Deficiency of NADPH Oxidase Components p47phox and gp91phox Caused Granulomatous Synovitis and Increased Connective Tissue Destruction in Experimental Arthritis Models

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Recent studies indicated that the nicotinamide dinucleotide phosphate oxidase (NADPH) oxidase-derived oxygen radicals plays a deleterious role in arthritis. To study this in more detail, gonarthrosis was induced in NADPH oxidase-deficient mice. Mice received an intraarticular injection of either zymosan, to elicit an irritant-induced inflammation, or poly-L-lysine coupled lysozyme, to evoke an immune-complex mediated inflammation in passively immunized mice. In contrast to wild-type mice, arthritis elicited in both p47phox^{-/-} and gp91^{-/-} mice showed more severe joint inflammation, which developed into a granulomatous synovitis. Treatment with either Zileuton or cobra venom factor showed that the chemokines LTB₄ and complement C3 were not the driving force behind the aggravated inflammation in these mice. Arthritic NADPH oxidase-deficient mice showed irreversible cartilage damage as judged by the enhanced aggrecan VDIPEN expression, and chondrocyte death. Furthermore, only in the absence of NADPH oxidase-derived oxygen radicals, the arthritic joints showed osteoclast-like cells, tartrate-resistant acid phosphatase (TRAP)-positive/multinucleated cells, extensive bone erosion, and osteolysis. The enhanced synovial gene expression of tumor necrosis factor- α , interleukin-1 α , matrix metalloproteinase (MMP)-3, MMP-9 and receptor activator of NF- κ B ligand (RANKL) might contribute to the aggravated arthritis in the NADPH oxidase-deficient mice. This showed

that the involvement of NADPH oxidase in arthritis is probably far more complex and that oxygen radicals might also be important in controlling disease severity, and reducing joint inflammation and connective tissue damage. (Am J Pathol 2003, 163:1525–1537)

Neutrophils and other myeloid cells produce superoxide as part of their bacterial killing. The nicotinamide dinucleotide phosphate (NADPH) oxidase multicomponent enzyme system catalyzes the production of superoxide and the active central role plays the transmembrane cytochrome b558 which is comprised of two subunits, gp91phox and p22phox. P47phox is the cytosolic component of NADPH oxidase complex that translocates to the membrane and associates with cytochrome b556 to form the active complex that catalyzes the reduction of oxygen to superoxide at the expense of NADPH.^{1–3} Functionally, p47phox increases the binding to cytochrome b556 of p67phox by approximately 100-fold and the binding of Rac by about 50-fold. Thus, p47phox appears to be a crucial adaptor protein of the NADPH oxidase complex essential for the regulation of the respiratory burst of neutrophils.⁴ Genetic defects in the NADPH oxidase system result in neutrophil dysfunction and cause chronic granulomatous disease (CGD) in humans.^{4–6} Mice with a disrupted p47phox gene or gp91phox gene do develop a chronic granulomatous disease.^{7,8}

Autoimmune disorders such as lupus and polyarthritis in CGD patients⁹ prompted us to study the role of NADPH oxidase in experimental arthritis. In arthritis patients, increased reactive oxygen species are present at the sites of inflammation.¹⁰ Circulating neutrophils and monocytes have increased NADPH oxidase activity in rheumatoid arthritis (RA) patients^{11,12} and the superoxide release by the neutrophils isolated from synovial fluid of these patients is even further increased.¹³ NADPH oxidase-derived superoxide, a highly reactive molecule, may have effector functions in RA, which are detrimental for the

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joint. One approach to elucidating the role of superoxide in experimental arthritis is to treat animals with superoxide dismutase (SOD). SOD has a beneficial effect on arthritis that is induced by streptococcal cell walls, adjuvant, or via immunization with collagen type II.^{14–16} However, we previously found that local treatment with cationic SOD has no effect on antigen- and zymosan-induced arthritis in mice.¹⁷ Recently, Lida and Saito¹⁸ found that endotoxin-free CuZn SOD has no effect in three arthritis models. Even trace amounts of endotoxin contamination of SOD do carry the anti-inflammatory effects found in previous studies. Therefore, to identify the role of superoxide derived from NADPH oxidase in arthritis, we used gene knockout (KO) models of CGD. Zymosan was used as an irritant to elicit arthritis since it is a potent activator of p47phox-mediated superoxide production in polymorphonuclear leukocytes and macrophages via activation of the toll-like receptor (TLR)-2, or through nonopsonic phagocytosis using the complement receptor type 3.^{1,19–21} The findings of ZIA are confirmed in the passive immune-complex arthritis (ICA) model in mice.²² Immune complexes stimulate neutrophil NADPH oxidase-mediated superoxide production via their Fc- γ receptors, a pathway distinct from TLR-2 signaling.²³ Experimental arthritis was compared between wild-type (WT) and p47phox gene KO mice. We found worsening of joint inflammation and granulomatous synovitis with extensive cartilage and bone erosion in the p47phox gene KO mice. This identifies NADPH oxidase-dependent superoxide as a possible negative feedback/anti-inflammatory mediator in arthritis, which is contrary to previous reports of the anti-rheumatic effects of SOD. Furthermore, this study unexpectedly showed that connective tissue destruction during arthritis can proceed and is even enhanced in the absence of NADPH oxidase-produced superoxide.

Materials and Methods

Mice

P47phox^{-/-} and gp91phox^{-/-} mice, generated as previously described,^{7,24} lack the cytosolic p47phox and membrane gp91phox subunits of the NADPH oxidase multicomponent system, respectively. The KO mice were backcrossed to the C57Bl6 background for 15 generations and C57Bl/6 (obtained from The Jackson Laboratory, Bar Harbor, ME) were used as controls. In some experiments p47phox^{-/-} mice of intercross progeny (C57Bl/6x129Sv) were used with their proper controls. Colonies were maintained at the National Institutes of Health (Bethesda, MD). All mice were housed under specific pathogen-free conditions during breeding and experiments. Mice received autoclaved chow and acidified water *ad libitum*. Only mice that were healthy were used in the experiments and were age-matched (10 to 20 weeks) and sex-matched for each set of experiments. To limit discomfort for the animals, experiments were terminated at day 7 of arthritis and mice were killed by cervical dislocation. All experiments were approved by local au-

thorities of Animal Care and Use Committee (DEC 98.22) and performed by personnel certified by the Dutch Ministry of Health, Welfare, and Sport.

