within or between proteins or as mixed disulfides with, for example, GSH. Low plasma and extracellular redox levels will influence the proteins expressed on cell membranes that are exposed to the oxidized environment. Despite the oxidizing environment, disulfide bonds in the extracellular domains of some cell surface proteins can be cleaved and exist as a thiol group. The best-characterized examples of proteins containing –SH groups on the cell surface are CD4, the integrin αIIbβ3, and the HIV-1 envelope protein gp120 (79).

Three possible mechanisms explain how disulfide bonds can be reduced in the extracellular environment. The first mechanism is by dithiol–disulfide exchange. This way of reduction is facilitated by oxidoreductases of the protein disulfide isomerase (PDI) superfamily. These are redox active proteins that have reducing activity when present on the outside of the cell membrane. PDI enzymes contain two cysteines that attack the disulfide bond in the substrate, thereby forming a mixed disulfide. This disulfide will undergo an intramolecular thiol–disulfide exchange, resulting in oxidation of the enzyme and reduction of the substrate disulfide bond. This appears to be the operative mechanism for CD4 to reduce its disulfide bonds. The second mechanism is alkaline hydrolysis in which a hydroxide ion reduces a disulfide bridge, thereby generating a cysteine thiol and cysteine sulfenic acid. This mechanism works most efficiently at alkaline pH. The final mechanism to reduce cell surface protein disulfide bonds is by acid-base assisted hydrolysis. This mechanism is theoretically feasible, although not yet experimentally confirmed (79). It should be emphasized, however, that we do not know yet the exact mechanisms whereby redox regulation operates, in particular since it is likely to occur in closed compartments with defined microenvironments. The NADPH oxidase complex has a very restricted localization in the lysosomal/endosomal membrane and in lipid rafts in the cell membrane, suggesting that it regulates the redox balance in enclosed compartments like lysosomes/endosomes and in intercellular synapses. In this way certain protein functions can be modulated in the sense of becoming deactivated or rather more active. It is likely that a defect in NADPH oxidase function alters the extracellular milieu in terms of oxidation levels since cells such as neutrophils cannot burst upon activation, which subsequently might affect the number of –SH groups on cell surface proteins (54). ROS produced into the extra- or intracellular compartment will oxidize the environment and are likely to affect the status of the thiol groups in certain amino acid residues on intracellular or membrane proteins. Previously, Sahaf *et al*. have described a method, how to measure the relative number of –SH groups on the extrafacial membrane of cells. Alexa fluorescent dyes (Molecular Probes, Leiden, the Netherlands) that are covalently linked to maleimide, are allowed to react with cells and will bind to available cell surface –SH groups (Fig. 4). Internalization of the dye is prevented by incubating at 4°C. Subsequently the amount of fluorescence per cell can be measured by flow cytometry as a measure for the number of cell surface –SH groups (153). Specificity of this assays was confirmed by pretreatment of the cells with either oxidized glutathione or *N*-acetyl cysteine that decreased and increased the number of cell surface –SH respectively, as anticipated.

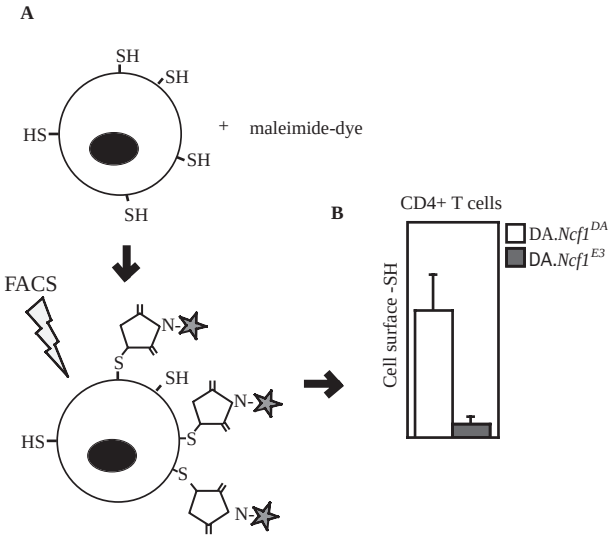


FIG. 4. Ncf1 mutated DA rats have higher numbers of T cell surface –SH groups. (**A**) Cell surface –SH groups can be detected by incubating cells with maleimide that binds to –SH groups, conjugated to a fluorescent probe. The fluorescent signal is quantified by FACS as a measure for the number of cell surface –SH groups at a specific cell type. (**B**) We previously showed that rats with mutated *Ncf1* (DA.*Ncf1DA*) have significantly higher levels of –SH groups on the surfaces of blood CD4+ T cells as compared to congenic DA.*Ncf1E3* rats. Based on Gelderman *et al*. (54).

VIII. THE NADPH OXIDASE, ROS, AND T CELL FUNCTION

The data obtained from our rat and mouse models with polymorphic *Ncf1* showed that T cells play an essential role in arthritis induction and indicate a role for ROS in T cell activation. In rats it was shown that the effect of the *Ncf1* polymorphism was transferable by CD4+ T cells (54, 80, 132). In the mouse model, the *Ncf1*-mutated mice had significantly higher anticollagen IgG levels as compared to heterozygous or wildtype mice after immunization (88). This indicates specific T cell help to B cells to induce this response. In addition, the *Ncf1*-mutated mice had a more severe delayed type hypersensitivity (DTH) reaction upon challenge with collagen in the ear. In search of a mechanism to explain how a decreased oxidative burst capacity could result in increased autoimmunity, we addressed the problem how T cell responses can be dictated by the Ncf1 gene, since we only observed background levels of ROS production in T cells and did not observe a difference between animals with different *Ncf1* alleles (54).

A. The effects of NADPH oxidase-derived ROS on T cell redox levels

If T cells do not or hardly produce NADPH oxidase-dependent ROS, they are likely to be influenced by ROS produced by other cells. Extracellular ROS might affect the extracellular redox level, in the sense of decreasing the number of cell surface –SH groups, as measured by the method of Sahaf *et al*.

(153). When measuring the number of cell surface thiols on T cells, we showed that both naïve mice and naïve rats with a decreased capacity to exert oxidative burst upon stimulation, due to a polymorphism in *Ncf1*, have higher numbers of cell surface –SH groups on CD4+ T (Fig. 4). Since T cells are critical for development of arthritis, explaining the difference in arthritis severity between mutated and wildtype animals, we investigated the role of different levels of T cell surface –SH groups on T cell activity and subsequent arthritis development. We showed that T cells produce increased levels of IL-2 after treatment with GSH, which increases the number of cell surface –SH groups, but cannot enter the cell (111). In the rat model, arthritis is transferable by lymphocytes from immunized rats with a mutation in *Ncf1*, but not from the *Ncf1* wildtype congenic DA.*Ncf1E3* rats. However, we showed that when lymph node cells were treated with GSH to increase the number of cell surface –SH groups, disease could be transferred by CD4+ T cells from the DA.*Ncf1E3* rats to naïve DA rats. The vice versa experiment showed results accordingly; oxidized glutathione (GSSG)-treated lymph node cells from immunized DA rats did not have the capacity to transfer disease anymore, which was due to a decrease in cell surface –SH groups. That the difference in cell surface -SH groups was present in naïve mice and rats, indicates that ROS production affects T cells already in naïve animals. Apparently, an oxidizing milieu prevents exaggerated T cell responses and might prevent autoimmunity in subjects with a proper burst capacity. However, where, when, and how T cell surface redox levels are determined is not known yet, but currently investigated. It might be the case that the reduced ROS production in these animals allows plasma proteins to be more reduced, because cells that are present in plasma produce less radicals. Indeed we observed lower levels of plasma protein thiols in mutated rats and mice, but we do not know if the redox status of plama affects T cell surface thiols or that cell–cell contact is necessary. Phagocytes in blood or tissue that encounter a pathogen or particle (*e.g.*, apoptotic cell debris) to be eaten up will produce ROS upon phagocytosis. It might be speculated that this is not a coincidental side-effect but rather a way to suppress other immune cells, like T cells, to become activated. If many pathogens are present, ROS production will exaggerate and lead to damaging oxidative stress. Constitutive levels of ROS production in the blood might prevent unnecessary inflammation by decreasing the number of T cell surface –SH groups. This effect might either be transferred via the serum, which thus has decreased numbers of –SH groups itself in the *Ncf1* wildtype rats (54) or via cell–cell contact between cells expressing a functional NADPH oxidase and T cells. It has been shown by others that ROS in the RA joint suppresses T cell activation by affecting LAT (linker for activation of T cells) (65). Although ROS are thought to have harmful effects in the joints, it has been shown that rather proteolytic enzymes mediate the cartilage degradation in RA instead of ROS and that ROS have a inhibiting effect on this process (161). This underscores our opinion that the most important role of ROS in arthritic joints is to downregulate the immune response and is not directly responsible for the observed destruction.

