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# Radical aspects on arthritis

the role of neutrophil generation of nitric oxide and superoxide in inflammatory conditions

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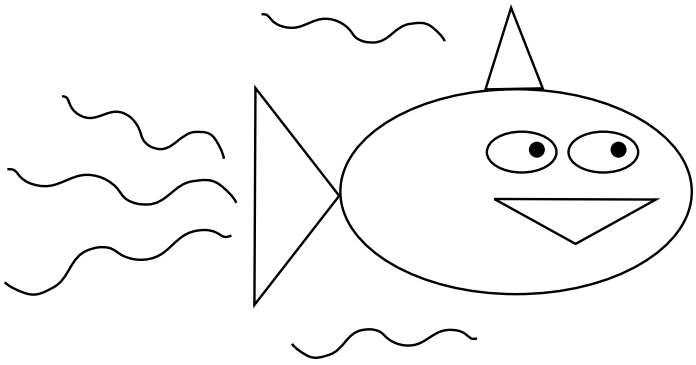
*To my surprise*

“Never forget that only dead fish
swim with the stream”

*Malcolm Muggeridge*



# Contents

# Abstract

The polymorphonuclear neutrophil granulocytes (neutrophils) are gaining renewed interest regarding their involvement in chronic inflammatory disorders, including rheumatoid arthritis (RA). Besides phagocytic and destructive capabilities, neutrophils have regulatory roles, *e.g.* by influencing responses from dendritic cells and lymphocytes. Several animal models have revealed that neutrophils are crucial for the initiation and maintenance of chronic inflammatory diseases. Neutrophil function is highly dependent on their ability to produce superoxide, an oxygen radical which can be further metabolized to other free radicals. Whether or not neutrophils are capable of producing the oxygen radical nitric oxide ($NO^{\bullet}$) has been a matter of debate.

In this thesis it was shown that freshly isolated neutrophils from the joint cavity of patients with RA, but not from other arthritis patients, had ongoing intracellular production of superoxide, indicating intracellular activation and processing of ingested material.

The finding that joint neutrophils, but seemingly not circulating cells, expressed the NO-producing enzyme iNOS, led to a series of experiments aimed to elucidate where in the exudative process this enzyme could first be detected. We could finally, for the first time, present evidence that human neutrophils actually express iNOS constitutively. Our data also suggest that neutrophil iNOS may be membrane associated, thus differing from the cytosolic location in other cell types. Since iNOS activity was not demonstrated in isolated cells, the notion that neutrophil iNOS is regulated primarily at the transcriptional level must be questioned. NO production from iNOS requires the presence of its substrate, L-arginine. To test the hypothesis that neutrophil arginase prevents neutrophil NO-production, we investigated whether arginase inhibition affects neutrophil NO-dependent oxidative function. Initial data revealed a difference in the effect of arginase inhibition comparing neutrophil stimulus with a soluble formylated tri-peptide (fMLF) and integrin-mediated stimulation with particle-bound collagen type-1. This led to the hypothesis that integrin-ligation on neutrophils induces extracellular liberation of arginase, which was confirmed both by measuring arginase and its enzyme activity. The findings in this thesis may be important not only regarding the role of neutrophils in chronic joint inflammation, but also as a link in the accelerated atherosclerosis observed in chronic inflammatory disorders, *e.g.* RA.

*Keywords:* Neutrophils, reactive oxygen species, arthritis, NOS, NO, arginase, integrins

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## Abbreviations

| | |
|---|---|
| **1400W** | $N^g$-mono-methyl-L-arginine |
| **ACPA** | anti-citrullinated protein antibodies |
| **ACR** | American College of Rheumatology |
| **ADP** | adenosine diphosphate |
| **ARG** | arginase |
| **ATP** | adenosine triphosphate |
| **B cells** | B lymphocytes ('bursocytes') |
| **$BH_4$** | tetrahydrobiopterin |
| **CAT** | cationic amino acid transporter |
| **CCP** | cyclic citrullinated peptide |
| **CD** | cluster of differentiation |
| **cGMP** | cyclic guanosine monophosphate |
| **CRP** | C-reactive protein |
| **DC** | dendritic cells |
| **DFP** | diisopropylfluorophosphate |
| **DNA** | deoxyribonucleic acid |
| **EIA** | enzyme-immunoassay |
| **ELAM** | endothelial leukocyte adhesion molecule |
| **eNOS** | endothelial nitric oxide synthase |
| **E-selectin** | endothelial selectin |
| **ESR** | erythrocyte sedimentation rate |
| **FAD** | flavin adenine dinucleotide |
| **FcγR** | receptor with affinity for the Fc-part of IgG |
| **FITC** | fluorescein-isothiocyanate |
| **fMLF** | formyl-methionyl-leucyl-phenylalanine |
| **FMN** | flavin mononucleotide |
| **G proteins** | guanine nucleotide binding proteins |
| **GTP** | guanosine triphosphate |
| **$H_2O_2$** | hydrogen peroxide |
| **HLA-DRB1** | Human Leukocyte Antigen DRB1 |
| **HNO/$NO^-$** | nitroxyl |
| **HOCl** | hypochlorous acid |
| **HPLC** | high performance liquid chromatography |
| **HRP** | horse radish peroxidase |
| **IC** | immune complex |
| **ICAM** | intercellular adhesion molecule |
| **IFN-γ** | interferon gamma |
| **IgG** | immunoglobulin G |
| **IL** | Interleukin |
| **iNOS** | inducible nitric oxide synthase |
| **kDa** | kiloDalton |
| **LACL** | luminol amplified chemiluminescence |
| **LFA** | leukocyte function antigen |
| **L-NAME** | NG-nitro-L-arginine methyl ester |
| **L-NMMA** | L-NG-monomethyl-arginine |
| **LPS** | lipopolysaccharide |
| **L-selectin** | leukocyte selectin |
| **$LTB_4$** | leukotriene $B_4$ |
| **MAC-1** | CD11b/CD18 |
| **MHC** | major histocompatibility complex |
| **MPO** | myeloperoxidase |
| **mRNA** | messenger ribonucleic acid |
| **$N_2O_3$** | dinitrogen trioxide |
| **NADPH** | nicotinamide adenine dinucleotide phosphate hydrogen |
| **Neutrophils** | Polymorphonuclear neutrophil granulocytes |
| **nNOS** | neuronal nitric oxide synthase |

| | |
|---|---|
| **$NO^{\cdot}$** | nitric oxide |
| **$NO_2$** | nitrogen dioxide |
| **NOHA** | $N^G$-hydroxy-L-arginine |
| **nor-NOHA** | N-omega-hydroxy-nor-L-arginine |
| **NOS** | nitric oxide synthase |
| **NOX** | NADPH oxidase |
| **$^{\cdot}O_2^-$** | superoxide |
| **$OH^{\cdot}$** | hydroxyl radical |
| **$ONOO^-$** | peroxynitrite |
| **PAD** | peptidylarginine deiminase |
| **PAF** | platelet activating factor |
| **Phox** | phagocyte oxidase |
| **P-selectin** | platelet selectin |
| **RA** | Rheumatoid arthritis |
| **RF** | Rheumatoid Factor |
| **RNOS** | reactive nitrogen oxide species |
| **ROS** | reactive oxygen species |
| **SOD** | superoxide dismutase |
| **T cells** | T lymphocytes ('thymocytes') |
| **TGF-β** | transforming growth factor beta |
| **TNF** | tumour necrosis factor |
| **β2-integrin** | an integrin containing CD18 |

## List of original publications

I

**Cedergren J, Forslund T, Sundqvist T, Skogh T**

Intracellular oxidative activation in synovial fluid neutrophils from patients with rheumatoid arthritis but not from other arthritis patients

*(Submitted)*

II

**Cedergren J, Forslund T, Sundqvist T, Skogh T**

Inducible nitric oxide synthase is expressed in synovial fluid granulocytes

*Clin Exp Immunol 2002;130:150-55.*

III

**Cedergren J, Follin P, Forslund T, Lindmark M, Sundqvist T, Skogh T**

Inducible nitric oxide synthase (NOS II) is constitutive in human neutrophils

*APMIS 2003;111:963-8.*

IV

**Cedergren J, Forslund T, Sundqvist T, Skogh T**

Oxidative activation of human neutrophils by type-1-collagen-coated particles is influenced by nitric oxide production and modulated by endogenous arginase

*(Manuscript)*

*The stage*

## RHEUMATOLOGY

It has been claimed by the Swedish Association against Rheumatism that approximately 1 million people in Sweden suffer from some kind of rheumatic disease. This might well be true, using a wide definition of rheumatology, namely medical disorders of the musculo-skeletal system. Fortunately, many of these individuals have mild disorders with minimal or no need for medication, physiotherapy or surgery. Other large groups have complaints due to chronic widespread pain or osteoarthritis. In Sweden these patients are usually taken care of by primary care physicians and/or orthopaedic surgeons, whereas Swedish rheumatology clinics normally restrict their commitments to systemic inflammatory rheumatic diseases, vasculitides, and arthritides, affecting <5% of the population. Patients with arthritis is by far the largest group in Swedish rheumatology care, with rheumatoid arthritis (RA) being the most common diagnosis.

### Rheumatoid arthritis

RA is a chronic inflammatory disease leading to joint damage and bone destruction, which can result in severe disability and increased mortality. The prevalence is 0.5-1% and the annual incidence 20-50 cases per 100,000 inhabitants in North European countries [Alamanos 2005]. RA is a multi-factorial disease, influenced by genetic, hormonal and environmental factors. Genetic factors include the presence of the 'shared epitope', i.e. a product of the HLA-DRB1 locus [Stastny 1978, Gregersen 1987]. A recently described polymorphism in PTPN22 (protein tyrosine phoshatase non-receptor type 22), a gene regulating T-cell activation, has also gained much attention [Hinks 2006]. Known risk factors among predisposed individuals include female sex, cigarette smoking and exposure to silica [Klareskog 2006, Oliver 2006].

The pathogenesis of RA is characterized by massive activation of the immune system. Although RA is often regarded as a disease limited to joints, it is common with other organ engagement (*e.g.* skin, eyes, muscles, kidneys and lungs). Therefore, RA should be viewed as a systemic disorder. In the joints, the synovium is invaded by T cells, B cells, macrophages and dendritic cells. An array of cytokines is secreted and the synovium becomes hyperplastic and invasive in its behaviour. Long regarded as a T-cell/macrophage-driven disease, the interest in B cells is now growing after the discovery of antibodies directed against citrullinated peptides and the therapeutic effects of an anti-B-cell antibody [Vossenaar 2004, Edwards 2004]. In contrast, the joint cavity is almost exclusively invaded by large numbers of polymorphonuclear neutrophil leukocytes (neutrophils). Neutrophil functions are described in more detail below.

The results of these inflammatory events are extensive. A recent study on employed RA patients showed that 66% had a disease-related work loss the previous 12 months [Burton 2006]. The likelihood of being work disabled varies in different studies, but is probably somewhere between 40 and 50% after 10 years disease duration [Burton 2006]. Adding the costs for work disabilities, to the costs for medication and operations, RA represents a huge economic burden for the society [Jonsson 2000, Hallert 2004]. In addition, RA leads to increased mortality. Even if there is an increased risk for lymphoma and maybe also for lung cancer in RA [Askling 2005, Baecklund 2006, Bernatsky 2006], the majority of the increase in mortality is explained by cardiovascular disease [Del Rincon 2001]. Interestingly, traditional risk factors for cardiovascular disease do not solely explain the increase in cardiovascular disease among RA patients. It has been proposed that inflammation per se is a risk factor and that additional mechanisms may be operative [van Leuven 2006, Gonzalez-Gay 2006].

## NEUTROPHILS

Even if the role of neutrophils has been studied extensively in infectious diseases, it has been neglected in rheumatoid arthritis and other autoimmune conditions for a long time. The neutrophil is the predominating, and potentially the most destructive, circulating human leukocyte and therefore a cornerstone in our defence against invading microorganisms. Thus, tampering with neutrophils and their functions may result in life-threatening immunosuppression. The widespread view on neutrophils as "rudimentary 'non-smart' creatures- crawling, eating and disgorging pre-packed enzymes and reduced molecules of oxygen" [Nathan 2006] - is a misconception. Recent literature has shown that it is time to re-evaluate this picture both in general immunology and rheumatology.

### Neutrophil production

In adults, the neutrophil progenitor pool is located almost entirely within the bone marrow. The turn-over rate is high since neutrophils survive only a few hours after leaving the bone marrow. This high rate of turnover calls for storage reserves and dynamic regulation of neutrophil production. In the bone marrow this is achieved by the neutrophil storage pool, which consists of non-dividing stages of the myelopoesis that rapidly can be up-regulated. An important regulatory pathway of neutrophil production was recently discovered by Stark et al [Stark 2005], who reported that the release of interleukin (IL-) 23 from tissue macrophages is depressed by ingestion of apoptotic neutrophils. This affects T-cell production of IL-17 in secondary lymphoid organs, which is a regulator of the production of 'granulocyte colony stimulating factor' (G-CSF) by stromal cells in the bone marrow. Thus, phagocytosis of apoptotic neutrophils acts as an inhibitory homeostatic feedback loop on neutrophil production.

### Neutrophils in circulation

As neutrophils mature they become more deformable, acquire new cell membrane receptors and demonstrate motility [Wallace 1987]. Known factors to modulate neutrophil migration to the bloodstream are IL-1 and complement factors such as C3e and C3d. Other agents that have been associated with increased neutrophil migration include high-dose intravenous immunoglobulin and glucocorticosteroids. After entering peripheral blood, neutrophils consist of a freely circulating pool and a marginated pool. Both pools are of approximately the same size. The marginated pool consists of cells adhering to the walls of small vessels. By the use of istope-labelled cells it appears that neutrophils selectively marginate in the lung, liver and spleen [Saverymuttu 1983]. Adhesion is modulated by the interaction of integrins, e.g LFA-1 (CD11a-CD18 complex), on the neutrophil surface with receptors (e.g ICAM-1, ICAM-2, ELAM-1) on

the vessel endothelium. If not activated within a few hours, neutrophils enter peripheral tissue and undergo apoptosis.

## Interactions with the endothelium

Neutrophils are the fastest cell in the body and arrive within minutes at inflamed tissues. When neutrophils are exposed to chemoattractants (*e.g.* C5a) and other active substances (*e.g.* TNF), they rapidly become much more adhesive to the endothelium whether or not the endothelium is stimulated. This is mediated by activation of the β2-integrins LFA-1 and MAC-1 (CD11b-CD18 complex) [Lo 1989, Kuypers 1990]. LFA-1 interacts with ICAM-2, which is normally expressed in unstimulated endothelium. MAC-1 is also important for this interaction but does not bind to ICAM-2. Interestingly, this early β2-integrin dependent adhesion results from a qualitative change in integrin avidity and does not require increased cell-surface expression [Vedder 1988].

Within minutes after the onset of an inflammation, stimulated endothelial cells start to express P-selectin on the cell membrane and after a few hours also E-selectin, ligands for neutrophil L-selectin and ICAM-1. Selectins interact with glycoproteins (*e.g.* P selectin glycoprotein ligand (PSGL-1)) on the neutrophil surface, resulting in a 'rolling' action of the neutrophils along the endothelial surface. The neutrophil-endothelial interactions trigger mobilization of secretory vesicles from neutrophils, resulting in enrichment on the neutrophil surface with MAC-1 and shedding of L-selectin [Borregaard 1994]. After interaction between neutrophil integrins and endothelial ICAMs neutrophil movement is stopped. This results in spreadening and flattening of the neutrophil that now is ready to migrate across the endothelial barrier and start moving towards the inflammatory site. Among several cytokines able to modulate this process it is worth mentioning PAF, which is released by the endothelium soon after the start of inflammation. PAF activates β2-integrins on adherent neutrophils, which leads to enhanced adhesion, chemotaxis, superoxide anion ($^{\cdot}O_2^-$) production and degranulation [Pinckard 1988].