Zymosan-Induced Arthritis (ZIA)

A homogeneous suspension of 300 mg of zymosan A (from *Saccharomyces cerevisiae*; Sigma, St. Louis, MO), dissolved in 10 ml of endotoxin-free saline was obtained by repeated boiling, followed by sonic emulsification. The suspension was autoclaved for and stored in 0.5 ml aliquots at -20°C. Arthritis was induced by intraarticular injection of 180 μ g of zymosan through the suprapatellar ligament into the joint cavity. In specified experiments the contralateral knee-joints received an equal amount of saline (6 μ l) as control.

Immune-Complex Arthritis

Immune complex arthritis was induced as described before.²² In brief, arthritis was induced by injecting 3 μ g of poly-L-lysine coupled lysozyme into the right knee-joint cavity of mice that had, the night before, received intravenously 0.2 ml specific rabbit antisera directed against lysozyme. To prevent infection both antigen and antisera were γ -radiated with 25kGy before use.

Depletion of Mice for Leukotrienes and Complement

To inhibit 5'-lipoxygenase, mice were treated with Zileuton (Abbott, Abbott Park, IL). A 600-mg tablet was emulsified in 100 ml of 0.5% hydroxyethyl cellulose in saline and stored at 4°C. Mice received by gavage an oral dose of 50 mg/kg daily, starting 1 week before induction of arthritis until the end of the experiment. To deplete animals of complement, cobra venom factor (CVF) (200U/kg; Quidel Corp., San Diego, CA) was injected *i.p.* the night before arthritis induction. The dose has previously been shown to reduce C3 levels in plasma to 3% of normal values within 4 hours after CVF administration and complement C3 values returned to normal 9 days later.²⁵

Assessment of Joint Swelling

Animals were injected subcutaneously with 14 μ Ci of ^{99m}TcTechnetium pertechnetate in 0.2 ml of saline in the neck region. After 10 to 15 minutes the mice were sedated by 4.5% chloral hydrate injected *i.p.*, 0.01 ml/g of body weight. The accumulation of the isotope due to increased bloodflow and edema in the knee was determined by external gamma counting using a fixed position of the Na-I-scintillation crystal. A ratio of the ^{99m}Tc uptake in the inflamed over contralateral knee-joint of >1.1 indicated joint swelling.

Histology

Knee joints were dissected, fixed in phosphate-buffered formalin (pH 7.4) for 4 days, decalcified in 5% formic

acid, and embedded in paraffin wax. Serial coronal (frontal) sections of whole knee-joint were made and stained with safranin-O and counterstained with fast green. Glycosaminoglycan depletion was scored using a linear scale from 0 (normal red staining) to 3 (complete loss of red staining) in the metabolically active upper-layer of articular cartilage. Inflammation was evaluated according to the extent and composition of the influx of inflammatory cells into the synovium (infiltrate) and joint cavity (exudate) and scored separately using a linear scale from 0 (no inflammation) to 3 (full-blown inflammation). Seventy-eight serial sections taken every 70- μ m apart were scored per joint.

NIMP-R14 Staining

Influx of polymorphonuclear neutrophils was analyzed on knee-joint sections. Briefly, sections were deparaffinized and preincubated for 15 minutes at room temperature with 20% normal rabbit serum. Thereafter, sections were incubated with rat monoclonal antibodies to mouse neutrophils (NIMP-R14, a 25- to 30-kd epitope mainly present on neutrophils²⁶) for 1 hour. After incubation with a second peroxidase conjugated rabbit anti-rat IgG antibodies for 30 minutes, sections were incubated with 3-amino-9-Ethylcarbazole (Vector Laboratories, Burlingame, CA) substrate in the dark at 37°C for 10 minutes. Thereafter, sections were stained with hematoxylin for 30 seconds. As a negative control, sections were incubated with normal rat Ig instead of NIMP-R14.

Tartrate-Resistant Acid Phosphatase (TRAP) Staining

Whole knee-joints were fixed for 2 days in 2% formalin, followed by decalcification in 10% ethylenediaminetetraacetate (EDTA) in 1 mmol/L Tris-HCl pH 7.4 for up to 2 weeks at 4°C. Decalcified specimens were processed for paraffin embedding. Staining of tissue sections (7 mm) for TRAP was performed by a leukocyte acid phosphatase kit, a cell-staining kit for the detection of TRAP (Sigma).

Immunohistochemistry of VDIPEN

Immunostaining of VDIPEN, a neo-epitope of a matrix metalloproteinase (MMP)-induced cleavage site in aggrecan, was performed as described.²⁷ In short, dissected knee-joints were fixed in 4% paraformaldehyde and subsequently decalcified in 10% EDTA in phosphate buffer (pH 7.4) for 7 days. Thereafter, joints were embedded in paraffin wax and coronal sections (7 μ m) were cut, dewaxed, hydrated and digested with proteinase-free chondroitinase ABC (0.25U/ml in 0.1 mol/L Tris-HCL, pH 8.0; Sigma) for 1 hour at 37°C. Sections were treated with Nakane's fixative, exposed to 1% hydrogen peroxide in methanol for 20 minutes, then 5 minutes with 0.1% TritonX-100 in PBS. After blocking with 1.5% normal goat serum for 20 minutes, sections were incubated with affinity-purified rabbit anti-VDIPEN IgG (a gift from Merck

Research Laboratories, Rahway, NJ) overnight at 4°C. This antibody has been extensively characterized and detects the VDIPEN C-terminal sequence in the core protein of aggrecan after cleavage by MMPs and cathepsin. Sections were then incubated with biotinylated goat anti-rabbit IgG, and the VDIPEN immunostaining was detected by avidin-peroxidase staining using nickel enhancement development. Orange G was used as a counterstain.