APC, comprising dendritic cells (DC), macrophages, and B cells do express a functional NADPH oxidase complex (9, 69, 159) and have close contact with T cells during thymic selection and during antigen presentation in the periphery. It is likely that APCs, by bursting during some point in the initiation of an immune response, influence T cells in their reactivity (Fig. 5). ROS might affect different processes in APC and thereby affect the immune reponse. It has been described before, that ROS affect phagocytosed proteins present in lysosomes or proteins in endosomes, derived from cellular sources (159). pH and redox circumstances in these cellular compartments will affect breakdown of these proteins and subsequent presentation to T cells via MHC. Next to this, it is likely that ROS produced by APC during antigen presentation, affects molecules on T cells or proteins linked to the membrane on the inside, thereby affecting signal transduction events and subsequent activation (8). Another possibility is that ROS affects membrane composition of the T cell, resulting in changes in membrane protein organization and thereby affecting intracellular responses (167). Clearly, regulation of the numbers of thiol groups on the T cell membrane is one of the key mechanisms regulating T cell activity *in vivo* and thereby also determines the development of autoimmune disease such as arthritis (54).

B. Do T cells produce ROS via NADPH oxidase?

So T cells are keyplayers in the increased autoimmunity as observed in animals with *Ncf1* alleles mediating a reduced ox-

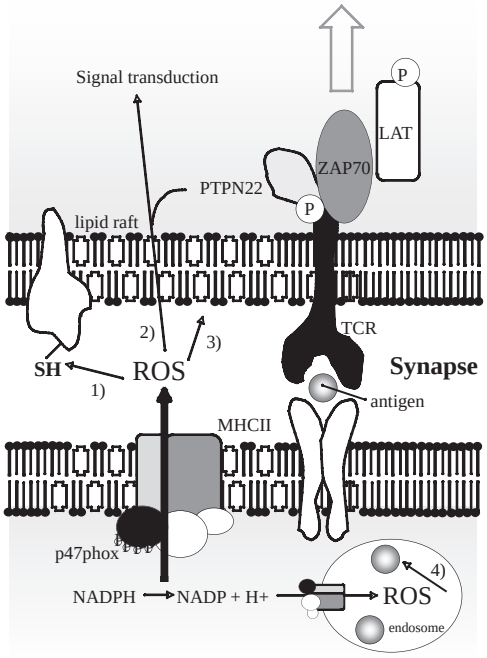


FIG. 5. The potential influences of ROS as produced in the immunological synapse on T cell activation. During antigen presentation ROS produced by the APC into the immunological synapse, might influence T cells in different ways. a). ROS might affect the number of –SH groups on T cell membrane proteins; b) ROS might affect the lipid raft formation in the T cell membrane; c) ROS might affect proteins present just on the inside of the T cell membrane, that play a role in signal transduction events; d) ROS might affect processing of proteins in endosomes, resulting in different antigen presentation.

idative burst, but the question remains if the leukocyte NADPH oxidase is expressed in the T cells themselves or that ROS are produced by other cell types that thereby influence T cell activation. T cells play a pivotal role in the initiation of immune responses and are essential cells in the pathogenesis of RA (173). As in other cells, mitochondria are the major source of cytosolic ROS in T cells. Mainly O2- is produced, which is scavenged by superoxide dismutase (SOD) to become H2O2 that can pass the mitochondrial membrane freely to go into the cytosol. Here it has a role in cellular signaling or in apoptosis induction. It has been shown that upon T cell receptor (TCR) stimulation, ROS is produced in peripheral blood mononuclear cells (197). It is likely that this ROS production does not occur via the NADPH oxidase, since T cells are thought not to express a functional NADPH oxidase comparable to that in phagocytes (144). Recently, however, two groups reported evidence for ROS production via an NADPH oxidase complex in T cells (68, 92). Jackson *et al*. (92) showed that T cells can produce ROS via a functional NADPH oxidase complex upon TCR stimulation with anti-CD3 antibody. To investigate this issue, they used primary T cell blasts from naïve *Ncf1* knockout mice as a comparison and stained for ROS using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) and dihydroethidium (DHE). In our mice with mutated *Ncf1*, however, we could not find any differences in T cell ROS production upon stimulation with anti-CD3 antibodies. We used another, more sensitive assay, staining ROS with dihydrorhodamine (DHR123), that gave the largest difference in staining of PMA stimulated phagocytes from *Ncf1* wildtype versus mutated animals. Jackson *et al*., however, expressed their data in percentage increase as compared to unstimulated controls, which leads to increases in ROS production of up to 400 percent, but might still be very low in absolute numbers. Others also argued for ROS production by T cells by a NADPH oxidase-like complex based on inhibition of an oxidative response *in vitro* by DPI, a specific inhibitor of this complex (35).

ROS production in T cells does not necessarily have to be derived from mitochondria or NADPH oxidase-like complexes; also certain cytosolic enzymes do produce ROS as a byproduct during their enzymatic reaction. Examples of such enzymes are cyclooxygenase, phospholipase A, and lipooxygenase (117). When we tried to measure oxidative burst in T cells from the rats or mice with a mutation in *Ncf1*, we could not observe any differences between mutated and wildtype animals despite efficient methods of stimulation like PMA or CD3 crosslinking, although we did see some low levels of DHR123 fluorescence (87) (Gelderman *et al*., unpublished results). It is difficult to be conclusive where these low levels of ROS derive from. Since radicals have the ability to cross membranes and some forms of ROS have a significant higher half-life as compared to O2.-, it is possible that the background levels of ROS are not at all produced by the T cells themselves. Especially if measuring T cell burst in whole blood or in suspensions from, for example, spleen or thymus, other cells are present that are much more potent in producing radicals via the NADPH oxidase, such as neutrophils or macrophages. The ROS they produce might diffuse into T cells and result in low staining levels. Also ROS produced in T cells by other sources such as mitochondria or specific enzymes, can be responsible for staining. This would explain the absence of different staining levels in *Ncf1* polymorphic animals. An additional explanation could of course be that ROS is produced by the NADPH oxidase complex but without usage of Ncf1. Although this has been shown to be possible in cell-free systems, as discussed above, it is not likely to happen in whole T cells. A way to circumvent contamination by ROS produced by other cells is by purifying the T cells. However, results should be interpreted with care since small amounts of contaminating cells might interfere with the result. ROS produced by these cells might diffuse into T cells and provide false-positive signals. As an alternative, cell lines are often used to investigate if certain cell types can exert oxidative burst and express NADPH oxidase complex members, but interpretation of data should be done carefully as well, since cell lines do not always precisely represent the *in vivo* cell type they are used for as a model.

Therefore, the data on ROS production by T cells via a leukocyte-type NADPH oxidase are still open to interpretation. Recent studies showed existence of a family of nonphagocytic NADPH oxidases (NOX and Duox proteins) that may play a role in receptor-stimulated ROS production in T cells, but exact expression and function of these oxidases are still to be addressed (110, 196)

C. ROS and T cell activation

Apart from the questions if and how T cells produce ROS themselves, there is solid evidence that they are affected by ROS. In RA, T cells are present in the joint, where they exhibit an activated phenotype, but they poorly respond to stimulation of the TCR. Due to this hyporesponsiveness, they stay in the joint and regulate inflammatory activity but can potentially also give help to macrophages that mediate joint destruction, instead of dying or migrating out of the joint (163). Hyporesponsiveness has thus been suggested to contribute to and perpetuate disease (65) and might be due to defective TCR signaling (122), although it might also relate to regulatory functions (23, 155). Since there is so much ROS produced in arthritic joints, many have tried to link T cell function with oxidative stress in RA patients. Remans *et al*. recently reported a possible mechanism on how oxidative stress could influence T cells in RA (143). They show that T cells from the synovial fluid have higher levels of intracellular ROS as compared to peripheral blood T cells from the same patient. Peripheral T cells were induced to produce equal levels of ROS by coincubating them with antigen presenting cells isolated from synovial fluid, which was contact dependent. They show that this effect can be inhibited by CTLA4-Ig that inhibits interactions between costimulatory molecules CD28 and CD80/86. However, no specific role for the NADPH oxidase was addressed in this study. In addition, the intracellular GSH levels in synovial fluid T cells are decreased, whereas thioredoxin is increased in the synovial fluid (122, 123). Treatment with *N*-acetyl-cysteine (NAC), a reducing agent, enhanced proliferative responses upon mitogenic stimulation as well as IL-2 production, confirming a role for redox regulation in this effect. Gringhuis *et al*. (65) subsequently studied whether early TCR signal transduction events were affected in synovial fluid T cells. They observed that the phosphorylation of the adaptor protein linker for activation of T cells (LAT), an essential component of TCR sig-

naling pathways, was severely impaired. This was shown to depend on the localization of LAT; membrane localization allowed phosphorylation and subsequent downstream signaling, whereas cytosolic localization prevented phosphorylation. Cytosolic localization was shown to be the result of a low intracellular glutathione (GSH) status, a hallmark of chronic oxidative stress. The tripeptide GSH (reduced form) and its oxidized from GSSG are important determinants of the intracellular redox status. Membrane localization of LAT could be restored upon treatment with NAC that restores the intracellular redox balance. Comparable findings were recently published for intestinal lamina propria T cells; the threshold of TCR signaling was shown to be tuned by the intracellular redox equilibrium (145), probably mediated by cysteine levels (169). Hehner *et al*. showed that TCR signaling was enhanced by oxidation of the intracellular thiol pool (71). In our Ncf1 polymorphic rats and mice with mutations in *Ncf1*, we could not detect differences in intracellular GSH contents in T cells, nor in other cells (54). This might mean that bursting by the NADPH oxidase complex does not affect the cytosolic redox balance. In a way this makes sense since the complex is localized in endosomal or cell membranes to produce ROS into the endosomes or to the extracellular compartment, although ROS can pass cell membranes and might go into the cytosol as well, if their half-lives allow so. It is likely that the intracellular redox balance is rather influenced by other systems or enzymes that produce ROS or directly via the environment.