## Chemotaxis (neutrophil migration)

Once neutrophils have left the circulation they encounter a complex array of extracellular matrix proteins, which activate their complement receptors or IgG Fc-receptors (FcγR) to enhance phagocytosis. This reversible adherence to a substratum, plus chemoattractant binding to plasma membrane receptors and reversible assembly of cytoskeletal elements (polymerization of globular actin (G-actin) monomers into actin filaments (F-actin)), are required for neutrophil chemotaxis. Examples of potent chemoattractants inducing neutrophil chemotaxis include N-formyl peptides, C5a, leukotriene $B_4$ ($LTB_{4)}$, IL-8 and PAF [Harvath 1991]. All chemoattractants have specific receptors predicted to couple to 'G proteins' in order to transmit signals to

the interior of the cell. After coupling, effector enzymes such as adenylate cyclase or phopholipase can be activated and generate second messengers.

## Phagocytosis and degranulation

Once on their way to inflammatory sites, neutrophil destruction of micro-organisms destruction may include oxidative (involving respiratory burst) and/or non-oxidative mechanisms. Non-oxidative killing involves the secretion of lysosomal enzymes (degranulation). In this warfare, no piece of the neutrophil armament is left unused to defeat microbial insults. Moving along the concentration gradient of a stimulus, the neutrophils can liberate the contents of two sets of granules (see below), activate a lethal shower at the plasma membrane, eject their nuclear proteins and ultimately throw up the cytosol. Neutrophils perceiving tissue damage plus infection, without rapidly encountering a micro-organism, will eject their arsenal extracellularly. In vitro, this control phase lasts for15-45 min [Nathan 1987]. Thereafter, pus is formed, cutting off microbial escape routes and trapping the microbes in a noxious cocktail. Under these circumstances, even the neutrophil nuclei contribute to host defense, where released chromatin forms extracellular nets decorated with proteases from the azurophil granules [Brinkmann 2004].

If an enemy or cell debris is encountered, neutrophils can phagocytose both non-opsonized and opsonized particles. The principal opsonin receptors of neutrophils, FcγRs and MAC1 (CD11b-CD18), bind to immunoglobulin or complement-coated particles respectively. The main FcγRs on resting human neutrophils are FcγRIIa (CD32) and FcγRIIIb (CD16), while the high affinity FcγRI (CD64) functions predominantly after neutrophils have been primed with gamma-interferon (IFN-γ) [McKenzie 1998]. These transmembrane receptors activate downstream protein and lipid kinases, inducing the polymerization of actin and the localized membrane remodelling that are essential for particle ingestion. While complement-opsonized particles are internalized by gently 'sinking' into the cell, FcγR ligation initiates the vigorous extension of pseudopods that surround and ultimately entrap the particle [Greenberg 2002].

The formed phagosome acquires its degradative and antimicrobial effects through fusion with secretory vesicles and granules, which contain a remarkably powerful arsenal of microbicidal peptides and proteolytic enzymes. There are two predominant types of granules, the azurophil and the specific. The azurophil granules largely contain proteins and peptides directed toward microbial killing and digestion, whereas the specific granules replenish membrane components and help to limit free radical production [Segal 2005]. Azurophil (or primary) granules contain myeloperoxidase and three predominant proteinases: cathepsin G, elastase and proteinase 3. Also, one third of the total lysozyme is found in these granules. More recently discovered factors include defensins, exhibiting antibacterial activity [Ganz

2003], and bactericidal/permeability increasing protein (BPI), first purified as a factor killing *Escherichia coli*. Inside the granule, these enzymes are resting in an acidic pH and strongly bound to a matrix of negatively charged sulphated proteoglycans [Kolset 1990]. Specific (secondary) granules contain unsaturated lactoferrin, which binds iron and copper; transcobalamin II, which binds cyanocobalamin; gelatinase; about two thirds of the lysozyme; and a number of membrane proteins. The latter include flavocytochrome $b_{558}$ of the NADPH oxidase (NOX), *see below* [Segal 1979]. Also found in these granules are the three matrix metalloproteinases MMP8, MMP9 and MMP25. By degrading laminin, collagen, proteoglycans and fibronectin, these MMPs are thought to have an important role in facilitating neutrophil recruitment and tissue breakdown [Faurschou 2003]. The term gelatinase (tertiary) granules refer to granules containing gelatinase but not lactoferrin. Secretory vesicles contain serum albumin and provide a valuable reservoir of membrane components. They replenish membrane proteins that are consumed during phagocytosis, but also contain flavocytochrome $b_{558}$. Neutrophils also have lysosomes containing acid hydrolases. Lysosomes normally release their contents into the phagocytic vacuole much later than the azurophil granules.

Changes in the free cytosolic $Ca^{2+}$ are required for granular fusion with the phagosome in neutrophils [Jaconi 1990]. The individual secretory compartments have different calcium tresholds for secretion. The changes in intracellular calcium levels leads to depolymerization of the periphagosomal actin coat, which permit docking and fusion of granules and vesicles [Bengtsson 1993]. While granules and secretory vesicles are capable of fusing throughout the plasma membrane when neutrophils are stimulated with soluble agonists, fusion is restricted to the phagosome membrane during engulfment of particles. Candidates for this regulatory mechanism are the SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors) family of proteins [Lee 2003].

A number of events are initiated after fusion of the phagosome with intracellular granules. The NOX is activated, creating reactive oxygen metabolites serving as antimicrobial agents, as well as regulators of the phagosomal pH. This serves to optimize the conditions for the liberated degradative enzymes, but also leads to ion fluxes ($H^+$, $K^+$ and $Cl^-$) and changes in the electrochemical gradient across the phagosomal membrane [Segal 2005, Lee 2003].

## The respiratory burst

Oxidative-dependent killing is mediated by oxygen metabolites generated upon activation of the neutrophil enzyme NOX (the respiratory burst). The enzyme is inactive until the neutrophil is stimulated by engagement of receptors for chemoattractants or receptors mediating phagocytosis or responses to various cytokines. NOX catalyses the following reaction:

**NADPH + $2O_2$ → $NADP^+$ + $2^{\cdot}O_2^-$ + $H^+$.**

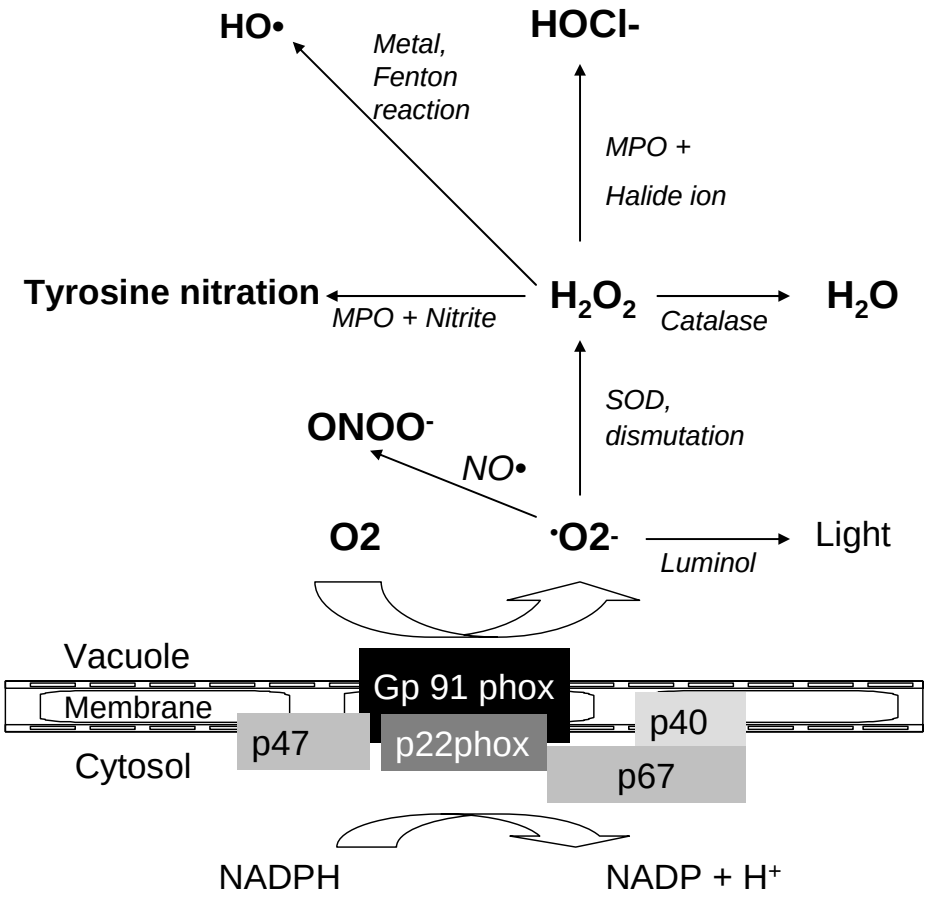


**Figure 1:** A simplified presentation of the neutrophil NADPH oxidase and some of the possible reactions following the production of superoxide ($^{\bullet}$O2-). NO•= nitric oxide, $ONOO^-$=peroxynitrite, SOD=superoxide dismutase, $H_2O_2$= hydrogen peroxide, MPO= myeloperoxidase, HO•= hydroxyl radical, HOCl-= hypochlorous acid. The membrane components gp91*phox* and p22*phox* is also referred to as cytochrome b551.

Increased levels of $NADP^+$ activate the pentose phosphate pathway and NADPH is regenerated. Concurrently with activation of the NOX, intracellular granules fuse with the plasma membrane or phagosomal/endosomal membranes to release a broad range of molecules. The generated $^{\bullet}O_2^-$ can rapidly combine (dismutation) to form $H_2O_2$, a process that can be accelerated by released superoxide dismutase (SOD). In the presence of iron, $^{\bullet}O_2^-$ and $H_2O_2$ react to generate hydroxyl radicals ($OH^{\bullet}$). In the presence of released myeloperoxidase (MPO), neutrophils use $H_2O_2$ and chloride to form hypochlorous acid (HOCl) (Fig 1). Post-translational chlorination of proteins, *e.g.* collagen, may increase their antigenicity/arthritogenicity [Westman 2006]. A number of other reactive oxygen species can also be found in inflamed areas, *e.g.* singlet oxygen by MPO-catalyzed oxidation of halide ions and ozone, formed by the reaction of singlet oxygen and antibodies bound to an antigen.

The neutrophil NOX has been well characterized. It consists of the catalytic subunit gp91phox, together with the regulatory subunits p22phox, p47phox, p40phox, p67phox and the small GTPase RAC. The combination of the two membrane bound subunits gp91phox and p22phox is often designated as flavocytochrome $b_{558}$ and contains the active site of the oxidase (Fig 1). The exact function of the cytosolic components has not been fully clarified, but they appear to serve as adaptor proteins at cytoplasmic side of the membrane-bound enzyme complex. A number of detailed reviews articles on the biochemistry of this system have been published [Cross 2004, Vignais 2002].

## Time for re-evaluation of the neutrophil?

As pointed out by Carl Nathan [Nathan 2006], it is easy to view the neutrophil as an unintelligent rapidly crawling cell, attempting to engulf and destroy microbes, and, if that is not possible, reacting according to the programme: "release all your weapons extracellularly, wait for reinforcements while you undergo apoptosis". If this statement were true, it is a bit distracting that neutrophils match every capability to kill nucleated cells that cytotoxic T-cells and natural killer cells exhibit. Neutrophils top it with the capability to kill and destroy non-nucleated cells and connective tissue. In fact, "neutrophils are the only cell licensed to liquefy any part of the body" [Nathan 2006]. With this authority in mind, it would not be wise to start our human immune defense with a non-differentiated response.

## Neutrophils as decision-makers

Several reports in recent years have highlighted the role of neutrophils as a cellular source of chemokines and their possibility to orchestrate early immune responses [Scapini 2000]. Neutrophils generate chemotactic signals and cytokines (*e.g.* TNF) that attract monocytes and dendritic cells (DC), and influence whether macrophages differentiate into a predominantly pro- or anti-inflammatory state [Chertov 1997, Bennouna 2003, Tsuda 2004]. Neutrophils generate chemerin, one of the few chemokines that attracts both

immature and mature plasmacytoid DC's [Wittamer 2005]. DCs are activated by cell-cell contact with neutrophils, in which carbohydrates on CD11b engage DC-specific ICAM3-grabbing non-integrin (DC-SIGN) [van Gisbergen 2005]. B-cell proliferation and maturation can be driven by neutrophil secretion of the TNF-related ligand B lymphocyte stimulator (BLyS) [Scapini 2005]. Moreover, neutrophils secrete IFN-γ, which helps to drive activation of macrophages and differentiation of T-cells, but neutrophils can also act as powerful suppressors of T-cells by impairing functions of the T-cell receptor [Ethuin 2004, Schmielau 2001, Munder 2006].

The presence of myeloid suppressor cells (MSCs) in chronic immune responses is well known [Bronte 2005]. These cells are commonly identified by the presence of MAC-1 and a small membrane-anchored antigen designated GR1. Even if neutrophils express both these antigens, MSCs generally appear as dendritic or macrophage-like cells. However, the recent finding that synovial-fluid derived neutrophils can undergo trans-differentiation to cells with dendritic-like characteristics further highlights the complex role of neutrophils in arthritis [Iking-Konert 2005].

In summary, it is plausible that the well-characterized role for neutrophils in innate immunity, in the future must be extended to include initiation and choice of suitable adaptive immune response.

## Therapeutic possibilities by targeting neutrophil NOX

Therapies aiming at tampering with neutrophils introduce obvious risks of causing serious side effects. Until recently it was thought that whatever activated a neutrophil strongly enough to make it undergo a respiratory burst also forced it to degranulate. This notion may have contributed to the reluctant attitude towards neutrophil-targeted modulation of inflammatory conditions. However, lately new insights have been achieved.

The TNF-mediated immediate rise of intracellular $Ca^{2+}$ in adherent neutrophils, has been found to activate soluble adenylyl cyclase and subsequently also NOX [Han 2005]. This discovery led to the identification of neucalcin, a compound able to block TNF-induced elevation of intracellular $Ca^{2+}$, preventing NOX activation only when initiated by soluble pro-inflammatory factors. Neucalcin does not block neutrophil migration, degranulation or killing of bacteria [Han 2005]. Since RA, as well as many other chronic inflammatory disorders, is characterized by high levels of TNF this may be an interesting future therapeutic approach.

The scenery around the production of reactive oxygen species (ROS), has undergone tremendous changes during the last years. Until the end of the 1980's, the only known example of deliberate production of ROS in mammalian cells was the NADPH oxidase in neutrophils and macrophages. During the 1990's the use of sensitive assays allowed the detection of low

amounts of ROS in various cell types. In 1999, the first homologue to gp91phox was described [Suh 1999] and subsequently designated NOX1, whereas the 'original' NADPH oxidase in phagocytes was named NOX2. Today, seven enzymes in the NOX family have been identified, including the dual oxidases (DUOX1 and DUOX2), which also contain a peroxidase domain that might mimic the function of myeloperoxidase [Lambeth 2004]. Enzymes belonging to the NOX family can be both constitutive and inducible, and have been found in a variety of tissues (*e.g.* colon, smooth muscle, kidneys, eyes, thyroid and lungs). In general, low levels of ROS are seen in these cells and have been implicated in cell signalling. A large body of evidence regarding different roles of ROS (*e.g.* cell growth, apoptosis, senescence and hormone function) was accumulated even before the discovery of the NOX family [Burdon 1995]. The field of NOX enzymes has been reviewed by Lambeth [Lambeth 2004].