Immunohistochemical Localization of Poly(ADP-Ribose) Polymerase (PARP)

Paraffin-embedded tissue sections were deparaffinized, and endogenous peroxidase was quenched with 3% H₂O₂ in 60% methanol for 30 minutes. The sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 minutes. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 minutes. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with 1:500 dilution of primary anti-PARP antibody (DBA) or with control solutions. Control included buffer alone or non-specific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase (DBA).

Radiology

At the end of the experiments, knee joints were isolated and used for X-ray analysis as a marker for bone destruction. X-ray photographs (Kodak Industrex film) were carefully examined using a stereo microscope. Bone erosions

Figure 1. Zymosan-induced joint swelling in WT and p47phox KO mice. A single dose of zymosan (180 μ g sonicated and sterile yeast particles) was injected intraarticularly into the right (R) knee joint. The left (L) knee joint was not injected and served as an intraanimal control. Joint swelling was measured at the indicated times after zymosan injection by the ^{99m}Tc-technetium uptake method (as described in Materials and Methods). Right knee joints with a R/L ratio of more than 1.1 are regarded swollen. Data are the mean $\pm$ SD of joint swelling measured in 12 animals at day 2, 7 animals at day 3, and 6 animals at day 7. At all timepoints measured the values of the p47phox^{-/-} mice were significantly different ($P < 0.0001$, by Student's *t*-test) as compared to WT mice.

were scored on a scale ranging from 0 to 5: 0, no erosions; 1, minor bone destruction, 1 spot; 2, moderate changes, 2 to 4 spots, 1 area; 3, marked changes, 2 to 4 spots, more areas; 4, severe erosions afflicting the joint; 5, complete destruction of joint, new bone formations. Osteolysis was also scored as loss of trabecular bone organization and bone density using a scale from 0 to 3, ranging from normal cortical and trabecular bone structure to complete loss of organization, cracks and occasional fractures.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction

RNA extraction and RT-PCR were performed as described.²⁸ Synovial biopsies were taken from tissue adjacent to the suprapatellar ligament with a biopsy punch (diameter of 3 mm) and immediately frozen in liquid nitrogen. RNA was isolated with TRIzol reagent according to the protocol of the manufacturer (Life Technologies, Breda, The Netherlands), and reverse-transcribed to complementary DNA (cDNA) using oligo(dT) primers and reverse transcriptase (Life Technologies). Equal amounts of cDNA, normalized by the housekeeping gene GAPDH, were used for standard PCR reactions using *Taq*-DNA polymerase (Life Technologies). Primers for murine MCP1, macrophage inflammatory protein (MIP)1 α , MIP2, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, IL-10, IL-1Ra, MMP2, MMP3, MMP9, tissue inhibitor of metalloproteinase (TIMP)-1, osteoprotegerin ligand (OPGL), and Osteoprotegerin (OPG) were designed by Primer3 (www.genome.wi.mit.edu/genome_software/other/primer3.html). Specificity was controlled using FASTA search databank.²⁹

Samples of the PCR reaction (5- μ l aliquots) were taken after an initial round of 20 cycles, and thereafter every 2 to 3 cycles until the end of the PCR reaction (cycle 35 to 40). The size of the PCR product was analyzed on an 1.6% agarose gel containing ethidium bromide. Photo-scanning analysis of the agarose gels was performed to determine the difference in the number of cycles between the expression in normal synovium (contralateral joint) with the inflamed synovium (zymosan-injected joint) of the same animal. Synovial tissue samples of six mice were evaluated individually and the mean difference in gene expression was presented.

Expression of apoptosis genes (BAX, Bcl-2, Bcl-xL, Bcl-xS, caspase-3) was evaluated by multiplex PCR using the mouse apoptosis genes set 2 (Apo-M152; AMS Biotechnology, Abingdon, Oxon, UK) according to the manufacturer's protocol. Products were run on 2% agarose gel and analyzed with the Multi-Analyst system (Bio-Rad, Hercules, CA).

Patella Assay

Patellae were dissected with a minimal amount of surrounding soft tissue and incubated in RPMI 1640 (Glutamax; Invitrogen, Carlsbad, CA) with 1% pyruvate (100 mmol/L) and Penstrep 100 IU/ml with or without murine recombinant IL-1 α (10 ng/ml) for 48 hours. Thereafter glycosaminoglycan (GAG) synthesis was assessed by incorporation of ³⁵S-sulfate (20 μ Ci/ml) during the last 3 hours. Patellae were fixed overnight (o/n) in 10% formalin and subsequently decalcified in 5% formic acid for an additional 4 hours. Thereafter, patellae were punched out of their surrounding tissue and dissolved in 0.5 ml Luma-solve (Lumac, Groningen, The Netherlands) o/n at 60°C. The ³⁵S-sulfate content of each patella was measured by liquid scintillation counting and expressed as counts per minute (cpm). More than 95% of the radiolabel was incorporated into the GAGs of proteoglycans. The concentration of NO₂⁻ (a stable breakdown product of NO) in the 24-hour culture medium was determined by the Griess reaction, using NaNO₂ as a standard. One hundred μ l of culture medium was mixed with 100 μ l of Griess reagent (0.05% naphthylene diaminedihydrochloride and 0.05% sulfanilamide in 2.5% H₃PO₄) in a flat-bottom microtiter-plate and adsorbance was read at 545 nm using an ELISA-plate reader.

Statistical Analysis

Statistical comparison between groups was performed with Student's *t*-test. Histological scores were analyzed using Mann-Whitney Rank Sum Test. Values of *P* < 0.05 were considered significant.