Another study by Snelgrove *et al*. describes the role of ROS in influenza infection, with similar results (175). They found that mice with a nonfunctional NADPH oxidase complex, due to a deficiency of Cybb (Cybb tm1 mice) made in 129 cells and backcrossed to C57BL/6 mice, or wildtype C57BL/6 mice treated with an antioxidant (MnTE-2-PyP) had higher levels of infiltrating immune cells in the lungs after induction of pulmonary influenza infection. This indicates a more active immune response rather than a defective response, similar to what we observed in the animal models for arthritis. This increased infiltrate resulted in an improved resolution of the infection and lung function that was shown to be mediated via macrophages and Th1 T cells. They argue that this increased infiltration might be due to a decrease in levels of ROS-dependent apoptosis or to a reduction of inhibitory signals from other immune cells, for example, through CD200. This hypothesis is underscored by the observation that CD200-deficient mice exhibit a similar phenotype as Cybb tm1 mice. The more pronounced Th1 environment, as seen in the absence of ROS, promoted activation of resident macrophages. In general, they conclude that ROS appear to play a homeostatic role in limiting macrophages activation. It might be the case that this is mediated during antigen presentation or selection. Also other groups provided evidence that the redox levels or the ability to produce ROS (which might not necessarily be linked) in APC steer the phenotypic response of T cells. It was shown in mice that low intracellular glutathione levels in APC induce a Th2 type immune response (137). In line with this, oxidative stress in human cells promotes differentiation towards a Th2 response (101), out of which could be deducted that a decrease in ROS production would lead to a Th1 skewed response and autoimmunity.

As discussed above, antigen presenting cells (APC) might determine T cell activation by producing ROS. Another way how APC can influence T cell redox status is by providing cysteine. In the oxidizing environment, cysteine mainly exists in its oxidized form cystine, whereas in the reducing intracellular milieu, cysteine is the most common form. Lymphocytes do need cysteine, but because of the lack of cystine in the environment they cannot take up cystine to produce cysteine from it and therefore they need other sources to retrieve their cysteine. This is one of the reasons why beta-mercaptoethanol is often added to lymphocyte tissue cultures. It has been shown that both macrophages (59) and dendritic cells (4, 38) produce cysteine that is required for T cell proliferation. Levels of cysteine production by APC might determine if an antigen-specific T cell is activated in response to that antigen. This is underscored by the fact that sulfasalazine is used as a treatment for inflammatory bowel disease and RA. Sulfasalazine inhibits a cystine transporter, which likely results in a decrease of cysteine production by APC and subsequent inhibition of T cell proliferation. Another argument for cysteine availability playing a role in RA is the increased levels of thioredoxin in the synovial fluid of inflamed RA joints (123).

Apart from these data that ROS levels determine activation in T cells, it has been shown that the type of T cell response is also influenced by ROS. King *et al*. (101) found that when T cells were stimulated with anti-CD3 and anti-CD28, in presence of either IL-12 or IL-4; this resulted in a Th1 and Th2 phenotype respectively, as expected. However, when this was done in the presence of low levels of superoxide, a clear Th2 phenotype was observed, despite presence of Th1 cytokines (101). In line with this, it was shown that depletion of intracellular GSH led to a shift to a Th2 response, characterized by increased IL-4 production and inhibition of IFN-γ and IL-12 production (137). An interesting finding in this light is the work of Snelgrove *et al*. who also observed a Th1 skewed phenotype in mice that lacked ROS production due to a targeted deletion of *Cybb* (175). These mice were shown to be less prone to infection with *Cryptococcus neoformans* and influenza infection, whereas most other pathogens are less efficiently cleared when the NADPH oxidase is not functional. This effect was shown to be mediated mainly via macrophages that were more active as compared to those in control mice. Taken together, these data suggest that the redox status of the environment affects T cell activation and the Th1/Th2 balance. Manipulation of the redox balance might therefore be a successful therapeutic strategy in diseases such as RA.

D. ROS and T cell selection/apoptosis

There are many publications on the role of oxidative stress on T cell activation and T cell death. In general, it is believed that ROS are partly responsible for induction of apoptosis and are also produced during apoptosis; but it is evident that additional stimuli are required for a cell to undergo apoptosis. However, most of this ROS have been shown to be produced via other ways than through the NADPH oxidase system (2, 22). Some reports, however, relate ROS produced by the NADPH oxidase and apoptosis induction. It has, for example, been described that T and B cell lines undergo apoptosis when targeted by anti-Fas antibodies and that ROS is released during this pro-

cess (181), which was inhibited with diphenylene iodonium (DPI), a NADPH oxidase inhibitor. GSH has been described to play a role in prevention of apoptosis induction; depletion of GSH renders the cellular environment more oxidizing and induced apoptosis (119). In general, oxidation leads to apoptosis, with necrosis upon intense oxidation events. Investigations towards the effect of *N*-acetyl cysteine, as a thiol antioxidant, on activation-induced death of a T cell hybridoma cell line reactive with myelin basic protein, seemed to confirm this. It was shown that NAC totally blocks activation-induced cell death and DNA fragmentation as seen during apoptosis, as did GSH (156). Nevertheless, these results were challenged by another group, who showed that NAC might inhibit activation-induced cell death, but that this effect was independent of redox regulation; intracellular GSH and GSSG levels did not change in NAC-treated apoptotic cells, and the isomer of NAC, which can not be converted to GSH, had similar effects (97). Nevertheless, an *in vivo* study, using transgenic mice constitutively expressing activated forms of human Rac2GTPase, suggested an important role of Rac2 in thymocyte apoptosis (116). These mice had significantly smaller thymi as compared to control mice due to lower number of both double and single positive T cells. However, these effects are not likely to be regulated via NADPH dependent ROS production, since T cells do not express the NADPH oxidase, although this could not be excluded, based on the experimental data.

Indeed if T cells express no or low levels of the leukocyte NADPH oxidase complex, it is not likely that this pathway will be of importance for T cell apoptosis. However, it is still possible that other cells initiate T cell apoptosis by releasing ROS on them. It could be hypothesized that during thymic selection the APC that mediates the selection (a thymic epithelial cell, thymic macrophage, or DC) starts bursting when the T cells recognizes a self antigen presented by this APC. In this way, self-reactive T cells might either become apoptotic or change their activation threshold. It could be possible that the fate of a T cell is dependent on the level of ROS produced during cellular contacts in the thymus or the periphery. It could determine both deletion of autoreactive T cells and/or the induction of regulating T cells. Very high levels induce T cell death, whereas intermediate or localized levels reduce T cell reactivity during autoantigen recognition, while absence of ROS might allow T cell activation. Also during resolution of an immune response, other cells, such as macrophages, might regulate T cell death by producing ROS during antigen specific interactions and prevent exaggerated inflammation. Although no real evidence is present for these hypotheses, it would be very interesting to address these issues, using the *Ncf1* polymorphic rodent strains as new tools.

IX. NADPH OXIDASE DEPENDENCY ON REDOX STATUS

An interesting question arising from the above discussed data, is whether the NADPH oxidase complex function is influenced directly by the redox status. It could either be possible that changes in cell surface proteins affect signal transduction pathways that affect NADPH oxidase assembly or that the membranes themselves are changed by different oxidation states. In addition, intracellular changes in the redox balance might also affect the function of the oxidase.