A novel and very interesting finding concerning NOX was described by the research group of Rikard Holmdahl in Lund. Searching for arthritis candidate genes, they identified Ncf-1 as a gene regulating arthritis severity in rats [Olofsson 2003]. The Ncf-1 gene encodes p47phox, i.e. a cytosolic factor in the NOX complex, and a functional single nucleotide polymorphism in this gene was associated with reduced oxidative response and survival of arthritogenic T-cells [Gelderman 2006]. The lower capacity to produce superoxide is associated with an increased number of reduced thiol groups (-SH) on T-cell membrane surfaces and increased T-cell reactivity [Gelderman 2006]. Treatment with phytol, a vitamin E derivative, increased the oxidative burst and corrected the effect of the genetic polymorphism in this rat model [Hultqvist 2006]. Mutations in the Ncf-1 gene have also been associated with encephalomyelitis and enhanced autoimmunity [Hultqvist 2004].

## Neutrophils in arthritis

Neutrophils were long considered to be devoid of transcriptional activity and thus not capable of performing substantial protein synthesis. However, it is now known that neutrophils, either constitutively or in an inducible manner, can synthesize and release a wide range of pro- or anti-inflammatory cytokines and growth factors [Cassatella 1999]. The pattern of cytokines produced by neutrophils depends on the stimulus [Casatella 1995]. Even if *in vitro* cytokine production by neutrophils is much lower compared to that by monocytes, this is less evident in vivo considering that neutrophils are the first to be recruited at the site of inflammation and largely predominate in number over monocytes. Apart from cytokines, a variety of neutrophil proteins are up-regulated in rheumatoid synovial fluid [Cross 2005]. The receptors FcγRI and MHC Class II are not normally expressed on the surface of circulating neutrophils, but can be detected on neutrophils exposed *in vitro* or *in vivo* to pro-inflammatory cytokines or isolated from inflammatory sites [Cross 2005, Iking-Konert 2005].

Although RA has long been considered to be a T-cell/macrophage driven disease, it cannot be denied that neutrophils have great capacity to inflict joint damage [Edwards 1997]. Indeed, neutrophils have been identified at the pannus/cartilage border [Bromley 1984, Mohr 1981], but their predominant location is within the joint cavity. Neutrophil counts in rheumatoid synovial fluid can exceed 100,000/$mm^3$, and the turnover can exceed one billion cells per day in a 30 ml joint effusion [Hollingsworth 1967]. Synovial fluid contains both pro- and anti-apoptotic signals, but Cross and co-workers recently showed that neutrophil apoptosis is delayed by synovial fluid, and dependent on local oxygen tensions [Cross 2006]. Very little is known about mechanisms concerning neutrophil entrance or departure from synovial fluid.

Rheumatoid synovial fluid contains no or little IL-2, IL-3, IL-4 or IFN-γ, which are derived from T-cells [Firestein 1987, Firestein 1988, Miossec 1990]. In contrast, synovial fluid contains high levels of TGF-β, IL-6, IL-8, IL-1 and TNF-α [Miossec 1990, Guerne 1989, Brennan 1990, Nouri 1984, Saxne 1988]. TGF-β is not produced by neutrophils but is one of the most powerful neutrophil chemoattractants [Brandes 1991]. Whether or not IL-6 is secreted by neutrophil is still debated. IL-6 is thougt to dictate the transition from acute to chronic inflammation and primarily exerts stimulatory effects on T- and B-cells. IL-8 is a powerful neutrophil chemoattractant, also capable of inducing degranulation, priming the respiratory burst and stimulating the production of other pro-inflammatory mediators. Neutrophils secrete significant quantities of IL-8 in response to IL-1 or TNF-α [Fujishima 1993]. Interestingly, chondrocytes are also a major source of IL-8. Although IL-8 lacked direct effects on cartilage breakdown, co-culture of neutrophils and cartilage explants in the presence of IL-8 caused rapid cartilage degradation [Elford 1991].

A cytokine that has attracted much interest in RA is IL-1β. Synovial fluid IL-1β is considered derived from synovial macrophages and chondrocytes. Neutrophils, however, are capable of synthesizing IL-1β, and considering their overwhelming numbers in rheumatoid synovial fluid, the cumulative IL-1 production by neutrophils may be significant [Lord 1991]. IL-1 effects on neutrophils include priming for phagocytosis, degranulation or superoxide generation, but may also be indirect via effects on endothelial expression of adhesion molecules.

TNF-α is a cytokine of central importance in RA. Several cytokines, including TNF-α itself and IL-1, are potent inducers of TNF-α secretion by neutrophils [Witko-Sarsat 2000]. TNF-α enhances expression of adhesion molecules, induces degranulation and primes oxidative activity in neutrophils. It has been shown that neutrophil-mediated cartilage injury is modulated by cytokines such as TNF-α [Kowanko 1990]. Edwards and Hallet actually proposed that neutrophils may be the main target of anti-TNF-α therapy

[Edwards 1997]. Their postulate concerning neutrophil migration was also confirmed by den Broeder and co-workers, showing that a single dose anti-TNF-α antibody markedly and rapidly decreased neutrophil influx to inflamed joints [den Broeder 2003].

With the previous descriptions of neutrophil functions in mind, it is also obvious that neutrophils can contribute to tissue damage in RA in other ways than being participants in the cytokine network. Fcγ receptors, mainly FcγRI, are up-regulated on synovial fluid neutrophils [Pillinger 1995]. Thus, neutrophils may bind to and endocytose soluble immune complexes (ICs), resulting in prostaglandin and leukotriene production, neutrophil degranulation, and the respiratory burst. Larger insoluble ICs/aggregates may result in incomplete closure of the phagocytic vacuole, and extracellular release of granular contents into synovial fluid. It has been shown that neutrophil degranulation in synovial fluid indirectly may contribute to articular damage by degrading synovial fluid hyaluronic acid [Greenwald 1980], but whether direct cartilage injury can be caused this way is a matter of discussion [Pillinger 1995, Edwards 1997]. However, several studies have shown increased superoxide production from synovial fluid neutrophils (discussed in paper I), and synovial-derived proteins show signs of sustained damage by reactive oxidants [Chapman 1989].

It has been reported that rheumatoid joint cartilage contains ICs embedded within its superficial layers [Ugai 1979 and 1983, Jasin 1985], and that these complexes include rheumatoid factor as well as anti-collagen antibodies providing an effective surface for neutrophil adherence and invasion. Thus, neutrophils may struggle to phagocytose the opsonized surface but, being unable to embrace it, ultimately discharging their contents directly onto the cartilage surface ('frustrated phagocytosis') [Pillinger 1995]. Sealed by the neutrophil plasma membrane, this compartment may restrict the availability of protective synovial fluid anti-proteases and proteins intervening with oxygene metabolites. *In vitro* studies have suggested an important role of reactive oxygen species in cartilage degradation, as reflected by their effects on matrix components (all of whom can be damaged by ROS) and chondrocyte behaviour [Henrontin 2003]. Chondrocyte defenses against such attracts include massive production of nitric oxide ($NO^{\cdot}$), which inhibits neutrophil superoxide production as well as neutrophil adherence by preventing actin polymerization. It has also been demonstrated that the inhibition of chondrocyte $NO^{\cdot}$ production exacerbates neutrophil-mediated cartilage destruction [Pillinger 1995].

As mentioned above, anti-rheumatic pharmacotherapy targeting TNF-α may function by preventing neutrophil recruitment to inflamed joints. Also many older anti-rheumatic agents, including non-steroidal anti-inflammatory drugs, glucocorticosteroids and gold compounds exert effects on neutrophils [Pillinger 1995, Hafström 1983]. Methotrexate, the most commonly used

disease-modifying anti-rheumatic drug, is a folate antagonist, but this mechanism is unlikely to explain its effects in RA. An alternative explanation may be the inhibition of endothelial adenosine release, which is mediated by methotrexate. Adenosine acts via neutrophil receptors to inhibit neutrophil functions, including adhesion and superoxide generation [Cronstein 2005]. In synovial fluid, however, the anti-inflammatory effect of adenosine may be counteracted by high levels of adenosine deaminase [Nakamichi 2003]. In a clinical study, the anti-rheumatic agents leflunomide and methotrexate, were found to significantly reduce neutrophil chemotaxis, joint swelling and pain [Kraan 2000].

Experienced rheumatologists have often noted that RA patients developing Felty's syndrome, i.e. the triad of RA, neutropenia and splenomegaly (due to consumption of circulating neutrophils), experience a marked decrease in inflammatory activity and number of swollen joints. A disease-modifying effect by elimination of circulating neutrophils is also supported by the positive results obtained by granulocyte apheresis in RA [Kashiwagi 1998, Mori 2004, Sanmarti 2005]. Furthermore, this notion gains support from animal models, *e.g.* the spontaneously occurring RA-like arthritis in K/BxN mice, where initiation and maintenance of arthritis is dependent on neutrophils [Wipke 2001]. In another murine arthritis model induced by anti-type II collagen antibodies and lipopolysaccharide, neutrophil depletion completely inhibited arthritis development, and ameliorated the disease in animals that had already developed arthritis [Tanaka 2006].

**PATHWAYS OF L-ARGININE**

## L-arginine

Arginine was identified already in 1895 as a component of animal proteins [Oliver 1895], and the discovery of the urea cycle by Krebs and Henseleit in 1932 led to the elucidation of the prominent roles of arginine in physiology and metabolic pathways [Krebs 1932]. Arginine is classified as a non-essential amino acid in healthy adults, but as an essential amino acid in young growing mammals, and in adults in cases of disease and trauma. The explanation for this classification is that exogenously (diet) supplied arginine accounts for about 10% of the daily flux of arginine in plasma [Wu 1998]. The major source of endogenous arginine production is protein degradation and turnover, while de novo synthesis in the liver, intestine and kidneys accounts for 5-15% of the daily arginine flux [Wu 1998]. A highly localized arginine biosynthetic pathway has also been shown in a number of NO$^{\bullet}$-producing cell lines. Citrulline, which is co-produced with NO$^{\bullet}$ under the action of NOS, can be recycled to arginine by the combined actions of arginine succinate syntethase and arginine succinate lyase. These enzymes, which are expressed to some degree in nearly all cell types, have been shown to be co-induced with iNOS (see below). Thus, regulation of the arginine recycling pathway represents a regulatory mechanism for inducible NO$^{\bullet}$ production. Studies in this field also support that regulation of arginine availability is an important control point for NO$^{\bullet}$ synthesis [Morris 1994, Xie 1997, Wu 1998].

Arginine transport systems may also regulate substrate availability for arginine-requiring enzymes. A family of cationic amino acid transporters (CAT) can be dynamically regulated in response to specific stimuli. Co-induction of CAT and iNOS has been shown in a variety of cell types. Arginine uptake by CAT, as well as the effect of some NOS inhibitors (*e.g.* L-NMMA) can be competitively inhibited by several other cationic amino acids (*e.g.* lysine, ornithine). Several other NOS inhibitors use the CAT system to be taken up, and may thus limit arginine availability besides inhibiting NOS. The cellular localization of CATs may also be responsible for the phenomenon designated 'the arginine paradox', referring to the observation that endothelial NO$^{\bullet}$ production can be regulated by altering the extracellular arginine concentration, despite normal intracellular arginine concentrations. Interestingly, it has also been shown in endothelial cells that CAT-1 is co-localized with NOS, indicating a directed delivery of extracellular arginine to NOS [McDonald 1997, Hatzoglou 2004, Wu 1998].

Most of the attention regarding arginine pathways during the last 15 years has focused on arginine catabolism (Fig 2). Of the four known enzymes using soluble arginine as a substrate, arginases and the NOS family will be

discussed in more detail below. The mitochondrial enzyme arginine:glycine amidinotransferase initiates arginine conversion to creatine, which is taken up by skeletal muscle and converted to creatinine after non-enzymatic dehydration. Since creatinine is filtrated by the kidneys, it is widely used as a clinical marker of renal function. The fourth enzyme, arginine decarboxylase, produces agmatine and $CO_2$ and is more recently identified in humans. The function of agmatine is not clear, but it has been proposed to act as a gating mechanism between the NOS *vs* arginase pathways, thus coordinating between inflammation and repair phases [Satriano 2003].

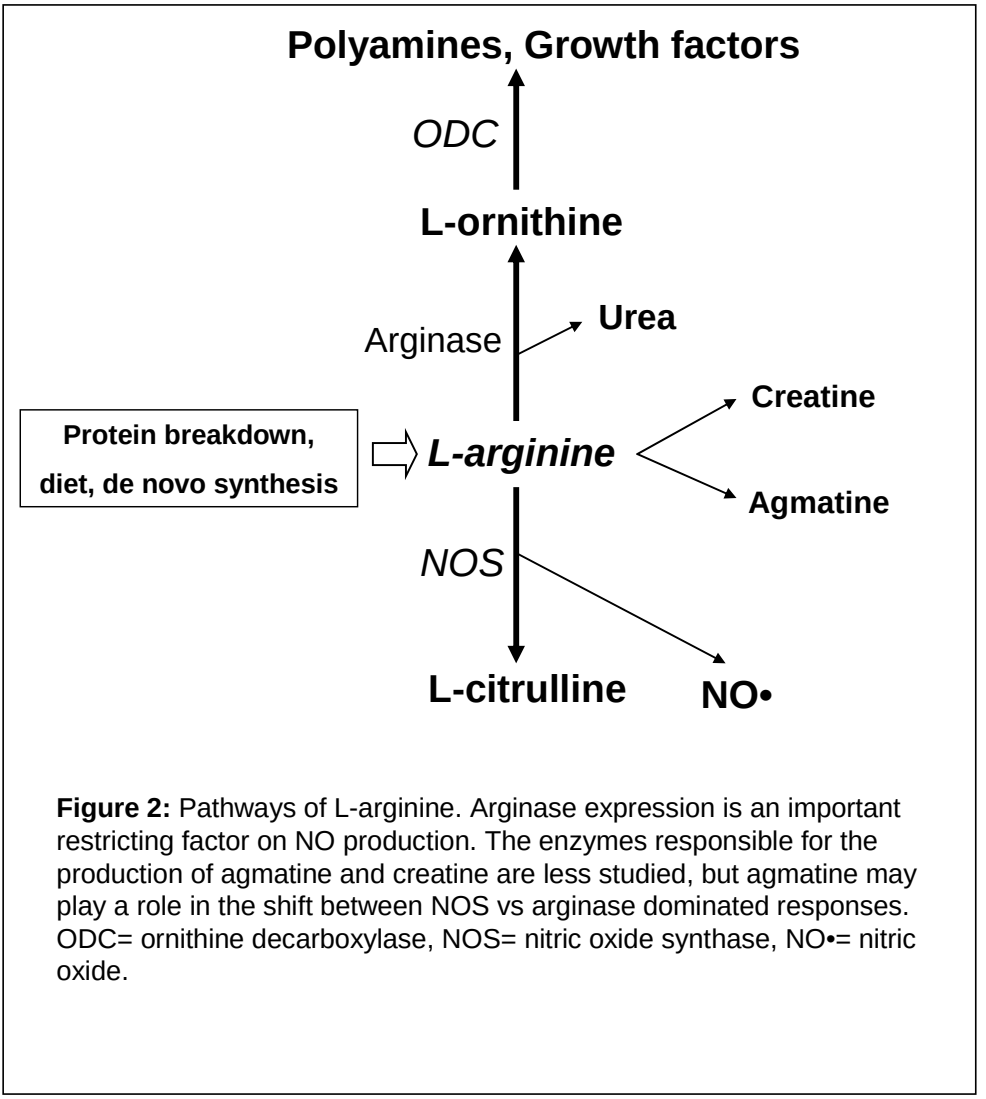


**Figure 2:** Pathways of L-arginine. Arginase expression is an important restricting factor on NO production. The enzymes responsible for the production of agmatine and creatine are less studied, but agmatine may play a role in the shift between NOS vs arginase dominated responses. ODC= ornithine decarboxylase, NOS= nitric oxide synthase, NO•= nitric oxide.