Results

Aggravated Joint Inflammation in NADPH Oxidase-Deficient Arthritic Mice

An intraarticular injection of sterile zymosan particles causes an acute joint inflammation and compared to WT mice, joint swelling was markedly increased (fivefold) in p47phox^{-/-} mice (Figure 1). Histological evaluation of the knees at day 7 of ZIA showed exudation of polymorphonuclear leukocytes (PMN) into the joint cavity residing in close proximity of the cartilage surfaces (Figure 2, E and F). Furthermore, zymosan-induced joint inflammation developed into a granulomatous synovitis in both p47phox^{-/-} and gp91^{-/-} mice (Table 1; Figure 2). Immunohistological staining of NIMPR4 confirmed that a large percentage (approximately 40%) of infiltrating cells were PMNs (Figure 2, C and D). Interestingly, the case-

Figure 2. Histological evaluation of joint pathology in WT (A) and NADPH-OX deficient (B to H) mice, 7 days after intraarticular zymosan injection. Frontal knee-joint sections ($\times 50$) stained with safranin-O: A, WT mouse; B, p47phox KO mouse, **arrowheads** indicate cartilage proteoglycan depletion, **open arrows** indicate sites of bone erosion. Zymosan caused granulomatous synovitis in p47phox^{-/-} mice (C, original magnification, $\times 100$) with a large number of PMNs as identified with NIMPR4 antibodies (D, representative figure of 6 mice). May-Grunwald Giemsa staining of a cytospin of exudate cells obtained from the joint cavity of p47phox^{-/-} mice showed the presence of PMNs (E, original magnification, $\times 400$). Exudate cells are in close proximity to the cartilage surface in p47phox^{-/-} mice (F, original magnification, $\times 400$). Glycosaminoglycan depletion as detected by loss of safranin-O staining and chondrocyte death in articular cartilage of patellae and condyles from p47phox^{-/-} mice (G and H, original magnification, $\times 200$). C, cartilage; F, femur; GP, growth plate; M, meniscus; P, patella, S, synovium; T, tibia.

Table 1. Histological Evaluation of Pathological Changes in the Murine Knee Joint at Day 7 of Zymosan-Induced Arthritis

Model/strain*	Infiltrate (0–3)	Exudate (0–3)	Cartilage proteoglycan loss (0–3)			Cell death [†] (0–3)	Bone erosion (0–3)
			Patella	Tibia	Femur		
Exp. 1: ZIA							
WT	0.6 ± 0.1	0.4 ± 0.1	1.7 ± 0.3	1.0 ± 0.2	1.6 ± 0.2	0.1 ± 0.02	0.1 ± 0.05
p47phox ^{-/-}	3.0 ± 0.0 [‡]	3.0 ± 0.0 [‡]	2.7 ± 0.1 [§]	3.0 ± 0.0 [‡]	1.7 ± 0.2	2.7 ± 0.1 [‡]	2.1 ± 0.2 [‡]
Exp. 2: ZIA							
WT	0.8 ± 0.2	0.4 ± 0.2	1.0 ± 0.2	0.1 ± 0.1	0.9 ± 0.2	nd	0
gp91 ^{-/-}	2.6 ± 0.3 [‡]	1.7 ± 0.4 [‡]	1.8 ± 0.2 [§]	1.3 ± 0.2 [‡]	1.1 ± 0.4	nd	1.8 ± 0.5 [‡]
Exp. 3: ICA							
WT	0.2 ± 0.1	0	0	0	0	0.1 ± 0.1	0
gp91 ^{-/-}	1.3 ± 0.7 [‡]	1.2 ± 0.5 [‡]	1.6 ± 0.7 [‡]	1.8 ± 0.8 [‡]	1.8 ± 0.8 [‡]	0.1 ± 0.1	0.5 ± 0.3

*Exp. 1: Knee joints from 14 WT and 15 intercrossed p47phox^{-/-} mice taken at day 7 of ZIA were analyzed with histology. Exp. 2: Knee joints from 8 C57Bl/6 and 8 gp91^{-/-} mice at day 7 of ZIA. Exp. 3: Knee joints from 8 C57Bl/6 and 8 gp91^{-/-} mice at day 7 of ICA. Total knee-joint sections were prepared and stained with haematoxylin and eosin. Infiltrate represents the amount of inflammatory cells residing in the synovial tissue. Exudate represents the amount of inflammatory cells (predominantly neutrophils) in the joint cavity.

[†]The empty lacunae, indicating chondrocyte death, in the articular cartilage matrix above the tidemark in both patella and femur (patellar groove) were scored arbitrarily. Joint sections stained with safranin-O to detect cartilage proteoglycan depletion in patellae, femoral condyles, and tibial plateaus, using hematoxylin-eosin-stained sections. These different histological parameters were scored arbitrarily by two independent observers in a blinded fashion using a scale of 0 (normal) to 3 (most extensive change). Data are presented as mean values ± SEM and statistically analyzed using the Wilcoxon rank sum test.

[‡]P < 0.001; statistically different compared to the control WT group.

[§]P < 0.01.

Exp., experiment.

ous necrotic centers of granulomata also stained positive for NIMP-R14, although cells could not be identified morphologically. A mononuclear cell layer (predominantly macrophages) which stained negative for NIMP-R14 surrounded the granulomas. The relative paucity of lymphocytes could suggest that the inciting zymosan was either an inert foreign body or that at day 7 an acquired immune response against zymosan had not yet developed. No granulomatous synovitis was seen in immune-complex mediated arthritis (ICA), although NADPH oxidase-deficient mice also showed a significant increased joint inflammation as compared to the WT littermates (Table 1).