Cysteine residues are important for the functioning of a wide variety of proteins, such as proteases, some tyrosine phosphatases, and also several redox enzymes. Ncf1 contains four cysteines. The status of the thiol groups in these cysteines might compromise protein function. It was observed by Park *et al*. in a cell-free system, that activation of the leukocyte NADPH oxidase was associated with the appearance of a membrane binding site on one of the cytosolic oxidase components, namely Ncf1 (135). This indicates that oxidase activation involves a conformational change in Ncf1 and suggests that Ncf1 is of importance for feedback regulation of oxidative burst via the NADPH oxidase complex. Similarly it was shown by Sumimoto *et al*. (179) that the SH3 domains of Ncf1, normally masked by another part of the protein near the C-terminus of the molecule, could be exposed by arachidonate (168). A few years later, Park *et al*. showed that this conformational change decreases the reactivity of cysteine C378 towards *N*-ethylmaleimide, a chemical derivative of maleic acid that irreversibly inhibits the formation of cystine linking in proteins (136). Inanami *et al*. continued on this finding and investigated the role of this and the three other cysteines in Ncf1 functioning (89). All cysteines were mutated to alanines, one at a time, and effects on NADPH oxidase activation were investigated. The largest effect was observed after mutating cysteine 196 to an alanine (C196A). The activity of the complex in the cells with this mutation was increased three to four times as compared to the wildtype protein. This effect is likely to be mediated by redox regulation, by, for example, interfering with a disulfide bond that is lost in the mutated Ncf1. Mutation of two of the other cysteines into alanines affected the timing of ROS production rather than the amount. ROS production was comparably delayed in these mutants, which might be due to an interaction between these two cysteines. The redox status inside the cell will determine whether the thiol groups of these cysteines will form disulfide bonds, changes in the intracellular redox balance might directly influence Ncf1 functioning. This would imply a certain feedback mechanism, where ROS produced by the NADPH oxidase increases the oxidation state inside the cell, which might in turn affect Ncf1 translocation and oxidase functioning. Although ROS are either produced outside the cell or into phagosomes, they can cross membranes and might be able to affect the redox status of the cytosol. Apart from directly affecting protein members of the complex, it could also be the case that ROS affect membrane composition. It is known that the NADPH oxidase complex often forms in so-called lipid rafts (67, 115, 207). Shao *et al*. showed in neutrophils that the cytosolic components of the NADPH oxidase complex are normally not present in lipid rafts, but are recruited to the rafts upon Fcγ receptor activation or activation with bacteria. They showed that rafts rather determine the onset but not the maximum amount of ROS produced by the oxidase (167). In contrast, others showed (51) that upon chemical disruption of lipid rafts, fMLP induced ROS production was abrogated in neutrophils, as were signal transduction pathways via ERK1/2 and protein kinase B (PKB)/Akt that are required to phosphorylate Ncf1. These data rather suggest that lipid rafts are

essential for functioning of the NADPH oxidase complex, although this might depend on how activation is induced.

X. ROS AND ANTIGEN PROCESSING

Apart from affecting the T cell, ROS production might also affect antigen processing or presentation on the site of the antigen presenting cell, resulting in modulated T cell activation and subsequent immune responses. Anthony Segal *et al*. suggested in the early 1980s that abnormal pH regulation within the phagosome of chronic granulomatous disease (CGD; explained below) phagocytes might have a role in defective killing (164). The basis of this assumption is that the initiation of superoxide production is normally accompanied by phagosomal alkalization as a result of the proton-acceptor function of superoxide anions. This pH change is proposed to be essential for the activation of granule enzymes within the phagosome. In CGD, however, the oxidase function is absent, and alkalization does not occur, resulting in impairment of the killing mechanisms. They showed that correction of phagocytic pH to more physiological values restored the ability of CGD neutrophils to kill *Staphylococcus aureus*. A paper published more recently by Savina *et al*. describes a role for NADPH oxidase-derived ROS in regulating the pH in dendritic cell phagosomes (159). They show that the cytosolic components of the NADPH oxidase complex (Ncf1/Ncf2/Ncf4) are recruited to immature DC phagosomes, causing active and sustained phagosome alkalinization by production of protons. In Ncf2-defective DCs, lacking a functional NADPH oxidase complex, phagosomal acidification and antigen degradation were increased, which caused a defect in crosspresentation to CD8+ T cells. In this way, ROS production thus directly influences antigen processing and subsequent presentation. Surprisingly, this effect did not operate in macrophages that are also known to be important antigen presenting cells. Still, this finding clearly adds another possible mechanism whereby redox regulation may operate. One could speculate that a decreased but not absent ROS production regulated by *Ncf1*, resulting in a slight decrease in phagosome pH in dendritic cells, increases antigen degradation resulting in a quicker and more efficient production of presentable peptides, resulting in increased T cell activation and autoimmunity as recently suggested by Amigorena and co-workers (159).

XI. MOUSE MODELS WITH A NADPH OXIDASE DEFECT

Several articles have been published on mice deficient in or with a deletion for one of the NADPH oxidase proteins, including *Ncf1*, *Cybb*, and *Ncf4* (Table 2). These mice have been created through a targeted deletion of the gene in a 129-derived ES cell and the resulting mice were backcrossed to C57BL/6 (B6). Experiments have been made after a variable number of B6 backcrosses. Although this is a standard procedure, there are several possible pitfalls when complex traits like those associated with redox pathways are studied. These problems have been previously discussed concerning osteopontin knockouts (17, 24). Osteopontin is encoded by a gene that is linked to the *Ncf1* gene in the mouse. In these knockout mice there were 129 derived genes present, linked to the fragment, as well as a genetic instability introduced by the insertion of new DNA that may influence the results. In addition, the B6 control mice could be quite different compared to the Jackson-derived strain that is different from many other colonies, unless littermates are used. Thirdly, these mice are usually complete knockouts, which means that the feedback mechanisms, activated by an oxidative burst or through a NADPH oxidase protein itself do not operate. Clearly, the development of CGD in humans usually occurs also in complete knockouts, but polymorphisms in NADPH oxidase proteins are not likely to induce CGD and their exact effects in humans are not known. Both the *Ncf1* (93) and the *Cybb* (139) knockout mice mimicked several aspects of human CGD (Table 2), whereas there is so far no evidence for this in the *Ncf1* mutated mice (Hultqvist *et al*., unpublished observation).

Table 2. Animal Models with Defects in NADPH Oxidase Proteins

Protein	*Species*	*Defect*	*Background*	*Phenotype*	*Reference*
Ncf1	Mouse	Polymorphism	B10.Q	Increased arthritis and EAE susceptibility	88
Ncf1	Mouse	Knockout	C57BL/6	Spontaneous infections, similar to CGD	93
Cybb	Mouse	Knockout	129S/V/C57BL/6	No spontaneous infections, but increased susceptibility to infections. Increased inflammatory response	128, 139
Ncf4	Mouse	Knockout	129S/V/C57BL/6	Decreased Ncf2 expression, decreased burst, decreased bacterial clearing	42
Rac2	Mouse	Knockout	129S/V/C57BL/6	Decreased burst, but upregulation to 100% after PMA or AA stimulation. Decreased inflammatory response, decreased bacterial clearing	146
Ncf1	Rat	Polymorphism	DA	Increased arthritis and EAE susceptibility	132

AA, arachidonic acid; CGD, chronic granulomatous disease; EAE, experimental autoimmune encephalomyelitis; PMA: phorbol 12-myristate 13-acetate.

The *Ncf1* knockout mouse was first described in 1995 by Jackson *et al*. (93). Exon 7 of the *Ncf1* gene was targeted in a 129-derived ES cell, since it was known to be essential for Ncf1 functioning. The *Ncf1* deficiency together with the linked 129 genetic fragment was backcrossed to the B6 background. These mice lacked phagocyte oxidative burst. The mice were fertile and did not show any physiologic differences compared to their wildtype littermates, until they started to develop severe infections. Most of these infections corresponded to those seen in CGD patients, for example, deep staphylococcal infections. *In vitro* killing of bacteria was impaired in these mice, whereas, interestingly, an enhanced inflammatory response, as recorded by the number of leucocytes after challenge with thioglycollate, was observed. It was concluded that the *Ncf1* knock out mice developed a spontaneous disease, resembling human CGD.

Another group developed Cybb (gp91phox) knockout mice by a gene targeting approach. It was shown that affected mice lacked phagocyte superoxide production and manifested an increased susceptibility to infection with *Staphylococcus aureus* and *Aspergillus fumigatus*. In addition, they had an altered inflammatory response in thioglycollate-induced peritonitis as had the *Ncf1* knockout mice (128, 139). However, spontaneous microbial infections were not observed in these mice. So they partially resemble human CGD due to a mutation in *Cybb*.

Recently, *Ncf4* knock out mice have been described as well (41). Targeted deletion of the gene resulted in absence of detectable protein levels in different cell types, indicating succesful deletion. Interestingly, Ncf2 expression levels showed a simultaneous decrease of 55%, whereas Ncf1 expression levels were not affected. Oxidative burst in cells from these mice was decreased up to 97%, dependent on which stimulus was used, but not totally absent. This reduced oxidative burst capacity resulted in reduced bacterial clearing capability; the extent of the *S. aureus* killing defect was as severe as that observed with neutrophils from *Ncf1* knock out CGD model mice. The reduced Ncf2 levels in *Ncf4* knock out neutrophils suggests that the steady state expression of Ncf2 is affected by Ncf4. This was supported by the observation that in NCF2-deficient CGD patients, NCF4 expression is reduced (185). Whether this regulation occurs at the level of protein stability, mRNA stability, or transcriptional/translational control is unknown.

In 1999, Rac2 knockout mice were developed and investigated for NADPH activity (146). These authors showed that superoxide production was severely reduced in these mice as compared to wildtype mice, although additional data suggested that Rac2 function could be taken over by another protein, likely being Rac1. Upon thioglycollate injection, to isolate exudate cells, it was observed that *Rac2* knockout mice exhibited deficient exudate formation. In addition, these mice were more susceptible to infection with *Aspergillus fumigatus*. However, foci of hyphae in the renal parenchyma of sick mice were surrounded by a significant neutrophil infiltrate, suggesting that the critical defect in host defense was either in the time required for neutrophil migration into the tissues or in neutrophil function upon arrival. Recently, it has been suggested that mice deficient for Rac2, can upregulate burst until at least normal levels upon stimulation by PMA and arachidonic acid (AA). Apparently, Rac2 is not required for translocation of Ncf1 and Ncf2. However, they showed that Rac2 is necessary for optimal activity of the assembled oxidase complex, an effect that can be replaced by exogenous AA, which may act directly or via an exogenous AA-induced mediator (100). If AA would exert a similar effect in the human situation, as suggested by Pompeia *et al*. (140), consumption of omega3 fatty acids, that prevent AA formation, may work vice versa as envisioned: decreased burst capacity might lead to increased susceptibility to autoimmunity, as suggested by our own results.