Another field of great interest to rheumatologists and immunologists is that of arginine-containing proteins and their immunogenic properties after enzyme-mediated conversion of arginine residues to citrulline. These enzymes, called peptidyl-arginine deiminases (PAD), become activated when intracellular $Ca^{2+}$ levels are sufficiently high, *e.g.* as a result of inflammatory activation or apoptosis. This explains the high expression of citrullinated proteins at sites of inflammation, *e.g.* in arthritic joints [Vossenaar 2003, Lundberg 2005]. Of the four known PAD isotypes, PAD-2 occurs in cell nuclei of monocytes/macrophages and PAD-4 also in the nuclei of neutrophils [Vossenaar 2003]. PAD has also been identified in the oral pathogen *Porphyromonas gingivalis*, making infection a possible initiator of protein citrullination [Rosenstein 2004]. Formation of anti-citrullinated protein antibodies (ACPA) is highly specific for rheumatoid arthritis and implicated as a pathogenic factor [van Venrooij 2006]. Since citrullinated proteins are abundant in the inflamed joint, it is conceivable that ACPA opsonizes soluble and surface-bound ICs in the rheumatoid joint, making them targets of phagocyte assault and activation in the synovium, cartilage and synovial fluid. Although a large number of potential target antigens (*e.g.* collagens, fibrinogen, vimentin and histones) can be citrullinated, a synthetic cyclic citrullinated peptide (CCP) is commonly used for diagnostic purposes. Not only is anti-CCP a sensitive and specific marker for RA, but its presence is also a predictor of an aggressive disease course and poor outcome [Kastbom 2004; Forslind 2004; Rönnelid, 2005; Huizinga 2005].

Methylation of arginine is another option for post-transcriptional protein modification. Recent studies have revealed that this is more common and widespread than previously thought, and a whole family of protein arginase methyltransferases (PRMT) is now recognized [Blanchet 2006]. In T-cells, stimulation of the co-stimulatory receptor CD28 induces arginine methylation of several substrates. Since demethylation has not been described, this is probably a long-lasting modification and may represent a form of storage of chemical information; 'signal memory' [Blanchet 2006]. Degradation of proteins containing methylated arginine may result in the formation of assymetric dimethylarginine (ADMA), which presently attracts much attention in cardiovascular research. ADMA can inhibit endothelial $NO^{\cdot}$ production, leading to endothelial dysfunction, atherosclerosis and increased risk of cardiovascular disease [Böger 2006].

## Arginase

Two arginase isoforms (ARG1 and ARG2), encoded by different genes, have been identified in mammals. The isoforms have 58% sequence identity at the amino acid level, but they have distinct tissue, cellular and subcellular distributions. ARG1 (also known as liver-type ARG) is located in hepatocytes and is an important component of the urea cycle. Cytokines can induce ARG1 expression in several biological systems, a process strictly linked to up-regulation of CATs [Bronte 2005]. Importantly, human ARG1 was recently also found to be constitutively expressed by neutrophils [Munder 2005]. This finding was very recently confirmed, although the exact

subcellular/granular localization of arginase is controversial [Jacobsen in press]. Besides participation in fungicidal activity, neutrophil arginase release can inhibit T-cell activation and T-cell proliferation [Munder 2006]. ARG2 (kidney-type ARG) is constitutively expressed in the mitochondria of various cell types, including renal cells, neurons, macrophages and enterocytes. ARGs are highly conserved between species, and bacteria and parasites sometimes exploit their arginases as a survival strategy by arginine starvation of the host [Vincendeau 2003].

After trimerization in the presence of $Mn^{2+}$, ARG hydrolyses arginine to ornithine and urea. Long recognized as the final reaction in the hepatic urea cycle, subsequent urinary excretion of urea is a regulatory mechanism of the body nitrogen balance. Outside the liver, however, ornithine production is the most important function of ARG. Ornithine is a precursor for the synthesis of polyamines by the ornithine decarboxylase (ODC), and for the synthesis of proline by ornithine aminotransferase. Polyamines are involved in cell growth and differentiation, whereas proline affects collagen production [Wu 1998, Bogdan 2001]. Accordingly, increased ARG activity has also long been detected in patients with colon, breast, lung or prostate cancer [Cederbaum 2004].

Theoretically, owing to their common use of arginine as a substrate, ARG and NOS (see below) can mutually limit substrate availability. However, although arginase-dependent limitation of substrate availability has been shown [Tenu 1999], this has not been demonstrated for NOS. Instead, $N^G$-hydroxy-L-arginine, the intermediate in $NO^{\cdot}$ synthesis, is also a potent arginase inhibitor [Daghigh 1994]. The interplay between ARG and NOS has been implicated in the Th1/Th2 paradigm, i.e. the shift between inflammation and healing [Hesse 2001]. Combined and regulated actions of ARG and NOS have also been shown, *e.g.* myelomonocytic suppressor cells in inhibiting T-cell responses to antigen [Bronte 2005].

With these data in mind, its not surprising that overexpression/overactivity of arginase has been implicated in the pathogenesis in a number of disorders, malignancies already mentioned. In psoriasis, keratinocyte hyperproliferation may be explained by arginase-mediated limitation of NOS activity [Bruch-Gerharz 2003]. In RA, both serum arginase protein levels and enzyme activity are increased [Huang 2001]. The role of arginase in vascular pathologies is a rapidly emerging field and arginase has been implicated in pulmonary hypertension, ischaemia-reperfusion, arterial hypertension, ageing, sexual arousal and atherosclerosis [Huynh 2006].

## Nitric oxide synthases (NOSs)

The production of NO˙ in the body is catalyzed by a family of enzymes called nitric oxide synthases (NOSs) (130-160 kDa). They all share between 50-60% homology [Alderton 2001]. Three distinct human isoforms have been isolated and cloned: eNOS (endothelial NOS, NOS I), iNOS (inducible NOS, NOS II), and nNOS (neuronal NOS, NOS III). nNOS and iNOS are considered soluble whereas eNOS is membrane bound [Liu 1995]. The eNOS and nNOS isoforms, designated 'constitutive NOS', are constantly present in resting cells, and are activated by calcium and calmodulin. NO˙ is synthesized in low concentrations by constitutive NOS, binds to haem iron of soluble guanylate cyclase to yield the second messenger 'cyclic guanosine monophosphate' (cGMP), which in turn modulates an array of mediators, including various ion channels, phophodiesterases and protein kinases, decreasing intracellular calcium levels, and allows smooth muscle relaxation [Ignarro 1992].

iNOS is normally not present in resting cells but can be induced by pro-inflammatory cytokines, bacterial products or infection of a number of cells, including endothelium, hepatocytes, monocytes/macrophages, mast cells and smooth muscle cells. Activation of iNOS generates NO˙ in high concentrations independently of intracellular calcium concentrations [Mayer 1997, Alderton 2001].

NO˙ formed by nNOS acts as a neuromodulator or neuromediator in some central neurons and in peripheral 'non-adrenergic non-cholinergic' nerve endings. nNOS-deficient mice develop preserved hippocampal long term potentiation, gastroparesis and muscle disorders [Huang 1993]. Besides the nervous system, skeletal muscle and vascular smooth muscle cells, nNOS has been identified in neutrophils [Wallerath 1997].

NO˙ produced by eNOS is responsible for maintaining low vascular tone and preventing leukocytes and platelets from adhering to the vascular wall [Ignarro 2002]. eNOS deficient mice develop hypertension, abnormal remodelling and increased intimal proliferation following vascular injury [Shesely 1996].

NO˙ synthesized under the influence of iNOS in macrophages and other cells plays multiple roles in the inflammatory defence (discussed below). Mice deficient of iNOS are more susceptible to inflammatory damage and tumours, but more resistant to septic shock [Mashimo 1999].

Regardless of the isoform, NOS catalyze the conversion of L-arginine and molecular oxygen to $N^G$-hydroxy-L-arginine (NOHA) and further to citrulline and NO˙. NADPH is used as an electron donor and haem, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin ($BH_4$) as cofactors through a reaction that consumes five electrons. iNOS is a bi-domain protein in which the C-terminal half of NOS

acts as a reductase domain containing the binding sites for calmodulin, NADPH, FAD and FMN; and the N-terminal half of the enzyme acts as an oxygenase domain that contains the binding sites for haem, $BH_4$ and L-arginine [Sennequier 1996]. The C-terminal reductase domain displays close homology with cytochrome P450 reductase and transfers NADPH-derived electrons that are required for reductive activation of molecular oxygen to the N-terminal oxygenase domain, resulting in oxygen insertion into L-arginine and NOHA [Alderton 2001]. The iNOS enzyme is active only in its dimeric form [Panda 2002]. This is initiated by the insertion of haem into the oxygenase domain, creating the binding sites for arginine and $BH_4$ [Siddhanta 1996]. $BH_4$ is a key cofactor bridging across the dimer interface, and is required for the active dimer. In the absence of $BH_4$, NOS produces $^{\bullet}O_2^-$, instead of $NO^{\bullet}$ [Guzik 2000, Alp 2003]. Likewise, at non-saturating arginine concentrations, a calmodulin bound NOS will produce reactive oxygen species such as superoxide and hydrogen peroxide [Stuehr 2001, Heinzel 1992]. Thus, an active NOS operating at a subsaturating arginine concentration can generate $NO^{\bullet}$ and $^{\bullet}O_2^-$ at the same time. Due to altered NADPH oxidation by iNOS, it has also been shown that certain arginine analogs can inhibit the production of reactive oxygen species by NOS, whereas others allow it or enhance it [Abu-Soud 1994, Sennequier 1996].

Traditionally, $NO^{\bullet}$-production by iNOS was considered mainly to be regulated at the transcriptional level by influence of pro-inflammatory mediators. Cytokines associated with Th1-like reactions (*e.g.* IL-1, TNF-α and IFN-γ) have been reported to up-regulate $NO^{\bullet}$ synthesis, whereas Th2-like cytokine patterns (*e.g.* IL-4, IL-10 and IL-13) down-regulate transcription of iNOS [Suschek 2004]. $NO^{\bullet}$ synthesis can also be regulated through effects on the substrate L-arginine, cofactors such as haem and $BH_4$, or the electron donor NADPH [Aktan 2004]. Regarding iNOS, the most studied of these factors is the availability of arginine. A number of data indicate that: (i) intracellular arginine biosynthesis from extracellular citrulline does not support maximal iNOS activity, (ii) endothelial iNOS-derived $NO^{\bullet}$ production is completely dependent on extracellular arginine concentration, and that (iii) normal physiological extracellular arginine concentrations may not support maximal iNOS $NO^{\bullet}$ production [Suschek 2004]. Considering also recent reports on increased arginase activity in inflammatory diseases [Vincendeau 2003, Suschek 2004], the sustained iNOS expression associated with many of these may in fact not reflect $NO^{\bullet}$ production in vivo.

## Nitric oxide ($NO^{\cdot}$)

$NO^{\cdot}$ is a small molecule (30 Da). The combination of one atom of nitrogen with one atom of oxygen results in the presence of an unpaired electron ($^{\cdot}$), thus $NO^{\cdot}$ is paramagnetic and a free radical. $NO^{\cdot}$ is less reactive than many other free radicals in that it cannot react with itself. It is uncharged and can therefore diffuse freely within and between cells across membranes. Therefore, $NO^{\cdot}$ can, on the one hand, act as a messenger molecule involved in physiological processes such as neurotransmission and control of vascular tone, and on the other hand, act as a mediator of cytotoxicity.

The $NO^{\cdot}$ reactions that are sufficiently rapid to have a significant role in vivo are relatively few and involve direct interactions with biologically relevant targets [Wink 1998]. Hence, low concentrations of $NO^{\cdot}$ formed by constitutive NOSs, involve reactions only with metals and oxygen radicals. The major metal-mediated reactions occur primarily with iron containing haem sites at haem proteins, a process termed nitrosylation. Basic regulatory reactions in this manner involve guanylate cyclase, cytochrome P450s, NOS, cytochrome c oxidase, peroxidases and haemoglobin. The best example of a direct effect in this fashion is $NO^{\cdot}$ generation in endothelial cells. Upon formation, $NO^{\cdot}$ migrates to guanylate cyclase in vascular smooth muscle cells, where alteration in protein configuration leads to conversion of GTP to cGMP, which mediates vaso-relaxation [Ignarro 1989]. In contrast to the $NO^{\cdot}$-mediated activation of guanylate cyclase, $NO^{\cdot}$ inhibits other haem mono-oxygenases including cytochrome P450 and NOS. Importantly, eNOS and nNOS are more susceptible to inhibition by $NO^{\cdot}$ than iNOS [Griscavage 1995].

The reactivity of $NO^{\cdot}$ with metals is not limited to just covalent interactions with metal ions. Various metal-oxygen complexes can rapidly react with $NO^{\cdot}$ e.g:

$Hb\ (Fe\text{-}O_2) + NO^{\cdot} \rightarrow metHb\ (Fe(III)) + NO_3^-$

This reaction provides the primary endogeous mechanism to eliminate $NO^{\cdot}$ as well as to control the movement and concentration of $NO^{\cdot}$ [Lancaster 1994]. Other reactions of $NO^{\cdot}$ with metal adducts is the rapid reaction between $NO^{\cdot}$ and metalloxo and peroxo species.

$Fe^{2+} + H_2O_2 \rightarrow Fe{=}O + H_2O$

Addition of $NO^{\cdot}$ reduces the hypervalent haem complex.

$Fe{=}O + NO^{\cdot} \rightarrow Fe^{2+} + NO_2^-$

These antioxidant properties of $NO^{\cdot}$ may be a primary mechanism by which $NO^{\cdot}$ protects tissue from peroxide mediated damage.

Low concentrations of $NO^{\cdot}$ may also yield reactions with other free radical species; the most studied being the reaction with oxyradicals formed during lipid peroxidation [Rubbo 1995]. Lipid peroxidation is an important reaction

in cell death, and results in the formation of a variety of lipoxy and peroxy adducts compromising cell membranes. $NO^{\bullet}$ reacts with these peroxy oxy radicals to terminate lipid peroxidation, and protect cells against peroxide-induced cytotoxicity [Padmaja 1993, Gupta 1997].

$NO^{\bullet}$ produced in high concentrations by iNOS have been associated with pathophysiological processes and diseases [Condino-Neto 1993, Abramson 2001, McCartney-Francis 2001, Kumada 2004]. The effects can be divided into nitrosation and oxidation chemistry. Nitrosation chemistry results primarily in the formation of nitrosothiols and nitrosamines. Oxidation chemistry can result in the oxidation of different macromolecules including DNA, proteins and lipids. These reactions are referred to as nitrosative and oxidative stress [Wink 1998]. The principle species formed by various reactions in biological systems are $N_2O_3$, $ONOO^-$, $HNO/NO^-$, and $NO_2$, commonly referred to as reactive nitrogen oxide species (RNOS). Under high fluxes of $NO^{\bullet}$, $N_2O_3$ can be formed by auto-oxidation ($2NO^{\bullet}+O_2$) of $NO^{\bullet}$. It has been shown that $N_2O_3$ is the most important RNOS for nitrosation. Since both $NO^{\bullet}$ and oxygen are more soluble in hydrophobic media, auto-oxidation occurs much faster in lipid membranes, and therefore nitrosation would be expected to occur predominantly among membrane associated proteins [Mancardi 2004].