NADPH oxidase complex plays an important role in the defense against microorganisms and for this mice were treated with trimethoprim-sulfamethoxazole (Methoxazol-T; Eurovet, Bladel, The Netherlands) as a precaution. Treatment with this antibiotic had no effect on the severity or course of zymosan-induced arthritis in either

WT or p47phox^{-/-} mice. Moreover, joint exudates taken from untreated arthritic p47phox^{-/-} mice were negative for microorganisms as tested on aerobic and anaerobic broth cultures. Furthermore, violet blue staining did not detect bacteria on exudate smear or histological sections of whole knee-joints from arthritic p47phox^{-/-} mice (data not shown). This proved that the aggravated joint inflammation in p47phox^{-/-} mice was not due to a local secondary septic joint inflammation.

The leukotriene LTB₄ and complement C3 are potent chemokines but are sensitive for NADPH oxidase-mediated oxidative degradation. Impaired inactivation of these chemokines might cause the enhanced inflammatory response in NADPH oxidase-deficient mice. Treatment with Zileuton, an inhibitor of 5'-lipoxygenase had no effect on the extent of zymosan-induced joint inflammation in either WT or p47phox^{-/-} mice (Table 2). Treatment of mice with CVF significantly suppressed infiltration

Table 2. Effect of 5'-Lipoxygenase Inhibitor Zileuton or CVF Treatment on ZIA

Mouse strain*	Treatment	Joint swelling (Tc ratio)	Infiltrate (0–3)	Exudate (0–3)	GAG [†] depletion (0–3)	Bone erosion (0–3)
Exp 1.						
WT	Vehicle	1.50 ± 0.08	0.8 ± 0.2	0.3 ± 0.2	2.3 ± 0.5	0
WT	Zileuton	1.43 ± 0.13	0.5	0	1.9 ± 0.1	0
p47phox ^{-/-}	Vehicle	3.75 ± 0.18 [‡]	3.0 [‡]	2.5 ± 0.2 [‡]	2.5 ± 0.2	2.3 ± 0.2 [‡]
p47phox ^{-/-}	Zileuton	3.45 ± 0.51 [‡]	3.0 [‡]	2.8 ± 0.2 [‡]	1.9 ± 0.2	2.5 ± 0.2 [‡]
Exp. 2						
WT	Vehicle	nd	1.1 ± 0.4	0.6 ± 0.4	1.3 ± 0.3	0
WT	CVF	nd	0.4 ± 0.2	0.1 ± 0.2	0.8 ± 0.2	0
gp91 ^{-/-}	Vehicle	nd	2.1 ± 0.5	1.8 ± 0.7	2.0 ± 0.4	1.1 ± 0.5
gp91 ^{-/-}	CVF	nd	3.0	1.6 ± 0.7	1.5 ± 0.3	2.4 ± 0.3

*Exp. 1: Each group consisted of 6 animals (p47phox^{-/-} backcrossed strain (N15), and C57Bl/6 control mice), and mice received a dose of 50 mg/kg/day of zileuton or an equivalent volume (0.2 ml) of vehicle orally. Treatment started 7 days before onset of arthritis and was continued thereafter. Exp. 2: Each group of 4 animals (gp91phox^{-/-} and C57Bl/6 wildtypes) received 200 U/kg of CVF intraperitoneally the night before arthritis induction. At day 7 of arthritis, joint swelling was measured and the knee joints were processed for histology. Joint swelling was measured externally by the uptake of circulating ^{99m}Tc uptake and expressed as a ratio of the arthritic over the contralateral non-arthritic joint.

[†]Proteoglycan depletion was scored in the patellar groove of the femur by loss of safranin-O staining. Data are presented as the mean ± SD (joint swelling) and mean ± SEM (histological parameters), and statically tested using Student's *t*-test and Wilcoxon rank sum test, respectively.

[‡]P < 0.001; significantly different compared to the control WT group.

Table 3. Suppression of Chondrocyte GAG Synthesis in Patellar Cartilage Exposed to IL-1 α in Vitro

Mouse strain	Treatment	GAG synthesis ³⁵ S-sulfate incorporation		Nitric oxide (μ M)
		(cpm)	(%)	
WT	None	1086 $\pm$ 62	100	1.0 $\pm$ 0.5
WT	IL-1 α	567 $\pm$ 99*	52	5.7 $\pm$ 0.7*
p47phox ^{-/-}	None	1240 $\pm$ 305	100	2.0 $\pm$ 0.6
p47phox ^{-/-}	IL-1 α	709 $\pm$ 118*	57	6.5 $\pm$ 0.5*

* $P < 0.01$, by Student's *t*-test.

Experimental groups consisted out of four patellae each. GAG synthesis was assessed 48 hours after IL-1 α (10 ng/ml) exposure by a 3-hour ³⁵S-sulfate chase. Media was changed after 24 hours and the nitrite content in the first 24-hour culture supernatant was measured using the Gries reagent.

(-64%) and exudation (-83%) in WT animals. The same CVF treatment, however, did not decrease joint inflammation in the p47phox^{-/-} mice (Table 2). This suggests that an impaired oxidative degradation of the chemokines LTB4 and complement C3 was not the underlying mechanism of the derailed inflammatory response during experimental arthritis in p47phox^{-/-} animals.

Increased Articular Cartilage Damage in Arthritic Joints from NADPH Oxidase-Deficient Mice

Whole knee sections from p47phox^{-/-} mice showed enhanced loss of glycosaminoglycan (safranin-O staining) content in the articular cartilage matrix of patella and

Figure 3. Immunolocalization of the neo-epitope VDIPEN in cartilage of WT (A and C) and p47phox KO (B and D) mice at day 7 of zymosan-induced arthritis. VDIPEN staining (black grains) was detected in cartilage at both the patella-femur junction and in the condyles of p47phox^{-/-} mice only. Representative figure of three experiments (total number of p47phox^{-/-} mice evaluated is 18 and an equal number of WT mice). Original magnification, $\times 100$. Sections were counterstained with Orange G.