XII. AUTOIMMUNE DISEASES IN NADPH OXIDASE-DEFICIENT ANIMALS

The various mouse strains with deficiency or mutations in NADPH oxidase component have been used to address the possible influence on inflammation and autoimmunity, however with some variable or possibly contrasting results.

One of the first reports to investigate the role of oxidative burst on autoimmune disease studied EAE, the most commonly used animal model for MS. It was shown that the Ncf1-knockout mice were completely resistant to EAE and also had a suppressed T cell response to the myelin oligodendrocytic glycoprotein (MOG) 35–55 peptide used for induction of the disease. This is in contrast to our genetic linkage data in the rat, in which EAE is enhanced by the mutated *Ncf1* allele associated with a lower oxidative burst. There are, however, important differences between these experiments. One difference is that the rats were injected with native myelin proteins, whereas in the mouse experiment the inducing agent was a myelin protein-derived peptide. Data confirming this interpretation came from experiments with the *Ncf1* mutated B10.Q mice, in which it could be shown that disease induced with MOG protein was higher in mutated mice, whereas it was lower when MOG peptides were used (88). This finding suggests that ROS affect the processing and maybe presentation of the autoantigen, as suggested above. In another experiment it was shown that macrophages and microglial cells isolated from the central nervous system in sick rats had higher levels of spontaneous and PMA-induced oxidative burst, whereas this difference could not be found in cells derived from peripheral blood (152). This indicates that disease locally upregulates ROS production that might lead to oxidative stress, which is then suggested to lead to increased inflammation and damage. Treatment of these rats with catalase, a H2O2 scavenger, markedly suppressed the severity of the disease and suggests that H2O2 plays a disease aggravating role in EAE as well. Also, leukocytes that were isolated from MS patients had increased spontaneous and induced burst, confirming these rat data (46). However, Koch *et al*. (104) investigated oxidative stress in serum and peripheral blood from MS patients but did not find a difference between patients and controls regarding antioxidative activity, which again fits with the rat data and argues for a local inflammatory effect of ROS rather than a systemic effect. It should be pointed out, however, that in all inflammatory conditions, oxidative burst promoting genes like *Ncf1* are upregulated in the target tissue, although it is not known whether this directly regulates inflammation or just reflects a response to the inflammatory mediators.

Investigations on arthritis development were reported by Van de Loo *et al*. who addressed the role of Ncf1 and Cybb in mice deficient for either of these proteins in a model for irritant-in-

duced arthritis (188). Zymosan was injected intra-articularly in the knee and it was observed that *Ncf1* knockout mice developed significantly more joint swelling. Levels of bone erosion and cartilage proteoglycan loss were increased in both Ncf1 and Cybb knockout mice. Their data indicate that NADPH oxidase-dependent superoxide ameliorates arthritis, tempers joint inflammation, and reduces cartilage and bone destruction. Importantly, these models are believed to be T cell independent and their data suggest that ROS in fact is protective in the inflammatory phase. However, the same group later published that NADPH oxidase-driven ROS production drives chondrocyte death and aggravates matrix metalloproteinases-mediated cartilage destruction in a mouse model of IFN-γ stimulated immune complex arthritis (191). Although these results resemble ours, the models only represent the inflammatory phase of arthritis: the immune priming of T cells by antigen presenting cells (APC) is thus not important in such models. Since we have shown that the effect of the mutation in *Ncf1* exerts its effect already during T cell priming, these data indicate that ROS produced by the NADPH oxidase complex can act at different phases of the immune response. Different models will yield different results, but can be informative for different phases or pathways in human disease.

Our data therefore show that ROS downregulate immune activation during the priming phase of an immune response (54). It is likely that ROS effects are dependent on the amount of ROS produced and the time when ROS is produced. In addition it is likely that ROS have different effects on different cell types as well, and cell–cell interactions might be involved, rather than a general effect mediated via the extracellular compartment. As mentioned above, a polymorphism in one of the NADPH oxidase proteins might rather result in lower levels of ROS than in total absence, resulting in different responses. It is likely that concentration differences affect the redox balance in particular cell types, resulting in different abilities to become activated or to activate other cells.

XIII. NADPH OXIDASE DEFICIENCY IN HUMANS

A. Chronic granulomatous disease

Chronic granulomatous disease (CGD) is an uncommon primary immunodeficiency affecting the innate immune system and is caused by mutations in any one of four genes encoding subunits of the superoxide-generating phagocyte NADPH oxidase, resulting in an absence or very low levels of enzyme activity. CGD patients are therefore highly susceptible to severe, sometimes fatal, bacterial and fungal infections presented in the form of pneumonia, abscesses, and lymphadenitis and often develop granulomas (53). The bacteria and fungi that commonly cause infection in CGD include: *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia*, and *Nocardia* and *Aspergillus* species. Rare infections with organisms only encountered in CGD, such as *Paecilomyces sp*. and *Trichosporon inkin*, suggest that patients with CGD have a unique susceptibility pattern (165). CGD is normally diagnosed in infancy and has an incidence of between 1 in 200,000 and 1 in 250,000 live births (75). When just discovered, this disease was described as 'fatal granulomatous disease of childhood', since children on average only reached an age of 4 years. However, improvement in drugs and diagnostics made the name change to Chronic Granulomatous Disease (CGD) and life expectancy was increased drastically. The most commonly used diagnostic tool is conversion of nitroblue tetrazolium to blue formazan by superoxide anion (O2-) by blood leukocytes, which fails in these patients (7). Because of the inability to produce ROS, the phagocytes of these patients can ingest bacteria, but have difficulties in killing them. CGD is a disease where bone marrow transplantation and gene therapy has proven to be successful (133).

Mutations leading to a CGD phenotype have been discovered in all of the phox components except for NCF4, which indicates that NCF4 is less critical for the activation of the complex (75). The most common form of CGD is the X-linked recessive form (X91), where mutations in CYBB, located on the X-chromosome, lead to a mutated, truncated, or deleted CYBB (gp91phox) protein. There are >350 mutations described for CYBB and a majority of them leads to a complete lack of the protein and consequently a nonfunctional NADPH oxidase complex, indicating the importance of the protein for ROS production (X-CGD database). In contrast, ~25% of all CGD patients are diagnosed with autosomal recessive *NCF1* CGD (A47) where, in contrast to X-linked CGD (mutation in CYBB), the same mutation is identified in 94% of the cases; a two base pair deletion in the beginning of exon 2 in NCF1 (148). This deletion results in a frame shift change which creates a premature stop codon and consequently results in production of a truncated protein. The two other forms of CGD are autosomal recessive CYBA (p22phox) (A22) and NCF2 (p67phox) (A67) CGD. Each of these forms are present in only 5% of the CGD patients and unlike A47, several different mutations have been reported (7). As for X91, most patients with A47, A67, and A22 CGD have no functional ROS production, yet the symptoms are often milder and the diagnosis is mostly only made later in life (31). This is especially the case in A47 CGD where relatives of diagnosed patients have been found to be homozygous for the two base pair deletion without showing any distinct symptoms (149). These findings indicate that an intact CYBB protein is crucial for NADPH oxidase activity but it also suggests that CYBB is involved in other cell types or systems as well. Only one patient with immunodeficiency originating from a mutation in *RAC2* has been described. The symptoms are however quite different from classical CGD (3). The importance of NCF4 is debated and several conflicting reports have been published on its importance for ROS production (41). No CGD patients have been found with mutations in NCF4, however a recent publication shows that knockout mice (*Ncf4-/-*) have a severely decreased bactericidal capacity and a disturbed regulation of the NADPH oxidase complex (41). Furthermore, also the level of Ncf2 was greatly reduced in the *Ncf4-/-* mice, which indicates that an absence of Ncf4 somehow alters the steady state of the Ncf2 protein in the cytoplasm. This illustrates the complicated interactions that exist between the proteins in the complex and also the need for the NADPH oxidase complex to be intact for full ROS production capacity.

