Published in final edited form as:

Free Radic Biol Med. 2011 June 1; 50(11): 1517–1525. doi:10.1016/j.freeradbiomed.2011.02.027.

Genetic ablation of phagocytic NADPH oxidase in mice limits TNF α -induced inflammation in the lungs but not other tissues

Wei-Jian Zhang^{1,2,*}, Hao Wei¹, Ying-Tzang Tien³, and Balz Frei^{1,2,*}

¹ Linus Pauling Institute, Oregon State University, Corvallis, OR 97331, USA

² Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, USA

³ Histopathology Laboratory, School of Medicine, University of Washington, Seattle, WA 98195, USA

Abstract

In vitro and limited *in vivo* evidence suggests that reactive oxygen species derived from NADPH oxidases (NOX-ROS) play an important role in inflammatory responses by enhancing the activity of redox-sensitive cell signaling pathways and transcription factors. Here, we investigated the role of NOX-ROS in TNF α -induced acute inflammatory responses *in vivo*, using mice deficient in the gp91^{phox} (NOX2) or p47^{phox} subunits of NADPH oxidase. Age- and body weight-matched C57BL/6J wild-type (WT) and gp91^{phox} or p47^{phox} knockout mice were injected i.p. with 50 μ g TNF α /kg b.w. or saline vehicle control and sacrificed at various time points up to 24 hrs. Compared to WT mice, gp91^{phox} $-/-$ mice exhibited significantly diminished ($P < 0.05$) TNF α -induced acute inflammatory responses in the lungs but not other tissues, including heart, liver, and kidney, as evidenced by decreased activation of the redox-sensitive transcription factor, NF κ B, and decreased gene expression of interleukin (IL)-1 β , IL-6, TNF α , E-selectin, and other cellular adhesion molecules. Similar results were observed in p47^{phox} $-/-$ mice. Interestingly, decreased lung inflammation in knockout mice was accompanied by increased leukocyte infiltration into the lungs compared to other tissues. Our data suggest that phagocytic NOX-ROS signaling plays a critical role in promoting TNF α -induced, NF κ B-dependent acute inflammatory responses and tissue injury specifically in the lungs, which is effected by preferential leukocyte infiltration.

Keywords

NADPH oxidase; reactive oxygen species; redox-sensitive cell signaling; NF κ B activation; lung inflammation; leukocyte infiltration

INTRODUCTION

The NOX family of NADPH oxidases, a highly regulated, membrane-bound group of enzyme complexes found in a variety of phagocytic and non-phagocytic cells, is a major biological source of superoxide radicals and hydrogen peroxide (1). In phagocytic cells the core enzyme consists of five subunits: p40^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, and gp91^{phox} (NOX2). In addition, two low-molecular weight GTP-binding proteins, Rap 1A and rac1/2,

*Correspondence and reprint requests should be addressed to: Wei-Jian Zhang and Balz Frei, Linus Pauling Institute, Oregon State University, 571 Weniger Hall, Corvallis, OR 97331, Phone: (541) 737-5075, FAX: (541) 737-5077, weijian.zhang@oregonstate.edu and balz.frei@oregonstate.edu.

are involved in activation of the enzyme. Mice that are genetically deficient in gp91^{phox} or p47^{phox} exhibit a reduced capacity to generate superoxide radicals (2–4).

Activation of NADPH oxidases takes place either at the plasma membrane, leading to release of superoxide radicals into the extracellular environment, or at intracellular membranes, leading to local formation of superoxide radicals and hydrogen peroxide in intracellular compartments. Phagocytic NADPH oxidase, originally described in neutrophils and macrophages, serves a critical function in host-defense against invading pathogenic microorganisms. The non-phagocytic NADPH oxidases, found in smooth muscle cells, synoviocytes, chondrocytes, endothelial cells, and epithelial cells, produce smaller amounts of reactive oxygen species (ROS), which may play a critical role in cell signaling and normal cell growth and function (1). However, the mechanisms underlying intracellular ROS production and the relative concentrations and activities of NADPH oxidases in each of the non-phagocytic cell types have not been fully determined.

In recent years there has been increasing evidence indicating that ROS, and in particular hydrogen peroxide, act as mediators in cell signaling in a broad array of physiological and pathophysiological responses (1, 5, 6). The regulation of gene transcription of pro-inflammatory cytokines, chemokines, and cellular adhesion molecules has been shown to be mediated by reduction-oxidation (redox) sensitive signaling pathways and transcription factors, such as nuclear factor κ B (NF κ B) and activator protein-1 (AP-1) (7, 8). NF κ B activation leads to upregulation of a wide array of inflammatory genes critical for neutrophilic inflammation and host defense against infectious agents, and may also cause the systemic inflammatory response syndrome (9, 10). A variety of *in vitro* studies has suggested that ROS act as second messengers affecting NF κ B activation (11–13). Thus, it is possible that NADPH oxidase-derived ROS (NOX-ROS) play an important role in regulating cell signaling and host inflammatory responses in both phagocytic and non-phagocytic cells. However, there is limited and controversial evidence from *in vivo* studies to support this notion (3, 4, 14–21).