The main target sites for nitrosation by RNOS are thiol-containing proteins, and low molecular weight thiols (such as glutathione and cystein), which form an intracellular pool that can protect the cell against oxidant stress. Nitrosation of thiols and the formation of S-nitrosothiols can inhibit a number of enzyme activities [Hausladen 1996], and has been shown to be involved in circulatory regulation as well as inflammatory and degenerative conditions [Zhang 2005]. Circulating low molecular weight S-nitrosothiols also provide a mechanism whereby $NO^{\bullet}$ can be transported in a stable form and exert effects for several hours. S-nitrosothiols have also been shown to have specific receptors and own metabolism [Rassaf 2005]. Under conditions where fewer thiol groups are available for interaction (*e.g.* oxidant stress), nucleophilic centres on DNA are also potential targets for $NO^{\bullet}$. $NO^{\bullet}$ has been shown to cause direct damage to DNA by deamination [Wink 1991]. Both DNA damage and $NO^{\bullet}$ itself activate polyADP-ribose synthetase. It has been suggested that this would lead to a futile cycle in which large quantities of ATP are consumed leading to energy depletion and cell death [Zhang 1994]. ADP-ribosylation of proteins, the covalent binding of ADP-ribose to acceptor amino acids (*e.g.* cysteine], is also promoted by $NO^{\bullet}$. Nitric oxide promotes the ADP-ribosylation of G-actin in human neutrophils and inhibits actin polymerization and thereby surface adhesion by neutrophils, endothelial cells and chondrocytes [Abramson 2001].

Oxidation refers to a substrate loss of electrons under physiological conditions. Under conditions of oxidative stress, molecules are oxidized by powerful oxidizing agents, resulting in for instance DNA strand breaks, lipid peroxidation and structural modifications of proteins. The three major RNOS mediating oxidative stress are nitroxyl ($HNO/NO^-$), nitrogen dioxide ($NO_2$) and peroxynitrite ($ONOO^-$). Among these, the interactions and physiologic relevance of the first two are still under discussion, while peroxynitrite has been more extensively elucidated [Mancardi 2004]. Interestingly, and illustrative of the complex radical biochemistry, one of the proposed possibilities for $HNO/NO^-$ production is oxidation of NOHA. This molecule can constitute as much as 50% of the metabolism of NOS [Buga 1996]. Besides being an antioxidant and modulator of arginine metabolism and transport, NOHA has been proposed to generate HNO after catalyzed oxidation by peroxidases [Pufahl 1995].

Peroxynitrite ($ONOO^-$) formed by $^{\bullet}O_2^-$ and $NO^{\bullet}$ has been shown to be a powerful oxidant. It can oxidize thiols, initiate lipid peroxidation, nitrate tyrosine, cleave DNA, nitrate and oxidize guanosine and oxidize methionine. Maximal oxidation through peroxynitrite is only achieved when $^{\bullet}O_2^-$ and $NO^{\bullet}$ are formed in a 1:1 ratio. In the presence of excess $NO^{\bullet}$ or $^{\bullet}O_2^-$, peroxynitrite is converted to nitrogen dioxide [Beckman 1994]. Under normal conditions, the cellular concentrations of $NO^{\bullet}$ and superoxide suggest that superoxide production determines the rate of peroxynitrite formation. Since the most important factor determining superoxide availability is the presence of superoxide dismutase (SOD), a potent protective mechanism against oxidative stress, SOD concentrations will determine if and where peroxynitrite is formed. SOD can also be induced by cytokines (*e.g.* TNF-α and IL-1) and may therefore represent a natural defence against toxic effects of $NO^{\bullet}$.

The formation of nitrotyrosin by peroxynitrite has been widely used as a marker of $NO^{\bullet}$ production. However, recent investigations have demonstrated that tyrosine nitration critically depends on the presence of myeloperoxidease or a related enzyme. Thus, the formation of nitrotyrosin appears to serve more as an indicator of granulocyte infiltration. The explanation for these findings is that peroxidases convert $NO_3$ to $NO_2$, which, possibly via intermediate formation of $N_2O_3$, mediates tyrosine nitration [Suschek 2004, Mancardi 2004]. A schematic illustration of possible $NO^{\bullet}$ effects are presented in Figure 3.

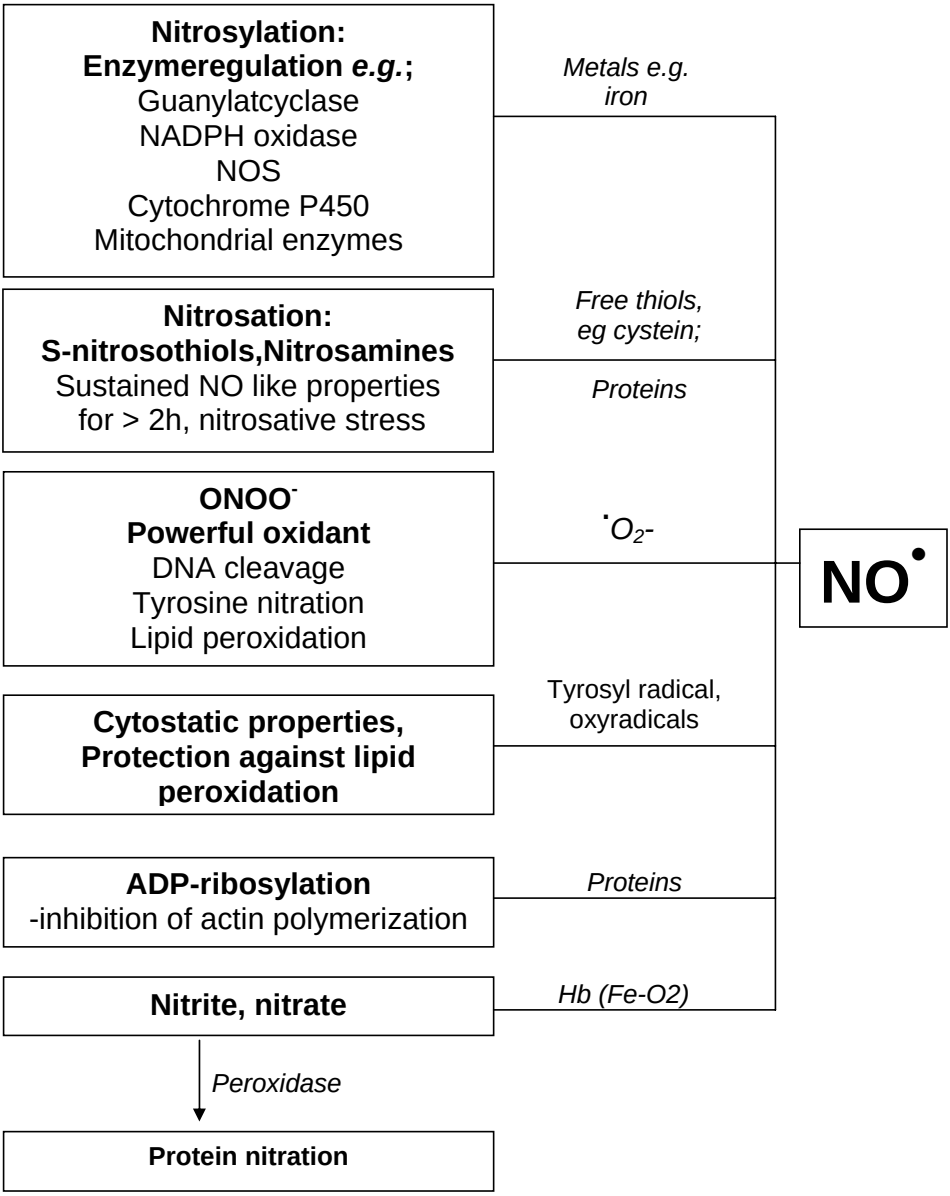


**Figure 3:** Summary of some of the proposed mechanisms by which $NO^{\cdot}$, or related nitrogen species, may exert effects.

## NO· and neutrophils

NO· as well as NO·-releasing compounds attenuate leukocyte rolling and adhesion to activated endothelium [Ou 1997, Kubes 1993, Kosonen 1999]. The underlying mechanism is not known, but it has been related to antioxidant mechanisms of NO· [Johnston 1996] and may be dependent on cGMP [Kosonen 1999]. NO· is also shown to down-regulate adhesion molecules that mediate interaction between leukocytes and endothelium, *e.g.* P-selectin [Lefer 1999], E-selectin [Kosonen 2000], and ICAM-1 [Lindemann 2000]. In *in vitro* studies, NO·/NO·-donors have been shown to inhibit degranulation, leukotriene production, superoxide anion generation and chemotactic migration of activated neutrophils [Moilanen 1993, Clancy 1992]. Several researchers found that NO· may have a biphasic effect (potentiating at lower concentrations and inhibitory at higher concentrations) on neutrophil functions though [reviewed by Sethi 2000].

Important differences exist between species regarding the neutrophil NO· synthesis. Rodent neutrophils are known to produce NO· through the iNOS pathway in response to pro-inflammatory stimuli such as lipopolysaccharide, TNF-α and IFN-γ [Kolls 1994]. These stimuli do not have the same action in human neutrophils, at least under *in vitro* conditions, and enzymatic production of NO· in *human* neutrophils remains somewhat controversial. The presence of iNOS mRNA in unstimulated human circulating neutrophils was reported by Amin et al [Amin 1995], whereas Miles et al found no signs of iNOS mRNA or protein expression [Miles 1995]. Many researchers have been unable to demonstrate NO· synthesis by human neutrophils [Yan 1994, Padgett 1995, McBride 1997, Miles 1995, Holm 1999], while others have [Salvemini 1989, Wright 1989, Zhao 1996, Stolarek 1998]. The reasons for these discrepancies are probably methodological, as reviewed by Sethi and Dikshit [Sethi 2000]. Neutrophils were reported to synthesize NO· *in vitro* at a rate similar to that of endothelial cells, thus indicating the presence of a constitutive NOS. This has also been shown by Wallerath et al, demonstrating a 150kDa protein corresponding to nNOS in human neutrophils [Wallerath 1997].

Despite the interspecies differences and conflicting results regarding human neutrophils *in vitro,* a number of indications point at neutrophil NO· production and/or iNOS expression *in vivo*. In neutrophils from septic patients, both iNOS mRNA and iNOS activity (conversion of arginine to citrulline and production of nitrite/nitrate) were induced [Tsukahara 1998, Hersch 2005]. Circulating iNOS-expressing neutrophils have also been reported in Kawasaki's disease and cardiac infarction [Yu 2004, Sanchez de Miguel 2002]. Increased NO· production from circulating neutrophils has also been reported to occur in patients with liver cirrhosis and hyper-dynamic circulation [Laffi 1995]. Exudated neutrophils from the oral cavity or from urine of patients with urinary tract infections were reported to express iNOS [Nakahara 1998, Takeichi 1998, Wheeler 1997]. Wheeler also

reported nitration of ingested bacteria by human neutrophils, a finding also described by Evans et al [Evans 1996]. A role for nitrogen intermediates in neutrophil microbe killing has also been suggested by Malawista et al [Malawista 1992]. Furthermore, histopathological evidence of iNOS-expressing neutrophils has been described in various disorders including inflammatory bowel disease [Singer 1996, Ikeda 1997], infected nasal polyps [Watanabe 2002], cerebral infarction [Forster 1999], and traumatic brain injury [Orihara 2001, Gahm 2002]. Moreover, recent publications have been able to present attenuation of neutrophil iNOS expression compared to controls, after intense exercise and in cancer patients [Sureda 2005, Jablonska 2005]. Thus, even if there are interspecies differences in terms of neutrophil NOS regulation, there is compelling evidence that neutrophil iNOS expression occurs under certain conditions *in vivo*. Since neutrophil activation normally occurs after extravasation, it seems more likely that iNOS-mediated NO production by neutrophils would occur in the extra-vascular space rather than in circulation.

The data presented above also indicate that human neutrophil iNOS, except the more complex regulatory mechanisms than rodent or murine neutrophils, may differ in other ways. In search of the constitutive NOS in human neutrophils, Bryant et al purified an NADPH/FAD/$BH_4$-dependent 130 kDa protein [Bryant 1992]. This protein co-purified with an NADPH/FAD-dependent 22 kDa protein associated with the superoxide-generating system. Since the sum of the two proteins is close to the molecular weight of nNOS (150 kDa), it could be argued that these two proteins represented fragments of nNOS [Sethi 2000]. In any case, the association of NOS with a superoxide generating fragment is interesting, and may explain the ability of SOD to enhance $NO^{\cdot}$ generation even in patients with chronic granulomatous disease (who have a defective NADPH oxidase) [Condino-Neto 1993]. Another interesting finding was reported by Wheeler et al who found iNOS activity in human neutrophils to be more than 90% membrane associated [Wheeler 1997], contrasting to rat neutrophils where iNOS is located in the cytosol [Yui 1991]. In further support of a non-cytosol localization in humans, Evans et al reported iNOS co-localization with myeloperoxidase, i.e. in the primary granules [Evans 1996]. Thus, these reports highlight the importance of examining membrane/granule fractions rather than cytosol, differences between detergents in ability to solubilize [Wheeler 1997], and differences in methods for cell disruption [Sethi 2000]. Using all these indications we were also able to demonstrate the presence of a constitutive iNOS in human neutrophils [paper III, Cedergren 2003]. Using the highly specific iNOS inhibitor $N^{g}$-mono-methyl-L-arginine (1400W), a recent publication also proposed a role for neutrophil iNOS in β2 integrin induced activation and adhesion [Jenei 2006].

## NO˙ and arthritis

It has been shown in animal models (adjuvant-induced, collagen-induced or streptococcal cell wall arthritis) that suppression of NO˙ production can have profound effects on disease initiation and progression [Stefanovic-Racic 1993, McCartney-Francis 1993, Connor 1995, Miesel 1996, Lawand 1997, van den Berg 1999]. Most of these reports were made using non-selective NOS inhibitors, but van den Berg et al reported that iNOS-deficient mice exhibited cartilage protection, although inflammation was hardly affected [van den Berg 1999]. This is in contrast to a report by McCartney-Francis et al, observing that selective inhibition of iNOS exacerbated erosive joint disease in the streptococcal cell wall model [McCartney-Francis 2001]. Thus, and contrary to previous views, it is important to recognize that all known isoforms of NOS may operate in immune/inflammatory responses [Bogdan 2001, Abramson 2001].

In RA patients, increased concentrations of nitrites have been demonstrated in synovial fluid and serum [Farrell 1992, Borderie 1999] and a relationship has been shown between serum NO˙ levels and disease activity [Ueki 1996]. More recent studies have confirmed increased serum NO˙ levels in RA, but questioned their association with disease activity [Choi 2003, Weinberg 2006]. Increased levels of both nitrotyrosine and nitrosothiols have been shown in synovial fluid and synovium [Kaur 1994, Sandhu 2003, Rocks 2005]. Peroxynitrite-modified collagen II was also recently discovered in serum of RA patients [Deberg 2005], and this NO˙-modified collagen was shown to induce inflammatory pathways, including iNOS expression, in human chondrocytes [Whiteman 2006].