B. Mutations in NADPH oxidase proteins and their effects in humans

Most of the mutations found in CGD patients have severe effects on the protein structure or level and consequently on the

ROS production capacity. There are also mutations that target critical binding sites disrupting the complex interactions between the different components of the NADPH oxidase complex or to the membrane (75). However, there are a number of single nucleotide polymorphisms in all of the genes encoding components of the NADPH oxidase complex that lead to no or only mild changes in the protein structure or expression levels (www.hapmap.org, accessed February 2007). Wyche *et al*. reported a lower oxidative burst capacity in neutrophils from individuals who are homozygous for the T allele of the C242T polymorphism (rs4673) in the CYBA gene (200). The same polymorphism has also been found to be associated with coronary artery disease in several independent studies (90). Hence, CYBA appears to be important in vascular control and is also reported to be associated with hypertension (126). These studies clearly show that even relatively mild genetic changes can result in a reduced burst capacity. However, it is important to point out that these changes do not make the complex completely dysfunctional, as is the case in CGD patients; it is the maximum capacity to produce ROS upon stimulation as measured *in vitro* that is reduced. This is the kind of effect found in our rat model. In the DA rat, the polymorphism in *Ncf1* does not lead to a decrease in protein expression. However, these rats have a decreased oxidative burst when the NADPH oxidase complex is activated *ex vivo*. This indicates that some conformational change occurs, leading to, for example, decreased binding of Ncf1 to the other members in the complex and thereby reduced function. Interestingly, it was shown that the difference in burst between DA and the congenic DA.*Ncf1E3* rats was much less pronounced when the rats had developed arthritis, indicating that arthritis upregulated the capacity to burst (87). Consequently, similar types of effects are expected in RA patients that have a defect in one of the NADPH oxidase proteins but do not have a CGD phenotype.

C. The genetic complexity of NCF1 in the human genome

The reason behind the extreme genetic homogeneity of A47 CGD is the genomic surroundings of the NCF1 gene in the human genome. Two pseudogenes (*ψ*NCF1) with a 98% sequence similarity to NCF1 have been found in the close proximity of NCF1 on chromosome 7. There are several polymorphic differences between the pseudogenes and NCF1 and in particular a GT deletion (ΔGT) in the beginning of exon 2, which leads to a premature stop codon, is characteristic of the *ψ*NCF1 genes (60, 78). The genomic region surrounding NCF1 contains several low-copy repeats (LCR) and is subject to chromosomal rearrangements leading to both segmental duplications and deletions (108). In patients diagnosed with the mental retardation syndrome William–Beuren syndrome (WBS) large regions, including NCF1, *ψ*NCF1, and several other genes, are deleted through nonallelic homologous recombinations between the LCR (5, 12). There is also evidence of copy number differences and inversions in healthy individuals and together with evidence of a high ALU-content indicate that this region is prone to genetic alterations (5, 25).

These dynamic properties also affect the NCF1 genes; as a result of unequal crossover or gene conversion between NCF1 and one of the *ψ*NCF1, A47 CGD patients have three genes containing the ΔGT deletion and no functional NCF1 (Fig. 6) (148). Two of the genes are the original *ψ*NCF1, whereas the third is a chimera of NCF1 and *ψ*NCF1, which has the ΔGT deletion sequence from one of the *ψ*NCF1. The chimeric NCF1, like the *ψ*NCF1, encodes a truncated protein, which leads to a significantly reduced ROS production in the A47 CGD patients. To detect A47 CGD carriers, methods have been developed to measure the ratio between *ψ*NCF1 and NCF1 through the presence or absence of ΔGT (76). In a normal individual (*i.e.,* a noncarrier), the ΔGT/GTGT ratio is 2:1, indicating two copies of *ψ*NCF1 and one of NCF1 on each allele. An A47 CGD carrier, on the other hand, has five copies of ΔGT containing genes and only one NCF1 on both chromosomes, giving a 5:1 ratio. Heyworth *et al*. also discovered two other ratios, 1:1 and 1:2, in a study of 50 individuals, which lead them to suggest that the functional NCF1 gene is duplicated in normal individuals (Fig. 6) (76). However, sequencing of the "duplicated" NCF1 gene revealed that it contains the GTGT sequence as well as several *ψ*NCF1-specific markers, and they therefore concluded that this "duplicated" gene is instead another chimera of NCF1 and one of the *ψ*NCF1 genes. Because the chimera NCF1 originates from *ψ*NCF1, it has been denoted Type-II *ψ*NCF1

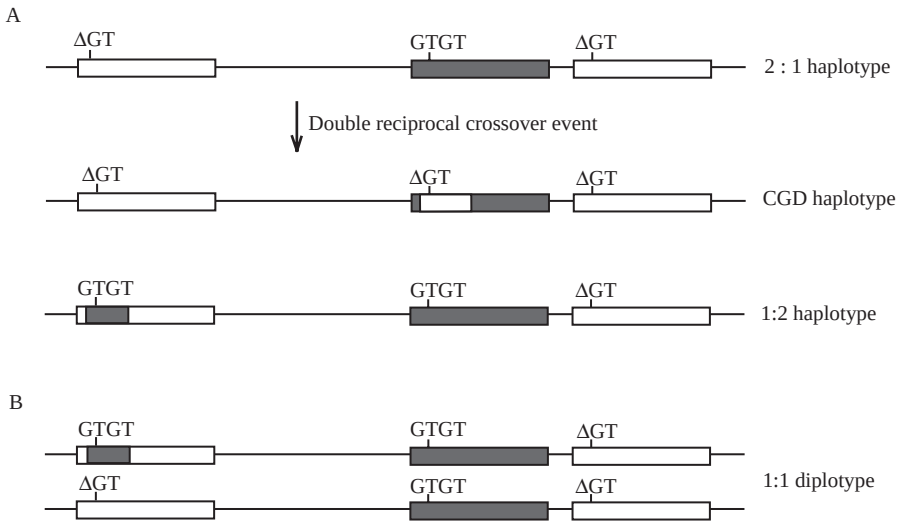


FIG. 6. The genetic organization of NCF1 in humans. Crossover events between *NCF1* and *ψNCF1* create two different *NCF1* chimera genes. (**A**) A double crossover event between *NCF1* and *ψNCF1*, involving a GTGT deletion in exon 2, gives rise to two different *NCF1* chimeras. The CGD haplotype contains two *ψNCF1* and one nonfunctional *NCF1* chimera, containing the GTGT deletion. The 1:2 haplotype contains one *NCF1* gene, one *ψNCF1* gene, and one functional *NCF1* chimera, not containing the GTGT-deletion. (**B**) The 1:1 ratio reflects the combination of one 1:1 haplotype and one normal 2:1 haplotype. Individuals who have the 1:1 ratio have three functional NCF1 genes.

(ψNCF1-II), even though it is not a pseudogene and has been shown to be fully transcribed (76). In fact it is most likely the opposite outcome of the same crossover event, which creates the chimeric *NCF1* gene containing ΔGT seen in A47 CGD patients (76). These findings indicate that NCF1 is under high recombination pressure and sequencing of the regions surrounding the GTGT sequence in exon 2 revealed a large number of ALU repeats (25), which could be the cause of the gene conversion.

D. NCF1 in humans and its possible connection to RA

Several of the genetic alterations detected in the NCF1 gene could have the same consequences on burst capacity as the Ncf1 polymorphisms found in the rat. The NCF1 chimera contains several SNPs that encode amino acid alterations and consequently the presence of the chimera could affect burst capacity. A recent study of WBS patients showed that even though the p47phox expression is higher in patients who have a 1:1 ratio, the NADPH oxidase activity is not significantly higher *in vitro* as compared to patients who have the 2:1 ratio (34). These data indicate that the presence of the chimera protein itself does not affect burst capacity. Although the same study shows that WBS patients who are hemizygous for NCF1 have a reduced burst capacity and also an increased risk of developing hypertension. These data show that dysfunction or a reduced expression of the NCF1 gene leads to a reduced burst which apparently can lead to pathological complications. The complex genetics of NCF1 allows a vast number of genetic alterations that could lead to differences in burst capacity, which, in turn, might influence immunological pathways. The complexity of this locus, and in particular the duplications, make it also very difficult to analyze the exact genotype of a given person. Such issues increase the difficulties in human genetics, in particular since it is likely that these properties are shared with a number of other loci of importance for autoimmune disease.

XIV. NEW THERAPEUTIC STRATEGIES FOR RA INTERFERING WITH THE REDOX BALANCE

A. Oxidants, antioxidants, and arthritis

The common general belief, among the public and among scientists, is that antioxidants are anti-inflammatory. This belief is, however, not well founded and obviously the exact role of ROS in autoimmunity and inflammation in general and arthritis in particular is not well understood. Numerous papers suggest that ROS have a damaging effect in the arthritic joint, when produced during the inflammatory phase of arthritis. Epidemiologic studies have indicated an inverse association between antioxidant levels and inflammation (77) and showed that RA preferentially occurs in previously healthy subjects who have low levels of antioxidants (32). Several reports state that RA patients have increased levels of ROS production, leading to increased levels of different markers reflecting oxidation and a decrease in antioxidant capacity. Increased ROS levels have been reported at inflammatory sites, and circulating neutrophils from RA patients have increased NADPH oxidase activity (15). A similar state with increased ROS production by phagocytes was observed in mice with CIA (124). An intense inflammatory process seems to generate an activation state in neutrophils and macrophages, which makes them prone to produce more ROS (142). Alternatively, the increase in ROS has also been explained by a defective antioxidant system (94). It was indeed shown that in RA patients the redox balance is shifted to oxidation rather than reduction, and antioxidant levels are often significantly lower than in healthy or osteoarthritis controls. Results from a selection of papers stating so are summarized in Table 3. The explanation to the discrepancy between these and our data can most likely be found in the fact that ROS may not only regulate RA and experimental arthritis and the initiation phase, but are also produced as a result of the inflammatory activity (Fig. 7). It was found in a microarray study on synovial tissue that NCF1 and CYBB were overexpressed in RA patients compared to osteoarthritis patients and also when severely sick RA patients were compared with patients with less severe disease (189, 190). This indicates that ROS might not only play a variable role in different phases of the immune response but also in different anatomic locations.