Tumor necrosis factor α (TNF α) is a major mediator of inflammation, immunity, and apoptosis, and has been implicated in the pathogenesis of a wide spectrum of human diseases (22, 23). It promotes inflammation by increasing blood neutrophil concentrations, enhancing expression of multiple inflammatory mediators, and inducing adhesion of neutrophils and monocytes to vascular endothelium and their subsequent tissue infiltration. Through binding to its cellular receptors, TNF α activates multiple signaling pathways (23, 24); ROS have been implicated in mediating TNF α signaling, although their source has not been fully established (5). Recent evidence indicates that NOX-ROS contribute to TNF α -induced NF κ B activation and adhesion molecule and IL-8 expression in cultured endothelial cells (14, 25–28). However, the role of NOX-ROS in cell signaling and gene expression in TNF α -induced acute inflammatory responses *in vivo* remain to be fully determined.

We have recently shown that genetic deficiency of gp91^{phox} or p47^{phox} does not diminish, but rather enhances, LPS-induced acute inflammatory responses *in vivo*, suggesting that NOX-ROS do not play a role in enhancing inflammation, but rather in its resolution (21). As the signaling pathways in TNF α -induced acute inflammation are distinct from those triggered by LPS, in the current study we investigated whether limiting NOX-ROS production by genetic deficiency of gp91^{phox} or p47^{phox} is associated with impairment or enhancement of TNF α -induced acute inflammatory responses *in vivo*.

MATERIALS AND METHODS

Animals and experimental procedures

Age- and body weight-matched female C57BL/6J WT, gp91^{phox}^{-/-}, and p47^{phox}^{-/-} mice at 10–12 weeks of age and weighing 20–22 g were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed under specific pathogen-free conditions and in a temperature and humidity-controlled environment (12-hr light/dark cycle) with unlimited access to tap water and food. Animals were acclimated for 7–10 days before initiation of experiments. All animal procedures were reviewed and approved by the Oregon State University Institutional Animal Care and Use Committee.

Wild-type, gp91^{phox}^{-/-}, and p47^{phox}^{-/-} mice were randomly assigned to receive i.p. injection of the vehicle HBSS (Sigma-Aldrich, St. Louis, MO) as controls, or 50 µg of murine recombinant TNFα (R&D Systems, Minneapolis, MN) per kg body weight (b.w.), and sacrificed 1, 3, 8, or 24 hrs after injections. Blood and various tissues were collected for further analysis.

Serum concentrations of inflammatory mediators

Serum concentrations of soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), and TNFα were measured by quantitative colorimetric ELISA kits from R&D Systems. The reported sensitivities of the assays are 5 pg/ml sVCAM-1, 29 pg/ml sICAM-1, 2 pg/ml MCP-1, and 5 pg/ml TNFα.

Tissue mRNA levels of inflammatory mediators

Total RNA was isolated from different tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was performed using the high capacity cDNA archive kit from Applied Biosystems (Foster City, CA). mRNA levels of E-selectin, VCAM-1, ICAM-1, MCP-1, TNFα, IL-1β, IL-6, myeloperoxidase (MPO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantitated by real-time PCR. All primers and probes were purchased as kits from Applied Biosystems. The assays are supplied as a 20x mixture of PCR primers and TaqMan minor groove binder 6-FAM dye labeled probes with a nonfluorescent quencher at the 3' end. TaqMan quantitative PCR (40 cycles of 95°C for 15 seconds and 60°C for 1 min) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in 96-well plates with the ABI Prism 7500 Sequence Detection System (Applied Biosystems). To obtain relative quantitation, two standard curves were constructed in each plate with one target gene and an internal control gene (GAPDH). Standard curves were generated by plotting the threshold cycle number values against the log of the amount of input cDNA and used to quantitate the expression of the various target genes and GAPDH gene in the same sample.

Bronchoalveolar lavage

Mice were sacrificed eight hours after HBSS (control) or TNFα injection. The left lung was tied off, and the right lung was lavaged by intratracheal injection of two sequential 0.6-ml aliquots of HBSS, followed by aspiration. Recovered fluid was pooled and centrifuged. Supernatants were preserved for measurement of total albumin concentration using an enzyme-linked immunoassay (Bethyl Labs, Montgomery, TX). The leukocyte pellet was resuspended in HBSS and cell numbers were counted using a hemocytometer.