Figure 4. X-ray photographs of whole knee-joints from WT (**A** and **B**) and p47phox KO (**C** and **D**) mice taken 7 days after zymosan injection. **B** and **D**: Mice treated with Zileuton (Table 2). Note the loss (osteolysis) of trabecular and cortical bone in the femur of p47phox^{-/-} mice. Representative figure of two experiments; first experiment consisted of five intercrossed p47phox^{-/-} mice and four WT mice, second experiment consisted of 12 backcrossed p47phox^{-/-} mice (N15) and 12 C57Bl/6 mice.

tibial plateau at day 7 of arthritis in both models (Figure 2, G and H; Table 1). The differences in GAG content of the patellar groove (femur) were statistically significant between WT and NADPH oxidase-deficient mice only in the ICA model (Table 1). We previously showed that IL-1 played a major role in the suppression of chondrocyte proteoglycan synthesis via a nitric oxide-dependent mechanism during experimental arthritis. We tested whether the articular chondrocytes with NADPH oxidase deficiency became more vulnerable to IL-1-induced inhibition of proteoglycan synthesis. Patellar cartilage was exposed to murine recombinant IL-1 α (10 ng/ml) for 48 hours. We found that p47phox deficiency did not affect IL-1-induced inhibition of chondrocyte proteoglycan syn-

thesis and did not significantly increase the level of nitrite (stable end-product of nitric oxide in aqueous solutions) in culture media (Table 3). More importantly, we observed enhanced expression of the neo-epitope VDIPEN, a MMP cleavage site in the core protein of proteoglycans, and increased chondrocyte death in the non-calcified cartilage layer (above tidemark) suggests that NADPH oxidase deficiency leads to irreversible cartilage damage during ZIA (Table 1; Figures 1 and 3).

NADPH Oxidase Deficiency Resulted in Pronounced Bone Destruction during ZIA

Histology showed pronounced erosions of the cortical bone in knee joints from gp91^{-/-} and p47phox^{-/-} mice at day 7 of ZIA (Figure 2B; Table 1). X-ray analysis showed significant changes of 2 to 4 erosion spots per whole knee-joint section in 9 out of 15 p47phox^{-/-} mice evaluated (mean 2.78 ± 1.48) and all 15 WT animals examined were without erosions (Figure 4). Furthermore, marked osteolysis (loss of trabecular bone) below the femoral growth plate in the femur was found in all p47phox^{-/-} mice at day 7 of arthritis (mean 2.1 ± 0.7) (Figures 4 and 5). The overall integrity of the bone structure was not compromised in the arthritic joints of WT mice, indicating a clear difference with p47phox^{-/-} mice. Histochemistry showed TRAP-positive osteoclast-like cells at the sites of bone erosion (Figure 6) in the p47phox^{-/-} mice. No osteoclast-like cells were present in the inflamed synovia and at the bone margins in the periosteum of the arthritic joints in WT mice. Only moderate bone erosion was observed in the arthritis joints of gp91^{-/-} mice with ICA (Table 1).

Increased Ratio of IL-1/IL-1Ra and MMP/TIMP mRNA Expression in Synovia of Arthritic p47phox^{-/-} Mice

Synovial tissues were dissected 24 hours after zymosan injection and mRNA expression was analyzed by

Figure 5. Sagittal plane of whole knee-joint sections stained for TRAP and counterstained with hematoxylin of WT (**A**) and p47phox^{-/-} (**B**) mice. **Black arrowhead** indicates the site of cortical bone erosion, **white arrowhead** indicates the areas with large numbers of TRAP-positive multinucleated cells in p47phox^{-/-} mice. Note the overall loss of trabecular bone structure in the femur. Representative figure of 4 backcrossed p47phox^{-/-} mice (N15) and 4 C57Bl/6 animals. Original magnification, $\times 50$.

Figure 6. Detection of osteoclast-like cells at sites of bone erosion at day 7 of zymosan-induced arthritis in $p47^{phox-/-}$ mice. Frontal knee-joint sections were developed for TRAP. TRAP-positive multinucleated cells were identified in close contact with cortical bone present in the bone matrix invading tissue (**A**) and in the synovium (**B** and **C**). Representative figure of 7 intercrossed $p47^{phox-/-}$ mice. **A** and **B**: Original magnification, $\times 200$; **C**, $\times 400$.

RT-PCR. Compared to the contralateral non-inflamed joints, the arthritic joints showed enhanced mRNA expression of chemokines (MCP1, MIP-1 α), cytokines (IFN- γ , TNF α , IL-1 α) and metalloproteinases (MMP-2, -3, -9) (Figure 7). The natural protective proteins IL-1Ra and TIMP were also up-regulated during inflammation. In the $p47^{phox-/-}$ mice, the expression of most of the above-mentioned genes was above the expression in WT mice (Figure 7). The shift toward a more severe process in NADPH oxidase-dependent superoxide deficiency was underlined by changes in IL-1/IL-1Ra mRNA expression ratio (0.6 in WT to 1.5 in $p47^{phox-/-}$ and the MMP3/TIMP or MMP9/TIMP ratios (1.4 in WT to 2.2 in $p47^{phox-/-}$ mice. Substrate polyacrylamide gel electrophoresis of synovial tissue extract detected enzymes with gelatinolytic activity in arthritic joints of $p47^{phox-/-}$ but not WT

mice (not shown). The genes for MIP2 and RANKL (also known as OPGL) were expressed exclusively in the arthritic joints of $p47^{phox-/-}$ mice. The antagonist OPG was not detected in the synovial mRNA samples. The above data underlined the misbalance of cytokines, enzymes, growth factors, and their antagonists in NADPH oxidase-deficient mice that could explain the exaggerated inflammatory response and marked connective tissue destruction during arthritis in these mice.