In human RA, synovial fibroblasts, synoviocytes, macrophages, osteoblasts and chondrocytes (superficial chondrocytes in particular), have been identified as intra-articular sources of NO˙ [Stichtenoth 1998]. Studying cells derived from synovial fluid, Grabowski et al could not demonstrate iNOS mRNA, whereas Borderie et al reported expression of iNOS in lympho-mononuclear cells [Grabowski 1996, Borderie 1999]. High amounts of intra-articular NO˙ production has been found to correlate to enhanced bone resorption and diminished bone proliferation, decreased proteoglycan synthesis, activation of metalloproteinases and chondrocyte apoptosis [Stichtenoth 1998]. Human chondrocytes derived from RA patients express iNOS and spontaneously produce large amounts of NO˙ when cultured *ex vivo* [Abramson 2001]. Possible up-regulators of chondrocyte iNOS expression include autocrine or synovial-derived soluble stimuli, but also interaction with matrix components such as collagen II or fragments of fibronectin or hyaluronan [Abramson 2001, Yasuda 2006]. Spontaneous high-output NO˙ production has been reported in synovial membrane cultures from RA patients [McInnes 1996]. The majority of iNOS expressing cells in the synovium are fibroblast-like, and since production of NO˙ and other reactive oxygen species in the synovium are associated with a higher

incidence of p53 (a tumour suppressor gene) mutations, this may contribute to the transformed-appearing phenotype of synovial fibroblasts [Abramson 2001, Valko 2007].

A microsatellite repeat polymorphism in the iNOS promoter gene was reported to correlate with an increased risk of developing RA [Gonzalez-Gay 2004]. Moreover, endothelial dysfunction and accelerated atherosclerosis in RA is associated with $NO^{\cdot}$. Newly diagnosed patients with RA have vascular dysfunction with impaired responsiveness to $NO^{\cdot}$, possibly due to endothelial iNOS expression [Bergholm 2002, Yki-Järvinen 2003]. Endothelial dysfunction was recently also described in rat adjuvant-induced arthritis [Haruna 2006]. It was shown that NOS was uncoupled due to $BH_4$ deficiency and produced superoxide instead of $NO^{\cdot}$. Substitution with NADH or arginine resulted in a significant increase in superoxide production, whereas $BH_4$ treatment reversed the endothelial dysfunction [Haruna 2006].

**AIMS**

The overall aim of this thesis was to study neutrophil mechanisms of possible relevance for arthritic conditions. This general objective was focused on the production of two oxygen radicals, superoxide and nitric oxide. Specific goals were to:

- Compare extra- *and* intracellular superoxide production in both blood and synovial fluid neutrophils from patients with different arthritic conditions. Compare β2-integrin *vs* fMLF mediated stimulation (Paper I).

- Evaluate if synovial fluid neutrophils express iNOS and thereby have prerequisites to contribute to intra-articular production of nitric oxide (Paper II).

- Based upon the results of paper II, determine if aseptic extravasation induces neutrophil iNOS expression (Paper III).

- Examine if neutrophil arginase exhibits functional properties that might interfere with the production of nitric oxide (Paper IV).

## MATERIALS AND METHODS

All laboratory work is described in more detail in each paper and will only be briefly summarized with focus on strengths and weaknesses in this section.

### Paper I

Blood samples were collected from 47 healthy blood donors and 25 arthritis patients in clinical remission. Sixteen of these patients fulfilled the ACR classification criteria for RA [Arnett 1988], 13 of whom were seropositive regarding agglutinating rheumatoid factor (RF). Remission was defined as no signs of ongoing synovitis together with ESR and CRP within normal limits. This material served as control and was compared with blood *and* synovial fluid samples from 26 patients with acute synovitis in the knee. Thirteen of these patients had RA according to the ACR criteria (11 RF-positive), and the remaining patients had other RF-negative athritides.

After neutrophil isolation, the cells were stimulated either with a formylated tripeptide (fMLF) as control or with non-phagocytoseable Sephadex beads coated with acid extracted collagen I (Cytodex 3). In the presence of a peroxidase (endogenous MPO or added horse radish peroxidase; HRP), the generated superoxide can react with added luminol and yield light (luminol amplified chemiluminescence; LACL). The resulting light emission was recorded continuously in a Biolumat LB 9505 (Berthold Co. Wildbad, Germany). Intracellular measurements were performed in the presence of SOD (which catalyses the dismutation of superoxide to $H_2O_2$) and catalase (which reduces $H_2O_2$ to water), which cannot pass the plasma membrane and therefore only exerts extracellular effects.

*Luminol-amplified chemiluminescence (LACL)*

Although sensitive and widely used in studies of the phagocyte NADPH oxidase, LACL has several limitations. It has been shown that luminol in some situations can act as an inhibitor of neutrophil NADPH oxidase activity [Fäldt 1999]. In that study, pre-incubation of neutrophils on a surface of aggregated IgG inhibited LACL, and it was assumed that luminol may interfere with intracellular pathways affecting the assembly and/or activation of the oxidase. Another possible limitation is the requirement of a peroxidase for luminol light enhancement. Neutrophils contain MPO in primary granules and NADPH oxidase in secretory vesicles, phagosomes or the plasma membrane. Thus, granule fusion or degranulation is mandatory for light emission by luminol. Extracellularly, this can be dealt with by the addition of a peroxidase (*e.g.* HRP), but this is not possible to achieve intracellularly, and granule secretion must therefore be considered in intracellular measurements. Also, MPO-deficiency occurs in ≈9% of the population [Kutter 1998], and this may influence LACL results. Finally, exudated neutrophils (*e.g.* in the joint) may be influenced by a number of

different inflammatory factors and, accordingly, also exhibit great variation in their oxidative response.

## Paper II

Blood samples from 10 healthy individuals were compared with blood and synovial fluid samples from 15 patients with acute synovitis in the knee. Eleven of the patients were diagnosed with RA, 8 of whom were RF-positive. iNOS expression in isolated neutrophils was investigated by flow cytometry. Plasma and synovial fluid nitrite/nitrate concentrations were analysed by the Griess reaction, and arginine/citrulline content by high performance liquid chromatography (HPLC).

*Flow cytometry*

Flow cytometry is one of the most widely used techniques to obtain high-precision quantitative and qualitative data on cell populations/ subpopulations in suspension [Chapman 2000]. In brief, cells in suspension are streamed in a carrier solution, passing laser or conventional light detectors enabling analysis of both physical properties, such as size and granularity, and multi-colour fluorescence properties, after binding of fluorochrome-tagged antibodies to cellular antigens. In paper II, we gated for size and granularity, and used fluorescein-isothiocyanate- (FITC-) and phycoerythrin-labelled antibodies directed against CD3, CD 14, CD 15 and CD 19 to identify different leukocytes. Two different anti-iNOS-antibodies were used: a polyclonal rabbit antibody raised against the amino terminus of human iNOS, and a monoclonal mouse antibody raised against the carboxy terminus of human iNOS. Blocking experiments were performed using non-labelled IgG and specificity experiments were performed by pre-incubation with blocking peptides recognized by the anti-iNOS antibodies. Conflicting and varying results obtained by flow cytometry usually relate to problems regarding cell preparation, fixation, permeabilization, or, choice and handling of antibodies. The concentration of fluorochrome-tagged antibodies is also important to consider, since too high fluorochrome-concentration can actually result in falsely low values due to 'self-quenching' [Ljunghusen 1990, illustrated in Fig 1b, paper I].

*Griess reaction*

The Griess reaction was originally described as a way to determine urinary nitrite content in case of urinary infections [Griess 1879]. After the discovery of human $NO^{\cdot}$ production, the method has been widely used to assess nitrite/nitrate content in different body fluids. A major problem using nitrite/nitrate content as an indicator of $NO^{\cdot}$ production is the individual variability due to dietary nitrite intake [Weinberg 2006]. Furthermore, nitrite is a small molecule with rapid equilibration between different compartments and metabolic pathways (*e.g.* peroxidases) that may interfere with the use of this assay. In this paper we also addressed the question of whether synovial fluid proteins could interfere with the absorbance measurements in the

assay. For this reason we dialyzed all samples against phosphate-buffered saline (PBS) using a semi-permeable membrane.

*High performance liquid chromatography (HPLC)*
HPLC is a sensitive method to analyse amino acids in body fluids. Thus, measurements of arginine and citrulline should primarily be judged in terms of local cellular environment, and both physiological and inflammatory activated metabolic pathways. These include cellular *de novo* synthesis of arginine from citrulline, protein breakdown, expression of CATs and intracellular concentrations. Therefore, extracellular content of arginine/citrulline provide only a rough estimation of the local possibilities of $NO^{\cdot}$ production.

## Paper III

Peripheral blood cells as well as exudated neutrophils from 8 healthy individuals were studied in this paper. Exudated neutrophils were obtained by the skin chamber method [Follin 1999]. iNOS expression was determined by flow cytometry and Western blot analyses. Evaluation of iNOS expression by flow cytometry was performed in a series of experiments evaluating influences of separation media, whole blood and haemoglobin. Neutrophil L-arginine uptake was assessed by liquid scintillation after incubation with $^{3}$H-L-arginine, and NOS activity was assessed by by measuring radioactivity derived from $^{3}$H-L-citrulline.

*The skin chamber method*
The method offers a way of achieving aseptic physiological skin extravasation of neutrophils collected in a milieu of autologous serum or plasma. The most obvious risk of bias is from bacterial contamination, but there were no signs of clinical skin infection in any of the tested individuals.

*Flow cytometry*
Discussed in paper II. Haemoglobin-dependent quenching of FITC-fluorescence is also illustrated in this paper.

*Western blotting*
Proteins in neutrophil homogenates were detected by a combination of sodium-dodecyl-sulphate (SDS-) gel electrophoresis and the use of specific antibodies to iNOS. The quality of these results depends on the purity of the neutrophil preparations, but neutrophils also present other challenges. First, neutrophils contain large amounts of proteinases. Cell disruption can therefore release and activate these enzymes and degrade the proteins. Detection of neutrophil proteins therefore requires potent proteinase inhibition. In the work preceding this study we had indications that commercial 'protease-inhibitor cocktails' of low human toxicity were insufficient to escape neutrophil proteinase effects on iNOS (unpublished data). In order to achieve efficient proteinase inhibition, we therefore treated

the cells with diisopropyl-fluorophosphate (DFP), a highly toxic but very potent protease inhibitor. Another advantage with DFP is its ability to pass cell membranes and inhibit proteases prior to cell disruption.

Secondly, another possible problem could be if human neutrophil iNOS is membrane-bound, and the antigen towards which the detection antibody is directed is 'hidden' in the membrane in contrast to animal cells, where iNOS is cytosolic. Wheeler et al found that deoxycholate was superior to other detergents for iNOS-extraction from neutrophils derived from patients with urinary tract infections [Wheeler 1997]. The non-published data preceding paper III therefore included comparisons of different detergents, where we found that the use of deoxycholate was a prerequisite to enable detection of iNOS.

*Liquid scintillation*
NOS conversion of $^{3}$H-L-arginine to $^{3}$H-L-citrulline is commonly used for evaluation of NOS activity. In an attempt to mimic the study by Wheeler et al, we examined $^{3}$H-L-citrulline production in neutrophils isolated from urine of patients with urinary tract infections. However, we found it impossible to address this question since the two types of bacteria in these cases, *Escherichia coli* and *Staphylococcus saprophyticus*, both very rapidly metabolized $^{3}$H-L-arginine to $^{3}$H-L-citrulline (non-published results). Therefore, exclusion of bacterial contamination is essential when using this method.

Prior to quantification of $^{3}$H-L-citrulline by liquid scintillation it was necessary to remove remaining $^{3}$H-L-arginine. This was achieved using cation exchange chromatography. We found this method dependent on the flow rate in the columns. Re-quantification of the same samples by liquid scintillation did not reveal any major differences, indicating that the measurement step was not a source of variance.

## Paper IV

Circulating neutrophils were obtained from healthy blood donors. Oxidative activation upon stimulation with fMLF or Cytodex-3 were investigated by LACL in the presence of L-arginine, L-NAME (a NOS inhibitor), and nor-NOHA (an arginase inhibitor). Possible influences of these substances on LACL were examined by a cell-free system using xanthin/xanthin oxidase. Engagement of integrins in Cytodex-3-mediated activation was evaluated by three monoclonal blocking antibodies. Neutrophil arginase secretion and enzyme activity were determined by an enzyme-immunoassay (EIA) and by arginine conversion to urea, respectively.

*Chemiluminescence*
Discussed in paper I. Luminol amplified chemiluminescence is abbreviated as LACL in this paper. The cell-free control system utilizing

xanthine/xanthine oxidase is useful for evaluating possible interference of used chemicals on luminol-enhanced light emission.

*Arginase EIA*
We used a commercially available kit for detection of human arginase I (HyCult biotechnology b.v., Uden, Netherlands). This assay does not cross-react with arginase II and allowed detection within a wide range of concentrations. The samples were run in triplicates.

*Arginase activity*
With slight modifications, enzyme activity was determined by measuring urea formation as previously described by Corraliza et al [Corraliza 1994]. Triplicate samples and re-reading of absorbance revealed a moderate method variance, but also a time-dependency in readings of the absorbance.

**RESULTS AND DISCUSSION**

## Paper I

As compared to healthy blood donors, circulating neutrophils derived from patients in remission showed a more pronounced (fMLF-stimulation) or more rapid (Cytodex-3 stimulation) both total and intracellular oxidative response. This is consistent with the findings reported by others [Biasi 1998, Crocker 2000], indicating that circulating neutrophils from arthritis patients in remission are primed. The finding may reflect the constitution of the arthritis patients, but could also be an effect of anti-rheumatic medication, for instance by preventing homing of primed cells to the joint.

Comparing blood and synovial fluid neutrophils from patients with active disease, several differences were noted between RA and non-RA subjects. In non-RA patients, compared to healthy donors, circulating neutrophils exhibited a significantly lower peak oxidative response, whereas synovial fluid neutrophils tended ($p<0.09$) to react with a more rapid response upon Cytodex-3 stimulation. This more rapid response was not seen in fMLF-stimulated samples. These findings may reflect active recruitment of primed cells to the joint and enhanced expression of integrins after joint entrance. In contrast to RA patients (see below), there were no elevations of baseline activity, neither in blood nor synovial fluid neutrophils from non-RA subjects.

The most interesting findings were noted in neutrophils isolated from patients with RA. Few previous studies in the field have performed comparisons between RA and other arthritic diseases, and we are not aware of any previous study comparing total and intracellular oxidative response. Circulating neutrophils from RA subjects did not differ from healthy blood donors comparing peak oxidative responses. Judging the total (HRP-enhanced) responses, however, there was a tendency towards a more prolonged response phase ($p=0.11$ at 15 min) in circulating neutrophils from RA patients. The explanation to this finding became apparent after analysis of intracellular activation patterns. Circulating neutrophils from RA patients displayed a significant increase in intracellular activation compared both to blood donors ($p<0.01$ at 15 min) and non-RA patients ($p=0.02$). Evaluation of synovial fluid neutrophils from RA patients further augmented this finding. This group was the only one with a significantly raised baseline activity, and comparison of total and intracellular oxidative response showed that this increase was almost exclusively intracellular, indicating that the cells were busy dealing with ingested material. RA synovial fluid neutrophils also exhibited an unusually rapid intracellular response after Cytodex-3 stimulation (Fig 4).