B. Antioxidant treatment

These data underscore the idea that antioxidant treatment might be a successful therapeutic strategy. The antioxidant Curcumin inhibits neutrophil function as well as synoviocyte function *in vitro* (91). Thioredoxin (TRX) that is constitutively expressed in most cells of the body and induced by a wide variety of cellular stresses (154) acts as a scavenger of ROS (85, 129). TRX has been shown to inhibit antibody-induced arthritis in mice when administered as recombinant TRX or when it was overexpressed as a transgene, although this study was not genetically controlled (184). Retinoids, derivates of vitamin A, have immuno-modulatory effects and prevent ROS production in human stimulated PMNs (52). Retinoids have been stated to inhibit CIA in mice (131) and AIA in rats (20). *N*-acetyl cysteine (NAC), which is a thiol supplier in conditions of diminished antioxidant defenses, decreases CIA in mice (109). A NF-κB blocking effect has also been seen by antioxidants like NAC (147, 177).

It is however not consistent that compounds with anti-oxidant properties possess anti-inflammatory effects *in vivo*. Melatonin, a hormone produced by the pineal gland has been shown to act as a radical scavenger (2), but this antioxidant has been shown to be pro-inflammatory and enhance the autoimmune response and arthritis both in physiological and pharmacological doses in experimental rodents (70, 95). CIA severity in mice is increased when mice are kept in darkness, an effect proposed to be mediated by higher melatonin production. Melatonin levels were indeed found to be higher in these mice. The involvement of melatonin in the development of CIA was confirmed when mice injected with this hormone showed increased CIA severity (70).

Other well-known antioxidants as ascorbic acid (vitamin C) and vitamin E have only low or no effectiveness (27). It has been reported that vitamin C is the first antioxidant to be oxi-

TABLE 3. REDOX LEVELS AND ANTIOXIDANT LEVELS IN RA PATIENTS IN DIFFERENT STUDIES

Measured variable	*Lower/higher in RA patients*	N	*Control*	N	*Reference*
Blood total thiols, GSH, Vitamin C	All lower	20	Healthy	20	94
Plasma sulfhydryl and carbonyl groups, TAC, hydroperoxides	Lower, higher, similar, higher	9	Healthy	22	47
Plasma protein sulfhydryl groups	Lower	21	Healthy	15	58
Total oxidative status, MDA level	Lower, higher	22	Healthy	18	134
TAC, MDA, antioxidant enzymes (GSH-Px and CAT)	Lower, higher, lower	24	Osteoarthritis	20	157
Serum carbonyl levels, serum thiols	Higher, lower	71	Healthy	30	114
Trx (protein level and activity), TrxR	Higher, lower	64	Healthy	27	113

MDA, malondialdehyde (result of lipid peroxidation); TAC, total antioxidant capacity; Trx, thioredoxin; TrxR, thioredoxinreductase.

dized after leukocyte stimulation, which makes it an important marker of oxidative stress (49). The most important derivate of vitamin E from a biological point of view, α-tocopherol, is consumed in the ROS scavenging process but can be regenerated by GSH and by ascorbic acid. Vitamin E also has an additional effect since it blocks arachidonic acid formation from phospholipids and inhibits lipoxygenase activity, thereby potentially possessing an anti-inflammatory capacity.

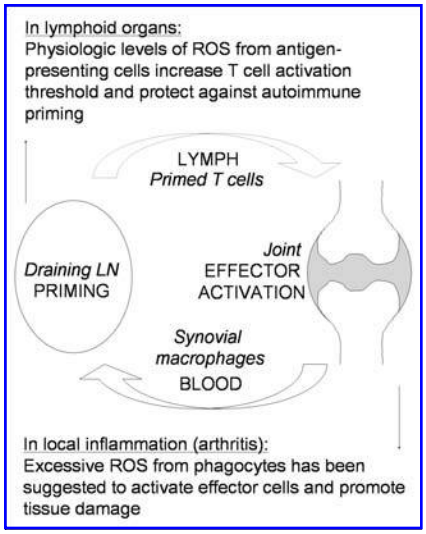


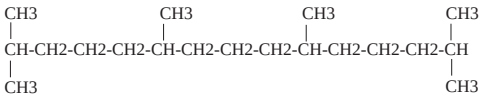
FIG. 7. A possible Yin-Yan effect of ROS on inflammation. ROS, produced in low levels, modulate immune activation and decrease T cell activation before disease onset. Deficient ROS production renders T cells more activated and allow them to transfer disease in the rat model. High levels of ROS as produced in the joint during the inflammatory phase of disease (oxidative stress) can confer damage to tissue and may locally modulate activation of effector cells.

The effect of supplementation of vitamin E (α-tocopherol), the major lipid-soluble antioxidant in human plasma and tissue, was tested in a spontaneous arthritis model in mice (KRN/NOD model) and was shown not to modify any clinical features of arthritis, such as articular index or body weight. But these authors observed that joint destruction, determined by qualitative and semiquantitative methods, was prevented (32). This indicates that Vitamin E in some way uncouples joint inflammation and joint destruction. Scavenging of ROS in the joint apparently directly prevents destruction of the joint, but does not influence the immune response itself. It could be that neutrophils are affected that are present in the joint but that do not affect the modulation of the immune response itself. When the redox status was determined in these mice by measuring the levels of reduced glutathione in whole blood samples, urinary isoprostane, or plasma hydroperoxide levels, no differences were observed between treated and control mice, indeed indicating that Vitamin E did not exert systemic effects. However, peripheral white blood cell capability to produce radicals was reduced in Vitamin E-treated mice, although this effect was only observed starting 3 weeks after treatment induction. This might indicate a feedback mechanism of levels of ROS produced and capacity to burst. Another study tested the effect of Vitamin E in human arthritis. They showed that treatment with Vitamin E had no effect on RA disease activity or on inflammatory parameters, although it improved pain, which rather suggests a role in analgesia mechanisms than in the inflammatory process itself (39).

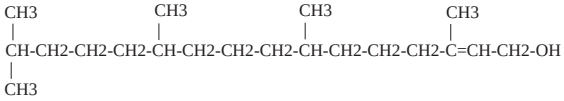
C. Oxidative burst-inducing substances

Despite the general dogma that ROS has a damaging effect, our data suggested that induction of ROS production could actually have a disease ameliorating effect (88, 132). After identifying the regulatory gene responsible for the arthritis protective effect seen in the congenic DA.Ncf1 rat (i.e, DA rats with

Pristane



Phytol



C8

CH3-CH2-CH2-CH2-CH2-CH2-CH2-CH3

C11

CH3-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH3

C16

CH3-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH2-CH3

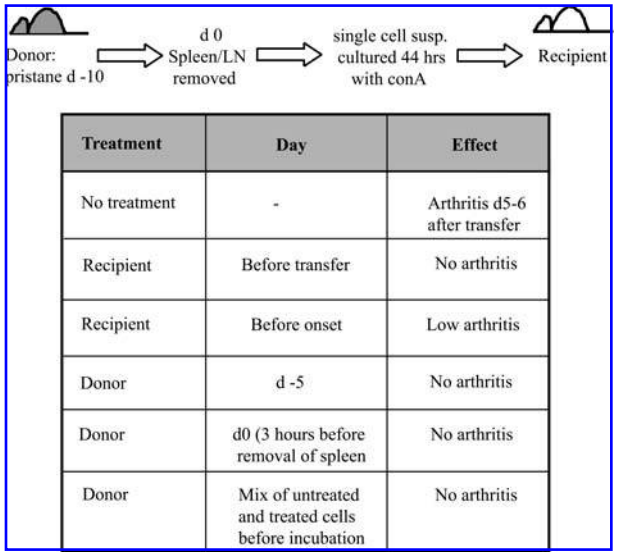
FIG. 8. Structural formulas of pristane and phytol. Pristane and phytol resemble each other structurally, despite being arthritis inducing in rats in the case of pristane and arthritis ameliorating in the case of phytol.

an E3 allele of *Ncf1*) (132), we started the quest of identifying substances that could activate the NADPH oxidase complex to produce ROS *in vitro*. We identified one compound that had a very efficient NADPH oxidase dependent burst -inducing effect in a neutrophilic cell line *in vitro*. This agent was phytol, the side chain of Vitamin E. When phytol was injected into naive DA rats that have a naturally low capacity to mount an oxidative burst, a restoration of the ROS producing capacity could be seen in granulocytes, as well as inhibition of PIA onset (132). This finding was surprising, since phytol had a structure similar to that of pristane (Fig. 8), used to induce arthritis in rats (193). Indeed, pristane also induced oxidative burst *in vitro*, although to a much lower extent than phytol. Importantly, however, pristane also had adjuvant effects leading to the development of arthritis, a capacity phytol is completely lacking. A closer comparative analysis of phytol versus pristane showed that the structures determining oxidative burst inducing capacity and adjuvant capacity were different, and we could determine that arthritogenic capacity was independent of the oxidative burst inducing capacity (87). The burst inducing effect was separated from the adjuvant effect of alkanes since only alkane oils with longer carbon chains than 15 carbons induced arthritis, whereas shorter alkanes were most potent in inducing oxidative burst. The adjuvant activity of alkanes longer than 15 carbons could also be blocked by a double bond or a hydrophilic group at the end. The simple alkane being most potent in stimulating ROS production (C11) was also found to inhibit PIA onset (Table 4)(87). We selected phytol as our prototype compound as this oil most effectively induced an oxidative burst. A single subcutaneous injection of phytol reversed the genetic effect on oxidative burst inducing capacity in the DA rats as compared with the congenic DA.*Ncf1E3* rat. The effect was systemic, affecting bone marrow cells within hours after injection and longlasting as it could be measured for more than a month.