Preparation and morphological evaluation of lungs

Mice were sacrificed eight hours after HBSS (control) or TNF α injections. The left lung was removed and fixed with 10% neutral-buffered formalin and embedded in paraffin. After dewaxing and rehydration, 5- μ m-thick paraffin sections were stained with hematoxylin and eosin. Neutrophils were counted from 10 randomly selected fields (20x magnification, 0.4 $\times$ 0.4 mm²) in each section. A total of 30 fields were screened from sections selected from the upper, middle, and lower parts of the left lung.

Western blots

Lung tissue samples were homogenized in 1 ml lysis buffer (Cell Signaling Technology, Danvers, MA) followed by centrifugation at 14,000 *g* and 4°C for 15 min. The supernatant containing solubilized proteins was recovered, frozen in aliquots, and stored at -80°C until analysis. Protein samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated overnight with mouse anti-MPO antibody (R&D systems) or rabbit anti- α -tubulin antibody (Cell Signaling), after blocking with 0.1% Tween 20 in Tris-buffered saline containing 5% skim milk powder. The membranes were incubated with HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-rabbit antibody (Cell Signaling) for 1 hr at room temperature. The proteins were detected by enhanced chemiluminescence (ECLTM, GE Healthcare, Buckinghamshire, UK).

Lung myeloperoxidase activity

Lung tissue samples were homogenized in 50 mM phosphate buffer (pH 7.4) and centrifuged at 10,000 *g* for 10 min at 4°C, and the resulting pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich). The suspension was subjected to three cycles of freezing and thawing and disrupted further by sonication (40 s). Subsequently, the samples were centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatant was used for measuring MPO activity with the Myeloperoxidase Activity Assay Kit from Northwest Life Science Specialties (Vancouver, WA). One unit of MPO activity is defined as the amount of enzyme that catalyzes the formation of 1 nM taurine chloramine from taurine at pH 6.5, 25°C, over 30 min in the presence of 100 mM chloride and 100 μ M H₂O₂. The MPO activity was normalized to the total protein concentration.

Activation of nuclear transcription factors

Nuclear extracts were prepared from different tissues using nuclear extract kits (Active Motif, Carlsbad, CA). For analysis of activation of nuclear transcription factors, ELISA-based assays (Active Motif) were used to determine the DNA binding activity of NF κ B (p65) and AP-1 (c-fos). The specificity of binding was confirmed by competition with either wild-type or mutant oligonucleotides. The wild-type oligonucleotides prevent NF κ B and AP-1 binding to the probe immobilized on the plates. Conversely, the mutated oligonucleotides should have no effect on NF κ B and AP-1 binding activity.

Statistical analysis

The data were calculated as means $\pm$ SEM and analyzed by unpaired student's *t*-test or ANOVA followed by Holm-Sidak post-hoc test. Statistical significance was set at *P*<0.05.

RESULTS

Genetic deficiency of gp91^{phox} or p47^{phox} does not diminish TNF α -induced increases in serum concentrations of inflammatory mediators

To assess the time-course of TNF α -induced acute, systemic inflammation in mice, serum levels of selected inflammatory mediators were measured up to 24 hrs after i.p. injection of TNF α (50 μ g/kg b.w.). As shown in Fig. 1, TNF α treatment caused a rapid increase in serum concentrations of sVCAM-1, sICAM-1, MCP-1, and TNF α : TNF α peaked at 1 hr, while the other inflammatory mediators were significantly increased at 3 hrs. sVCAM-1 and sICAM-1 remained elevated up to 24 hrs, while MCP-1 and TNF α returned to baseline at 8 hrs. Interestingly, these TNF α -induced changes in serum levels of inflammatory mediators were indistinguishable between gp91^{phox}^{-/-} and WT mice at all time points examined (Fig. 1). Similar results were observed in serum of p47^{phox}^{-/-} mice 3 hrs after TNF α injection (data not shown). These data suggest that NADPH oxidase activity is not required for normal TNF α -induced systemic inflammatory responses.

Genetic deficiency of gp91^{phox} or p47^{phox} significantly diminishes TNF α -induced upregulation of inflammatory gene expression in the lungs but not other tissues

To assess TNF α -induced gene expression of inflammatory mediators, their mRNA levels were measured in lung, heart, liver, kidney, small intestine, and spleen. As expected, lung mRNA levels of all inflammatory mediators increased in a time-dependent manner following TNF α administration to WT mice (Fig. 2). VCAM-1, ICAM-1, and IL-6 mRNA levels were increased substantially 1 hr after TNF α injection and remained elevated for at least 2 hrs before declining to baseline after 8 hrs. E-selectin, TNF α , and IL-1 β mRNA levels peaked at 1 hr and then rapidly declined at 3 hrs, returning to baseline after 8 hrs (Fig. 2).