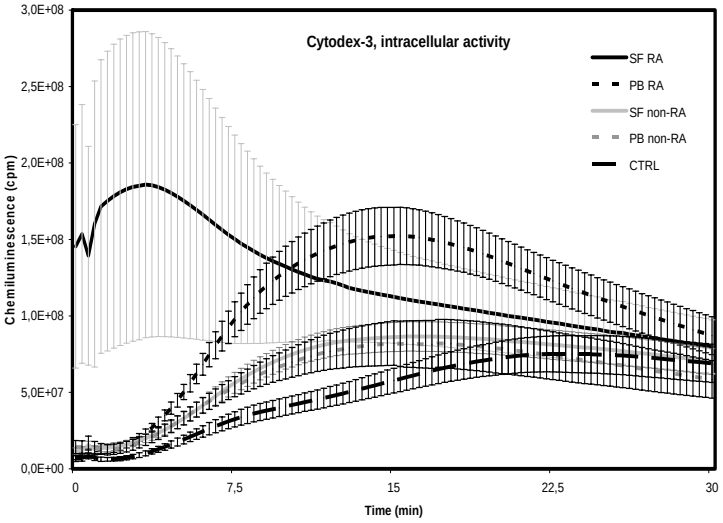


**Figure 4:** Neutrophil chemiluminescence in synovial fluid (SF) and peripheral blood (PB) samples from patients with rheumatoid arthritis (RA) or other arthritides (non-RA). CTRL= peripheral blood neutrophils from blood donors.

We did not observe any differences comparing blood neutrophils from RA and non-RA patients in remission. Thus, priming of circulating neutrophils seems to be a non-specific phenomenon in patients with inflammatory joint disease, or possibly an effect of anti-rheumatic medication. Further studies are needed to shed more light on this observation. The *diminished* oxidative response by circulating cells from patients with active non-RA disease could be the logical consequence of recruitment/extravasation of primed neutrophils from the blood to the joint. Why this was not the case in RA patients is open to speculation. Being a more aggressive inflammatory disease, neutrophil trafficking to inflamed joints is probably more intense. Therefore, a higher neutrophil turnover in RA, compared to non-RA joint disease, could result in higher numbers of circulating primed neutrophils in parallel with continuous recruitment of primed cells to the joints. Another, and much more provocative thought, regards the natural fate of neutrophils in the joint cavity. As mentioned in the introduction, turnover in a 30 ml joint effusion can exceed 1 billion cells per day, but data concerning neutrophil clearance are lacking. Normally neutrophils undergo apoptosis in tissues and are scavenged by macrophages. This clearing mechanism may not be operational in synovial fluid since neutrophil apoptosis is down-regulated in synovial fluid [Cross 2006], as evident in our setting by the lack of increased annexin V expression. Therefore, neutrophil re-entry into circulation can be considered in the case of joint inflammation. Although never accepted as a possible neutrophil behaviour, this could also explain the absence of diminished oxidative response and the increased intracellular response in circulating neutrophils from RA patients. Some support for this

hypothesis has also been published by Granfors et al, reporting the persistence of *Yersinia enterocolitica* antigen in circulating neutrophils from patients with reactive arthritis [Granfors 1998].

There are two plausible explanations for the increased intracellular superoxide production in synovial fluid neutrophils; apoptosis or ingestion. However, as already discussed, it has recently been shown that synovial fluid is anti-apoptotic [Cross 2006], and since we did not find any signs of increased Annexin V expression in synovial fluid neutrophils compared to blood neutrophils, apoptosis is an unlikely explanation. Thus, the most probable explanation to the raised baseline intracellular oxidative activation is processing of ingested material. Being professional phagocytes, resting neutrophils express FcγRIIa and FcγRIIIb, and after priming with IFN-γ also the high affinity FcγRI (CD64). Synovial fluid from RA patients, but not from other subjects, can induce oxidative activation due to the presence of IgG-immune complexes (ICs) [Dularay 1990]. The presence of rheumatoid factor (RF) is known to modulate the interaction between ICs and Fc-receptors [Gale 1985]. In the present study 11/13 patients with RA were RF-positive, but none of the non-RA group.

Although not addressed in this study, antibodies against citrullinated proteins are of interest when discussing the findings reported in this paper. Although anti-CCP antibodies are highly specific for RA, and although several citrullinated proteins have been identified in rheumatoid joints, including synovial fluid [Koivula 2005, Takizawa 2006, Kinloch 2006], those/that (if any) of particular aetio-pathogenetic interest remain(s) to be revealed. Thus, ICs formed between ACPA of IgG class and citrullinated proteins in synovial fluid may be targets for invading neutrophils as well as a possible explanation to the increased intracellular activity in synovial fluid neutrophils. Furthermore, activated neutrophils theoretically have the capacity to activate nuclear PAD4 [Vossenaar 2003] and confer citrullination of their own proteins, *e.g.* histones [Arita 2006], which themselves could become targets for neutrophil attack.

Integrin-mediated stimulation of neutrophils was in this study achieved by large Sephadex beads coated with acid-extracted denaturated collagen I. Although collagen II has attracted great interest as a target antigen in RA, the distribution of collagen I corresponds well to the organ involvement seen in RA (*e.g.* bone, tendon, skin, eyes and organ capsules). Degradation products from collagen I correlate with activity markers such as ESR and CRP [Sassi 2003] and, like other ACPA family members, antibodies directed against citrullinated collagen I have shown high specificity (99%) for RA [Suzuki 2005]. Apart from acting stimulatory on neutrophil integrins, collagen I is one of the few proteins known to be fragmented by superoxide anion. Thus, it is theoretically possible for a self-perpetuating vicious circle between collagen I breakdown and neutrophil activation to emerge.

In summary, the results in paper I indicate that neutrophils derived from RA patients are activated in a disease-specific manner. This involves an unusually pronounced intracellular oxidative response indicating processing of ingested material.

## Paper II

During the work presented in paper II, Borderie et al published a similar study on iNOS expression in synovial fluid cells. Since their results opposed our preliminary findings, we carried out a number of control experiments to reinforce our conclusions. We found that the mode of permeabilization (extraction of membrane lipids) was important to enable detection of iNOS with the same monoclonal mouse anti-iNOS antibody as that used by Borderie et al (directed against the carboxy terminus), whereas this was not as critical using the polyclonal rabbit anti-iNOS antibody (raised against N-terminus antigens). Considering the possible anchoring of iNOS to granule membranes vis-à-vis the fine specificity of the two anti-iNOS antibodies used, the permeabilization step may be of particular importance. Non-permeabilized cells did not give rise to iNOS fluorescence, and pre-incubation with FITC-labelled normal rabbit IgG, did neither block the binding of rabbit anti-iNOS antibodies nor give rise to unspecific fluorescence. Pre-incubation of anti-iNOS antibody with blocking peptide inhibited iNOS fluorescence by 60%. Thus, lines of evidense confirmed specific binding of the rabbit anti-iNOS antibody, and also indicated that the distribution of iNOS in human neutrophils may differ from other cells.

Different cell populations in blood and synovial fluid samples were identified by their forward and side scatter appearance, and their surface expression of different CD molecules. Even if the lymphocyte/monocyte populations showed a statistically significant elevation of iNOS fluorescence in synovial fluid samples compared to paired blood samples, this was less pronounced than in neutrophils ($p<0.001$, Figure 5). This finding was reproduced in all of the 15 patients examined.

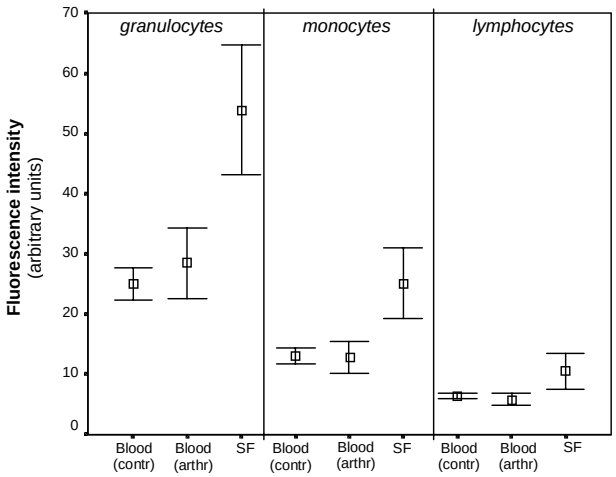


**Figure 5**: iNOS expression in different cell populations from control blood, patient blood and synovial fluid (*SF)*. Mean values ± standard deviations are given.

The results of nitrite/nitrate and citrulline/arginine analysis are presented in the table below:

| | Controls | Plasma | Synovial fluid | Plasma *vs* SF |
|---|---|---|---|---|
| **Nitrite** | **14.5±3.3** | **9.1±3.5** | **9.0±5.9** | **ns** |
| **Nitrite+nitrate** | **28.5±22** | **8.6±6.5** | **19.2±20.7** | **0.054** |
| **L-citrulline** | **31.3±2.7** | **26.2±8.9** | **33.4±11.4** | **ns** |
| **L-arginine** | **237±39** | **176±65** | **257±78** | **0.015** |

The significant elevation of arginine in synovial fluid *vs* plasma may be a result of protein breakdown, active transportation into synovial fluid, or *de novo* synthesis by inflammatory activated cells. More importantly, this demonstrates that in the presence of an active NOS, this prerequisite for NO˙ production exists in synovial fluid. Although the sum of nitrite and nitrate almost reached a statistically significant elevation in synovial fluid, we did not find any significant elevations of bi-products from NO˙ production. This is not very surprising, however, concerning recent data indicating that nitrite/nitrate content is highly influenced by dietary intake [Weinberg 2006]. Also, nitrite/nitrate are very small molecules and slight elevations would rapidly become equilibrated between different compartments. Furthermore, nitrite can be metabolized in the presence of peroxidases [Suschek 2004], thus making detection difficult. In this study we also eliminated excess protein in synovial fluid to avoid absorbance interference in the analysis. This may be an explanation to some previous studies reporting increased levels of nitrite/nitrate in rheumatoid synovial fluid.

Analysis of L-citrulline in different compartments revealed no significant differences. Since citrulline can be further metabolized, for instance recycled to arginine, this may not be a relevant indicator of NO˙ production. However, while a trend towards a weak correlation was observed between arginine levels in synovial fluid and blood (0.55, p=0.12), we observed no correlation whatsoever between citrulline levels in synovial fluid and blood (-0.002, p=0.995). This may actually indicate local joint production of citrulline in individual patients. In conclusion, pre-requisites for NO˙ production, i.e. iNOS-expressing cells and L-arginine, are present in synovial fluid, but we could not present definitive proof of ongoing NO˙ production.

## Paper III

Like synovial fluid neutrophils, all examined samples of skin-chamber exudated neutrophils showed signs of flow cytometric iNOS expression compared to autologous whole blood samples ($p<0.01$). Since iNOS expression could be detected also in freshly isolated blood neutrophils, a series of experiments were performed to evaluate possible influence of the preparation procedures. Centrifugation of whole blood or incubation with separation medium did not induce iNOS expression in neutrophils. As shown in Fig 6, resuspension of isolated neutrophils in whole blood or cell-free whole blood lysate, inhibited anti-iNOS antibody fluorescence staining.

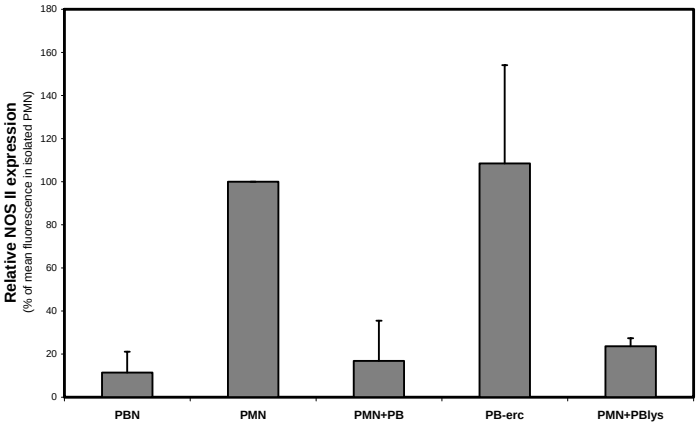


**Figure 6:** Neutrophil NOS II expression in whole blood (PBN), after isolation from blood (PMN), in isolated neutrophils resuspended in blood (PMN+PB), in saponinized and centrifuged/washed whole blood (PB-erc), and in isolated neutrophils resuspended in cell-free saponin-lysate (PMN+PBlys). Mean values (SD) from ≥3 individuals.

Thus, we concluded that quenching of FITC-fluorescence by haemoglobin explained our results, and hypothesized that iNOS protein was expressed already in circulating neutrophils. Therefore, in a new series of experiments we examined iNOS content in circulating neutrophils by Western blotting. To extract neutrophil proteins detectable by anti-iNOS antibodies we found deoxycholate to be superior to other detergents in the protein extraction procedure. The next issue was the size of the proteins detected by iNOS antibodies. Being too small to correspond to iNOS, we suspected insufficient proteinase inhibition. Accordingly, in samples treated with the very potent protease inhibitor DFP, we could show a 130kDa protein detected by both mouse and rabbit iNOS-antibodies (Fig 7).

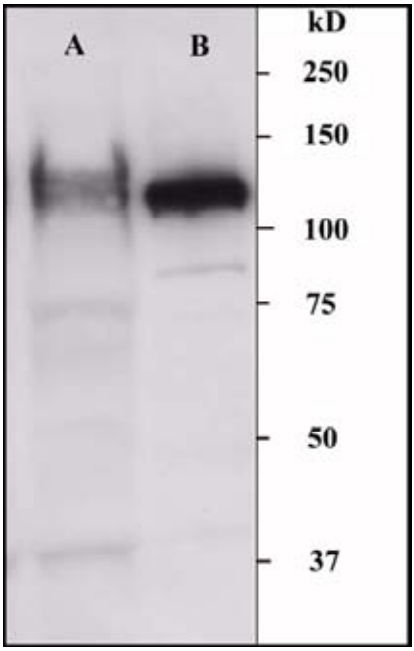


**Figure 7:** Western blot of a whole cell lysate from freshly isolated neutrophils from a healthy blood donor (Lane A). Lane B is a positive control with purified NOS II, molecular weight 130 kD.

In the whole cell assay for enzymatic activity we found no evidence of $NO^{\cdot}$ production by freshly isolated neutrophils. This was not due to low uptake of L-arginine, since cell-associated $^{3}$H-L-arginine increased linearly with time and could be completely inhibited by competition with unlabelled L-arginine. However, these findings are not surprising, since a constitutive neutrophil iNOS would represent a dangerous weapon if activated for instance in the circulation. Constitutive expression of iNOS by neutrophils is not unique in human biology. Both retinal cells and airway epithelium have been shown to express the inducible NOS isoform constitutively [Park 1994, Watkins 1997]. In retinal tissue two types of iNOS mRNA have been described, possibly indicating structural diversity of the iNOS gene in various tissues [Park 1996]. Therefore, the findings in this and some previous studies challenge the paradigm of iNOS as a high-output cytosolic enzyme regulated only at the transcriptional level. A very recent publication by Jenei and co-workers lends further support to this notion[Jenei 2006]. They found that integrin-mediated neutrophil adhesion to a fibrinogen-coated surface was abolished if the neutrophils had been pre-treated with the highly specific iNOS inhibitor 1400W. Thus, they concluded that physiological levels of $NO^{\cdot}$ produced by an activation of iNOS in response to β2 integrin engagement, promote neutrophil adhesion. Moreover, signalling via tyrosine kinases, PI 3-kinase, protein kinase C and calcium mobilization was critical for the generation of $NO^{\cdot}$, but the link between these signalling molecules and activation of iNOS remains to be elucidated. Jenei and co-workers suggest relocalization of iNOS to a specific compartment and/or interaction with its binding partners

as possible control points. Hence, these data indicate an important functional role for the constitutive neutrophil iNOS, instead of the traditional un-regulated, high-output, anti-intruder view traditionally implicated with the presence of iNOS.