In the inflamed synovial tissue from $p47^{phox-/-}$ mice, the increased expression of the pro-apoptotic Bax gene is counterbalanced by an increase in the anti-apoptotic Bcl-2 gene, which can form heterodimers. The decreased expression of caspase-3 and Bcl-xS gene points in the direction of decreased apoptosis during arthritis in the NADPH oxidase-deficient mice (Figure 8). Immuno-

Figure 7. Gene expression in inflamed synovia of WT and p47phox KO mice at day 7 of zymosan-induced arthritis. Synovia were dissected and total RNA was extracted and subjected to RT-PCR using specific oligonucleotide primers for the indicated genes. Equal amounts of mRNA (normalized by their GAPDH content) were used and the expression in the inflamed synovia was set against the normal contralateral joint (Δ PCR cycles). Data represent the mean mRNA expression of 3 intercrossed p47phox^{-/-} and 3 WT mice.

detection of poly (ADP-ribose) polymerase (PARP), another marker of apoptosis, showed comparable staining intensity in the inflamed synovial tissue between both mouse strains (Figure 9). This suggests that the enhanced inflammation in the NADPH oxidase-deficient mice was probably not due to diminished apoptosis.

Discussion

In this study we investigated the role of NADPH oxidase in experimental arthritis using gene KO mice. As a consequence of NADPH oxidase deficiency these mice developed chronic granulomatous synovial lesions in response to intraarticular zymosan. Granuloma formation in inflamed synovia is a rare event in murine arthritis models but occurs more frequently in RA patients (incidence is 40%) and is also reported in patients with systemic lupus erythematosus and Crohn's disease. The NADPH oxidase-deficient mice also developed extensive bone erosions and osteolysis after zymosan injection while WT mice did not. This suggested that, NADPH oxidase dependent superoxide ameliorates arthritis, tempers joint inflammation, and directly or indirectly reduces cartilage and bone destruction.

These results in NADPH oxidase-deficient mice are distinct from those obtained by experiments using reactive oxygen species (ROS) inhibitors, indicating that the use of various KO mouse models will help in delineating the role of NADPH oxidase-derived oxygen radicals.¹⁴⁻¹⁶ It is possible that the acute loss of NADPH oxidase-

derived superoxide production was compensated by the action of other mediators. For instance, in the absence of gp91phox, NO may provide some resistance to indigenous bacteria.³⁰ The latter could be of importance, as the combined inhibition of superoxide and NO was by far more effective in the treatment of experimental arthritis.³¹ We did not study possible compensatory mechanisms in detail, but NO production as induced by IL-1 in synovial explants was not further increased with p47phox deficiency. We previously showed that NO derived from inducible NOS does not significantly contribute to zymosan-induced joint inflammation.³² It is therefore unlikely that unchecked NO in the absence of superoxide in p47phox^{-/-} mice was responsible for worsening of arthritis.

The superoxide scavengers used in previous studies failed to discriminate between the recently discovered new members of the NADPH oxidase family like the gp91phox homologues called Nox/Duox present in non-neutrophil cells. Although the rate of oxidant production is quite low by these other cell types, evidence is emerging that the Nox/Duox-derived oxidants serves a cell signaling function, eg, angiogenesis^{33,34} but others are speculated.³

The exacerbated joint inflammation in the NADPH oxidase-deficient mice could be caused by impaired phagocytosis of the immune complexes and zymosan used to elicit arthritis. Neutrophils from CGD patients are impaired in the phagocytosis of immune-complexes. The formation of granulomatous synovitis also suggests that zymosan in the NADPH oxidase-deficient mice was not cleared properly from the joint due to impaired phagocytosis. Evidence to support this is the exuberant splenic granulomata formed in p47phox^{-/-} mice after administration of *Schistosoma mansoni* eggs.³⁵ Excessive inflammatory response to sterile, heat-killed fungal particles in the absence of a respiratory burst is reported in the lung. In X-linked (gp91phox^{-/-}) CGD mice, killed *Aspergillus fumigatus* injected into the lung causes significantly more alveolar neutrophils and increases expression of IL-1 β and TNF α .³⁶ Enhanced local levels of MIP2 and keratinocyte-derived chemokine also accompanied neutrophil sequestration in gram-negative lung inflammation in gp91phox^{-/-} and p47phox^{-/-} mice.³⁷ We found that the exacerbated zymosan-induced inflammation in p47phox^{-/-} mice was accompanied with increased gene expression of TNF α , IL-1 α , MIP1 α , and MIP2. Although ROS are able to provoke cell signaling by the activation of NF- κ B, Erk, and the JAK-STAT pathway,^{38,39} our study suggests that this is probably not the major route of cell activation during arthritis.

In vitro, reactive oxidants can inactivate proinflammatory chemotactic factors, including leukotrienes and C5a.^{40,41} We previously showed that in p47phox^{-/-} mice, the enhanced thioglycollate-induced peritonitis was in part due to reduced oxidant-induced LTB4 inactivation.⁴² Pretreatment of the p47phox^{-/-} mice with zileuton to prevent LTB4 generation did reduce the thioglycollate-induced peritonitis but had no effect on zymosan-induced joint inflammation. CVF is known to bind factor B of the alternative complement pathway, forming

Figure 8. Expression of genes involved in the apoptotic process in the inflamed synovia of WT and p47phox-deficient mice. Total mRNA obtained from non-inflamed (pooled samples of 2 mice) and inflamed synovia (pooled samples of mice) were subjected to multiplex PCR. The included positive apoptosis gene set (M) showed the migration from top to bottom of GAPDH (532 bp), BCL-XL (371 bp), caspase-3 (320 bp), Bax (272 bp), Bcl-2 (235 bp), and BCL-XS (183 bp). The PCR product of caspase-3 was very faint in all of the samples.