Phytol worked prophylactically to prevent development of arthritis when injected before onset of PIA or CIA. Administration of phytol to rats with an acute or chronic arthritis decreased disease severity significantly. Phytol also suppressed the autoimmune response since the anti-CII antibody levels and delayed type hypersensitivity reactions were suppressed. The total level of antibodies however was not altered arguing against a general immunosuppressive effect (87). Even though preliminary data from oxidative burst capacity suggested that the main effect of phytol seemed to be on granulocytes we wanted to investigate how the increased ROS production from these cells could affect arthritogenic T cells. To study this we used transfer models of arthritis (Fig. 9). Since it has been shown that CD4+T cells transfer arthritis in rats, this model is an excellent tool to study the impact of phytol on these cells. Phytol injection inhibited arthritis development upon adoptive transfer of arthritogenic T cells when injected either in donor rats before taking spleens for T cell transfer or in the recipient rats that were given the T cells. This effect was rapid since injection of phytol in donor rats up to 3 h before sacrifice prevented arthritis development. We believe that this effect is due to downregulation of arthritogenic T cells and not mediated via

TABLE 4. THE EFFECT OF SMALL STRUCTURAL CHANGES OF CARBON CHAIN LENGTH ON NADPH OXIDASE STIMULATING CAPACITY AND ARTHRITOGENICITY

Alkane	*Burst* in vitro	*Arthritogenicity*	*Preventive effect (on PIA)*
C8	−	−	ND
C9	−	−	ND
C10	+	−	ND
C11	++	−	+++
C12	++	−	+++
C13	++	−	+++
C14	+	−	ND
C15	+	+++	ND
C16	−	++	−
C17	−	++	ND
Phytol	+++	−	+++
Pristane	+	+++	−



Treatment	Day	Effect
No treatment	-	Arthritis d5-6 after transfer
Recipient	Before transfer	No arthritis
Recipient	Before onset	Low arthritis
Donor	d -5	No arthritis
Donor	d0 (3 hours before removal of spleen	No arthritis
Donor	Mix of untreated and treated cells before incubation	No arthritis

FIG. 9. Adoptive transfer of arthritogenic T cells. Spleen or LN cells from pristane immunize rats are cultured *in vitro* and injected in naïve or phytol treated recipients. The effects of phytol treatment on PIA development are depicted in the table. Based on Hultqvist *et al*. (87).

ROS producing cells since co-incubation of cells from treated donors together with cells from an untreated donors did not protect from arthritis. The preventive effect is presumably not due to transfer of regulatory T cells since the recipients developed PIA in a normal fashion after pristane injection. From this study we conclude that increased ROS from granulocytes or macrophages affect T cells possibly via an overall increased oxidation state.

These findings reveal possibilities not only for a new type of therapeutic treatment for humans by itself but also for a new strategy for identifying new therapeutic substances that can be used for treatment of the human disease. Importantly, this therapeutic strategy can not only help RA patients with a defective oxidative burst but most likely also patients with a functional NADPH oxidase complex. This is deduced from the observation that rats with a normal oxidative burst suffering from arthritis (87) could be treated with this strategy. This treatment strategy also seemed to be at least as efficient as standard treatments used for RA today when compared to methotrexate (MTX) and the soluble TNF-α receptor Etanercept (Fig. 10). Furthermore, the possibility for NADPH-activating pharmaceuticals to treat autoimmune conditions such as RA also open up the possibility for combined therapy together with other DMARDS like anti-TNF treatment.

XV. GENERAL CONCLUSIONS

RA is a quite common autoimmune disease that at least partly has a genetic basis. Animal models for RA allow discovery of genes underlying disease pathways. One such gene found by linkage analysis in a rat model for RA is the *Ncf1* gene, which was later confirmed in mice. A single amino acid change in *Ncf1* results in a decreased capacity to exert oxidative burst via NADPH oxidase and renders rats and mice more susceptible to arthritis. A decreased burst capacity affects the redox balance and it has been shown that changes in the redox balance affect T cell activation. Interfering with the redox balance and thereby decreasing T cell activation is a promising therapeutic approach to treat RA, although it might not be as easy as it sounds. The effects of ROS on inflammatory responses seem to be dependent on the amount of ROS produced and the place, where, and the time when ROS are produced. In addition, it is likely that ROS have different effects on different cell types and that cell–cell interactions might be involved, rather than a general effect mediated via the extracellular compartment. It is therefore clear that the general dogma that ROS are destructive and antioxidants act as anti-inflammatory needs to be revised. Instead, we are facing a more complex situation in which an induced oxidative burst and the redox potential may have regulatory properties, determining both the immune response and the inflammatory response. In fact it is likely they have an important general function in cellular communication.

An important message is that the study of such a complex phenomenon as redox regulation benefits dramatically from genetic studies, as we could show for a selected polymorphism in *Ncf1* resulting in low capacity to burst that was shown to be in-

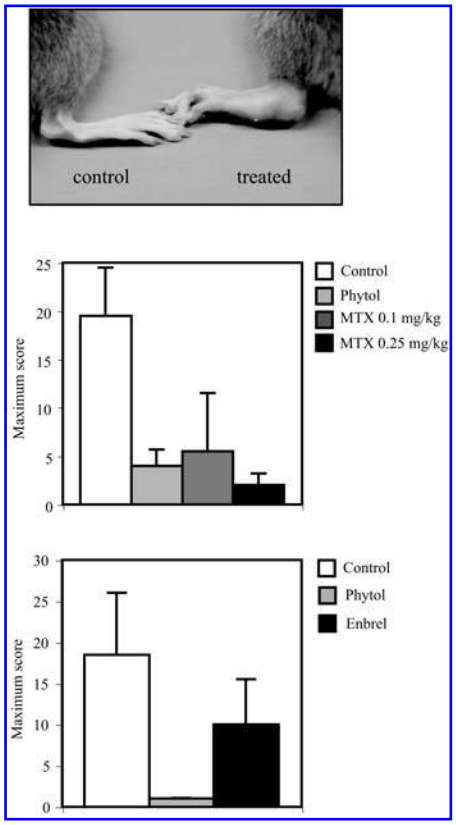


FIG. 10. Phytol is an effective therapeutic of PIA in rats. Treatment of rats with active PIA (pristine-induced arthritis) with phytol is as effective as conventional therapeutics for RA. Arthritis severity is scored with a quantitative scoring system where the rats can maximally receive 60 points. Phytol is administered 8, 10, and 12 days after immunization with pristane. Based on Hultqvist *et al*. (87).

volved in redox regulation. This provided the opportunity to study the complexity of the role of ROS in immune regulation in an experimental setting. Importantly, this *Ncf1* polymorphism resulted in lower levels of ROS rather than total absence, which had different effects on the immune response. It is likely that concentration differences affect the redox balance in particular cell types, resulting in different abilities to become activated or to activate other cells. The currently known data, as discussed in this review, reveal that ROS, as produced by the NADPH oxidase complex, do play an essential role in arthritis. However, whether ROS have a protective and immune modulatory role or rather a damaging effect seems to depend on the time and place of ROS production. In the priming phase of the immune response ROS is likely to play an immune downregulatory role. Further studies are necessary to state conclusively how, when and where ROS mediate these effects.

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ABBREVIATIONS

AA, arachidonic acid; CGD, chronic granulomatous disease; CIA, collagen induced arthritis; COX, cyclooxygenase; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DA, Dark Agouti; DC, dendritic cell; DMARD, disease modifying antirheumatic drug; FACS, fluorescent activated cell sorter; GC, glucocorticoids; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; IFNg, interferon gamma; LAT, linker for activation of T cells; MHC, major histocompatibility complex; MS, multiple sclerosis; MTX, methotrexate; NAC, N-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate; Ncf1, neutrophil cytosolic factor; NSAID, nonsteroidal anti inflammatory drug; PADI4, peptidyl arginine deiminase, type IV; PIA, pristane induced arthritis; PMA, phorbol 12-myristate 13-acetate; PTPN22, protein tyrosine phosphatase, nonreceptor type 22; QTL, quantitative trait locus; RA, rheumatoid arthritis; ROS, reactive oxygen species; TCR, T cell receptor; TNFa, tumor necrosis factor alpha; Th1/2, T helper 1/2; TRX, thioredoxin.

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