Even if an endogenous neutrophil-derived NOS inhibitor has been described [Dembinska-Kiec 1995], the two most widely discussed post-transcriptional points of NOS regulation is inhibition of NOS dimerization and substrate availability. As discussed in the introduction, both haem and $BH_4$ is necessary for NOS dimerization, and although shortage of $BH_4$ has been shown to have major effects on endothelial NOS and dimerization of iNOS, not much is known about $BH_4$ in neutrophils. NOS substrate availability is dependent on CATs, and the neutrophil uptake of $^3$H-L-arginine indicate that this control point would not limit $NO^{\cdot}$ production.

Active arginases, depleting an active NOS of its substrate, is a well known rate limiting factor for $NO^{\cdot}$ production [Mori 2000]. Therefore, the recent description of constitutive arginase I expression in human neutrophil azurophil granules may be of importance in discussions regarding iNOS regulation [Munder 2005]. Interestingly, azurophil granules were also the site of neutrophil iNOS expression described by Evans et al, after neutrophil ingestion of bacteria [Evans 1996]. Azurophil granules may provide an arginine-depleted environment suitable for a pre-formed iNOS. This position would also allow for the combined action of NOS and NADPH oxidase, making the production of peroxynitrite into the phagosome possible. Furthermore, granule association of neutrophil iNOS could explain the methodological problems concerning detergents and protease inhibitors previously discussed. However, it should be mentioned that, in a very recent study by Jacobsen and co-workers, human neutrophil arginase I was found exclusively in gelatinase granules [Jacobsen in press].

## Paper IV

Chemiluminescence was used to evaluate neutrophil oxidative response to Cytodex-3 stimulation in the presence of L-arginine, L-NAME (a NOS inhibitor), or nor-NOHA (an arginase inhibitor). To verify the influence of integrins in neutrophil stimulation, the cells were pre-incubated with monoclonal antibodies to CD11a, CD11b or CD18 prior to stimulation (Fig 8).

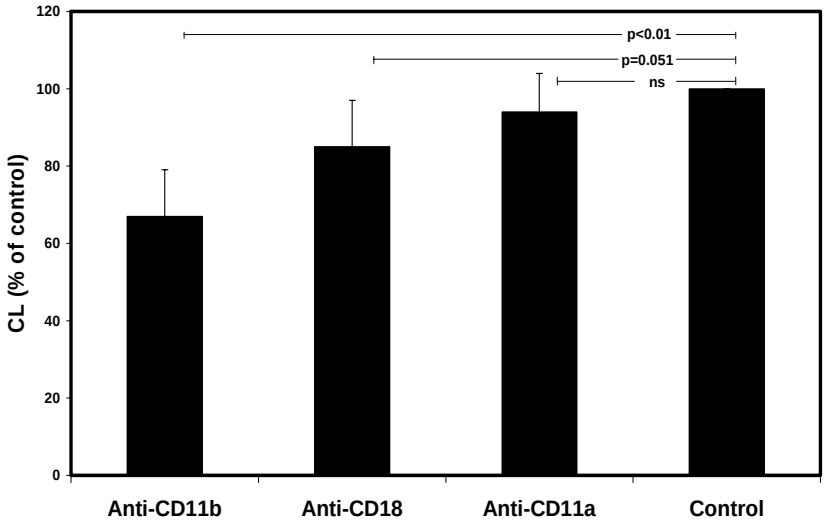


**Figure 8:** Neutrophil LACL response to Cytodex-3 after pre-incubation with three different monoclonal anti-integrin antibodies. Mean ± standard deviation, n=6.

Thus, even if previous studies have shown the importance of CD11a/CD18 integrin in collagen mediated neutrophils stimulation [Garnotel 1995, Forslund 1994], also CD11b seem to be of importance. This is not surprising since Cytodex-3 stimulation essentially represent a collagen I coated surface to which neutrophils can adhere, and CD11b/CD 18 is the most important adhesion molecule. The fact that the response is attenuated and not blocked is most probably due to additional operative mechanisms, but may also be explained by the up-regulation of vesicular storage integrins to the cell surface during stimulation.

Possible influences of the chemicals L-arginine, L-NAME and nor-NOHA on the LACL were evaluated using a cell-free system producing superoxide in the presence of xanthine/xanthine oxidase (Fig 9):

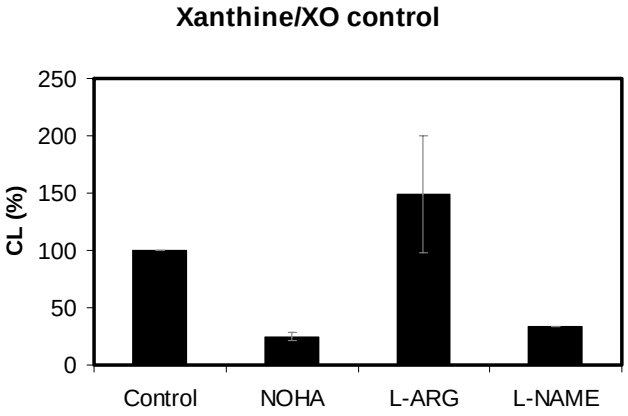


**Figure 9:** The cell free control system for luminol-amplified chemiluminescence (CL) using xanthine/xanthine oxidase. Values are expressed as percentage of the positive control (mean ± standard deviation).

Since NO˙ can contribute to neutrophil chemiluminescence by the formation of peroxynitrite [Carreras 1994, Fukuyama 1996], previous studies have assumed that effects of L-arginine and L-NAME on LACL could be interpreted as interference with neutrophil NO˙ production [Kudoh 1999, Braga 2005]. These data clearly illustrate that such conclusions must be drawn with caution due to the significant effects exerted by these substances on LACL in a cell free system. Furthermore, from these data we would also expect a diminished LACL in neutrophil samples treated with nor-NOHA. If, on the other hand, limited arginine availability is a restricting factor for NO˙ production, arginase inhibition by nor-NOHA could increase NO˙ production and enhance LACL.

With the described system bias in mind, we found no differences in fMLF-stimulated HRP-treated samples that could not be explained by this bias. In the intracellular assay, the expected attenuation by nor-NOHA was absent and LACL equal to controls. In Cytodex-3 stimulated samples we observed an opposite relationship after treatment with nor-NOHA; no deviations from that expected in the intracellular assays and augmented LACL in the HRP-treated samples (Fig 10):

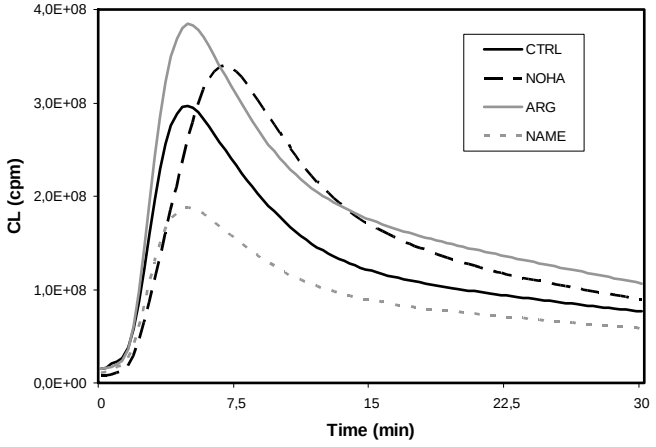


**Figure 10:** LACL from Cytodex-3 stimulated, HRP-enhanced samples. Curves represent mean values, n=12.

These data indicate stimulus-dependent differences regarding arginase-mediated effects, and we hypothesized that integrin-, as compared to fMLF-, provoked neutrophil stimulation would lead to a greater extracellular release of arginase, explaining the effects of arginase inhibition in HRP-treated samples. Another finding in favour of this notion was that addition of L-NAME to nor-NOHA treated samples completely abolished the nor-NOHA enhancement in HRP treated samples (unpublished results). This outrules other unspecific nor-NOHA effects and indicates actual effects on NOS. Furthermore, since neutrophil adhesion is dependent on iNOS activity [Jenei 2006], pre-treatment with L-NAME will diminish adhesion to Cytodex-3, thereby limiting oxidative activation and degranulation. Hence, attenuated LACL and less extracellular arginase available for inhibition would be expected, thus being a possible explanation for these results.

Measurements of extracellular arginase I protein and arginase activity both revealed a significantly higher arginase content in Cytodex-3 stimulated samples compared to fMLF-stimulated samples ($p<0.01$). We therefore conclude that ligation of β-integrins induce extracellular release of preformed arginase from human neutrophils. Since endothelial $NO^{\cdot}$ production is dependent on extracellular L-arginine levels, we propose that this might be a way for human neutrophils to reduce L-arginine availability, diminish local $NO^{\cdot}$ production, and facilitate adhesion and extravasation. Chronic inflammatory states such as RA, with high numbers of neutrophils continuously leaving the bloodstream, can therefore serve as models to test this proposal. Indeed, it has been shown that both arginase protein and arginase activity are increased in RA, and that serum levels of L-arginine are depressed [Huang 2001, paper II]. Increased arginase activity could lead to increased in urea production, but since circulating urea levels also depend on protein breakdown and kidney function, this is not a specific reflection of arginase activity. However, raised circulating urea levels have been reported in RA and also associated with poor outcome [Fleming 1976]. Since endothelial integrity is dependent on NOS activity, and since endothelial production of arginase has emerged as a leading actor in the atherosclerosis process, neutrophil release of arginases may also be a key to the accelerated atherosclerosis and increase in cardiovascular events in RA [Predescu 2005, Yang 2006, Kaplan 2006].

## CONCLUDING REMARKS

Besides being a cornerstone in innate immunity and our first line of defence against invading intruders, a more diversified picture of the role of neutrophils in autoimmune diseases is emerging. Previously regarded as a terminally differentiated cell with little or no capacity of protein synthesis, neutrophils are now known to synthesize and release a number of important cytokines and modulate second line defences by affecting the responses of lymphocytes, macrophages and dendritic cells. Accordingly, initiation of arthritis in several animal models has also been shown to be dependent on neutrophils. Being professional phagocytes, neutrophils can phagocytose both opsonized and non-opsonized material. With the recent discovery of immunization to citrullinated proteins as a key event in RA, the novel findings in paper I indicating a disease-specific intracellular activation of synovial fluid neutrophils from RA patients may be of great importance. Even if the causes of neutrophil accumulation in the joint cavity are unknown, it is possible that they become engaged in the handling of opsonized target antigens, *e.g.* citrullinated protein present in the synovial fluid, thus explaining the massive amount of neutrophils in the joint cavity. Since the intracellular activation is indicative of processing of ingested material, further studies on this issue are warranted.

In a series of experiments described in paper II and III we finally managed to show the presence of a constitutive iNOS in human neutrophils. Although the subcellular localization and regulatory mechanisms remain to be clarified, it is obvious that the properties and functions of the human neutrophil iNOS isoform in many ways differ from the prevailing views established in other, both human and animal, cells. In a very recent publication, the human neutrophil iNOS has been claimed to be essential for neutrophil adhesion. This may be a clue to the neutrophil accumulation in synovial fluid, since the massive amounts of $NO^{\cdot}$ produced by chondrocytes could inhibit neutrophil iNOS, thereby preventing adhesion.

The extracellular release of arginase following integrin ligation described in paper IV is exciting, regardless of the intracellular source of arginase. It is possible that stimulation with collagen-I coated Sephadex beads could represent a model of neutrophil 'frustrated phagocytosis'. This is, however, contradicted by the fact that extracellular release of arginase was also, but to a lesser extent, noted after stimulation with fMLF. Neutrophil release of arginase in response to integrin ligation (or other stimuli) could represent an exciting new regulatory mechanism and speculatively be an important link between inflammation and accelerated atherosclerosis.

In summary, generation of oxygen radicals by the NOS family and the phox family both represent evolutionary well preserved mechanisms for cell signal transduction and cell regulation. The principal targets for this regulation, i.e.

metals and thiols, are also present in intruders, and high-output systems may have evolved in host defence to interfere with the metabolism of such external threats. The principal findings in this thesis; the intracellular activation of neutrophils in RA; the presence of a constitutive iNOS; and the arginase release from human neutrophils, may have implications for physiological immune and inflammation regulation, autoimmune conditions, infectious diseases, and cardiovascular disease.

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# Sammanfattning på svenska

Vid reumatiska ledinflammationer ansamlas mycket stora mängder polymorfkärniga neutrofila granulocyter (neutrofiler) inne i den vätskefyllda ledhålan. Neutrofiler har kraftfull destruktiv potential och anses kunna bidra till uppkomst av skada i leden. Då flera djurmodeller av ledinflammation har visat sig omöjliga att initiera i frånvaro av neutrofiler, har intresset för denna celltyp åter ökat efter att de under lång tid har stått i skuggan av andra typer av vita blodkroppar. En viktig del i avdödning av mikroorganismer och cellsignalering är förmågan att bilda fria syreradikaler, t.ex. superoxid ($^{\cdot}O_2^-$) och kväveoxid ($NO^{\cdot}$). Denna avhandling belyser aspekter kring produktionen av dessa reaktiva syreprodukter och mekanismer av potentiell betydelse vid ledinflammation.

I det första arbetet visas att neutrofiler isolerade ur ledvätska från patienter med ledgångsreumatism (RA) har ett unikt beteende avseende superoxidproduktion jämfört med motsvarande celler från patienter med andra reumatiska sjukdomar. RA-neutrofiler från ledvätska (men inte från blod) producerar superoxid intracellulärt redan i vila och stimulering via vidhäftningsmolekyler ger en snabb ytterligare ökning av denna aktivitet. Fyndet antyder att cellerna är engagerade med hantering av endocyterade partiklar och/eller immunkomplex/immunaggregat.

I de båda nästkommande arbetena undersöktes förekomst av det $NO^{\cdot}$-producerande enzymet iNOS i neutrofiler. En rad tidigare publikationer har rapporterat motsägelsefulla resultat i denna fråga. Efter en serie experiment kunde vi konstatera att humana neutrofiler uttrycker iNOS konstitutivt, men att både dess cellulära lokalization och reglering skiljer sig från andra celler.

Neutrofiler har nyligen även visats innehålla arginas, ett enzym som konkurrerar med iNOS om bindningen till L-arginin och som därmed kan hämma $NO^{\cdot}$-produktion. I det fjärde arbetet undersökte vi därför om hämning av arginas påverkade neutrofilernas funktion och produktion av superoxid. Vi fann att effekterna av arginashämmning var större hos celler som stimulerats genom vidhäftning av kollagenklädda partiklar jämfört med en löslig formylerad tri-peptid (fMLF). Med stöd av dessa fynd kunde vi i påföljande försök bekräfta hypotesen de att extracellulär frisättning av arginas är större efter vidhäftning av kollagen-partiklar än med fMLF-stimulering. Fysiologiskt är fyndet logiskt då det skulle medföra ökade vidhäftningsmöjligheter för neutrofilen inne i blodbanan genom att begränsa blodkärlets egen $NO^{\cdot}$ produktion. Fyndet är också förenligt med den ökade frekvensen hjärt- och kärlsjukdomar vid RA, då en intensiv kontinuerlig utvandring av neutrofiler skulle medföra ökad arginas frisättning, sänkta argininnivåer och endotelial dysfunktion.

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