Figure 9. Immunolocalization of PARP in synovium of WT (A) and p47phox^{-/-} (B) mice at day 7 of zymosan-induced arthritis. Comparable positive staining was seen in both mouse strains. Figure is representative of 15 intercrossed p47phox^{-/-} and 18 WT mice. No immunoreactivity was found in the non-arthritic control knees (not shown). Original magnification, ×200. B, bone; JC, joint cavity.

a stable C3bBb complex and a markedly depleted C3, preventing the chemoattractive activity of C3b and C5a. Zymosan activates complement via the alternative pathway and in this study we showed that CVF pretreatment of mice significantly reduced zymosan-induced joint inflammation in WT mice. However, CVF treatment of the p47phox^{-/-} mice failed to suppress the exacerbated zymosan-induced joint inflammation, showing complement independence. This showed that the enhanced joint inflammation in NADPH oxidase-deficient mice was not due to an impaired inactivation of the chemotactic factors LTB₄, C3b, and C5a at the site of inflammation by lack of reactive oxidant inactivation.

Diminished ROS-mediated apoptosis of inflammatory neutrophils may also account for the derailment of the inflammatory reaction in the arthritic NADPH oxidase-deficient mice.⁴³ Reactive oxygen intermediates, especially in combination with NO, can damage lipids, proteins, and nucleic acids causing cells to die by apoptosis. Our finding that NADPH oxidase-deficient mice developed gaseous necrotic centers of granuloma and that reduced expression of caspase-3 and Bcl-xS suggests that there is reduced apoptosis in the arthritic tissues of gp91phox and p47phox deficient mice. The unchanged expression of PARP, an enzyme involved in DNA repair,⁴⁴ does not support this conclusion.

Frustrated phagocytosis of neutrophils due to large foreign bodies or insoluble immune-complexes trapped on cartilage leads to an increased respiratory burst and degranulation.⁴⁵ Phagocytosing neutrophils from CGD patients releases 2 to 3 times more active lysozyme and β -glucuronidase.⁴⁶ It is also shown that granulomatous tissue exerts a strong cartilage destructive effect. Implantation of cotton-wrapped cartilage into a murine air-pouch results in granuloma formation and increased gelatinase activity, and accelerates loss of proteoglycans and collagen from cartilage.⁴⁷ We also found increased MMP

expression and gelatinolytic activity in the synovial tissue of p47phox^{-/-} mice. The levels of neutrophil-derived proteinases probably exceeds the threshold raised by antiproteinases present in the synovial fluid. Furthermore, the neutrophils in the arthritic joints of p47phox^{-/-} mice were in close proximity to cartilage (Figure 2F), increasing the chance that neutrophil products (eg, secreted (metallo)proteinases) can escape scavenging and inhibition.⁴⁵ In the p47phox^{-/-} mice, this resulted in the generation of the VDIPEN neo-epitope, a MMP cleavage site in aggrecan. Previously, we reported that VDIPEN expression only occurred in experimental arthritis models in which immune complexes were involved (eg, antigen-, immune-complex-, and collagen-induced arthritis) but not in the non-immunologically mediated zymosan-induced arthritis.^{27,48,49} A possible explanation is that immune-complex deposition on the cartilage surface brings neutrophils in close contact with cartilage and directs destruction there. Furthermore, our study supports previous reports on oxygen-independent cartilage destruction evoked by neutrophils or conditioned synovial media *in vitro*.^{50,51}

We previously showed that endogenous IL-1 suppresses chondrocyte GAG synthesis and this contributes significantly to articular cartilage damage seen during experimental arthritis.⁵² NO-mediated the suppression of GAG synthesis by during arthritis.⁵³ This study showed that NADPH oxidase-dependent superoxide played no part in the suppression of GAG synthesis. Although we only studied arthritis until day 7, it is highly unlikely that the cartilage would recover in the p47phox^{-/-} mice at later stages, as there was extensive chondrocytes death (Table 1). The study of Blanco et al⁵⁴ shows a balance in NO/ROS for maintaining cell viability and that inhibition of IL-1-induced superoxide causes chondrocyte apoptosis, and inhibition of NO leads to necrosis.

The bone resorbing activity of the specialized, highly differentiated osteoclasts mediates bone erosion. We found TRAP-positive multinucleated cells, characteristic of osteoclast-like cells, at sites of trabecular (osteolysis) and cortical bone erosion in NADPH oxidase-deficient mice. Furthermore, we found increased osteoprotegerin ligand (OPGL/RANKL) expression in the inflamed synovium of p47phox^{-/-} mice, a key mediator in osteoclast differentiation.^{55,56} The NADPH oxidase complex, including the p47phox protein, is present in the ruffled border of osteoclasts actively resorbing bone.⁵⁷ Superoxide does not stimulate bone resorption directly but leads to hydrogen peroxide production, which in turn stimulates osteoclast formation and enhances bone-resorbing activity.⁵⁸ However, osteoclast from gp91 KO mice produces equal amounts of superoxide and these mice are not osteopetrotic, indicating normal bone turnover. Yang et al⁵⁹ showed that NOX4, a member of the newly discovered Nox/Duox family of NADPH oxidases in the osteoclasts generates superoxide and mediates bone resorption. Another contributor to the osteoclast-generated superoxide is TRAP. TRAP is a binuclear metalloenzyme that can react with hydrogen peroxide to generate ROS, which can destroy collagen and other proteins.⁶⁰ Physiologically, TRAP is required for normal bone matrix resorption, as mice lacking TRAP have disrupted endochondral ossification and develop mild osteopetrosis.⁶¹ In this study we found that TRAP-positive cells are present in the areas of bone resorption, and bone resorption occurred in the absence of functional NADPH oxidase. Our study showed that for full osteoclast activity leading to bone resorption, NADPH oxidase is not required.

It is clear from our study that in non-infectious joint inflammation elicited by an irritant (zymosan) or immune complexes, the NADPH oxidase-derived oxidants might well serve to self-limit the disease process. Future research to define the mechanism of the control of inflammation by this crucial inflammatory mediator should indicate novel pathways for therapeutic exploration.

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