Interestingly, TNF α -induced upregulation of inflammatory gene expression in lung was significantly diminished in gp91^{phox}^{-/-} mice compared to WT mice (Fig. 2). Hence, mRNA levels of E-selectin at 1, 3, and 24 hrs, IL-1 β at 1 hr, and VCAM-1, ICAM-1, TNF α , and IL-6 at 3 hrs were significantly lower in gp91^{phox}^{-/-} mice compared to WT mice ($P < 0.05$, $n = 4-5$). These data indicate that genetic deficiency of gp91^{phox} decreases TNF α -induced acute inflammatory responses in the lungs, consistent with the notion that NOX-ROS play an important role in this process.

Exposing WT animals to TNF α for 3 hrs strongly up-regulated expression of all inflammatory genes measured in all tissues examined (Table 1). Compared to WT mice, inflammatory gene expression was significantly lower in the lungs of gp91^{phox}^{-/-} and p47^{phox}^{-/-} mice (Figs. 2 and 3) but, interestingly, not any other tissues (Table 1). Hence, in gp91^{phox}^{-/-} mice, lung mRNA levels of E-selectin, VCAM-1, ICAM-1, IL-1 β , IL-6, and TNF α were diminished by 72%, 63%, 61%, 35%, 57%, and 51%, respectively ($P < 0.05$, $n = 4-5$) (Fig. 2). Similar results were observed in lung tissue of p47^{phox}^{-/-} mice (Fig. 3). However, lack of gp91^{phox} or p47^{phox} did not diminish TNF α -induced upregulation of these inflammatory genes in any other tissues (Table 1). To the contrary, TNF α -induced IL-6 and TNF α gene expression seemed to be further enhanced in liver of gp91^{phox}^{-/-} mice (Table 1A).

Genetic deficiency of gp91^{phox} or p47^{phox} attenuates TNF α -induced NF- κ B activation in the lungs but not other tissues

It is well documented that the redox-sensitive transcription factors, NF κ B and AP-1, play critical roles in TNF α -induced upregulation of many inflammatory genes. We observed that TNF α treatment increased NF κ B-DNA binding activity in a time-dependent manner in the

lungs of WT mice. NF κ B binding activity increased substantially 1 hr after TNF α injection, peaked at 3 hrs, and returned to baseline after 8 hrs (Fig. 4A). Interestingly, TNF α -induced NF κ B activation was significantly lower at both the 1 and 3-hr time points (by 43% and 57%, respectively) in the lungs of gp91^{phox}^{-/-} mice compared to WT mice ($P < 0.05$, $n = 4-5$) (Fig. 4A and B). Treatment of WT mice with TNF α for 3 hrs also strongly induced NF κ B activity in various other tissues (Fig. 4B and C). However, TNF α -induced NF κ B activation was not significantly lower in non-lung tissues of gp91^{phox}^{-/-} mice (Fig. 4B), as well as p47^{phox}^{-/-} mice (Fig. 4C), compared to WT mice ($P < 0.05$, $n = 4-5$). Lack of gp91^{phox} and p47^{phox} did not, however, diminish TNF α -induced activation of AP-1 in all tissues examined compared to WT mice (data not shown).

Genetic deficiency of gp91^{phox} attenuates TNF α -induced acute lung injury

Bronchoalveolar lavage (BAL) fluid was used for assay of total albumin concentration and total neutrophil count as independent assessments of acute lung injury and alveolar inflammation. The TNF α -induced increase in albumin concentration in BAL fluid was significantly less in gp91^{phox}^{-/-} mice compared to WT mice (231 ± 23 μ g/ml v.s. 306 ± 20 μ g/ml, $P < 0.05$, $n = 5$). In contrast, a significant increase in lavage neutrophil count was observed in TNF α -treated gp91^{phox}^{-/-} mice compared to WT mice ($21.2 \pm 3.3 \times 10^4$ cells v.s. $7.4 \pm 0.9 \times 10^4$ cells, $P < 0.05$, $n = 5$), while there was no significant difference between HBSS-treated (control) gp91^{phox}^{-/-} mice and WT mice ($4.6 \pm 1.0 \times 10^4$ cells v.s. $4.4 \pm 0.3 \times 10^4$ cells, $n = 5$).

Genetic deficiency of gp91^{phox} or p47^{phox} does not affect TNF α -induced neutrophil infiltration into the lungs

Since polymorphonuclear neutrophils (PMNs) and other phagocytic cells, such as monocyte-macrophages, play critical roles in acute inflammation and tissue injury, the specific enzyme marker, myeloperoxidase, was measured in various tissues after TNF α challenge. As shown in Fig. 5A, TNF α treatment induced time-dependent upregulation of MPO gene expression in the lungs of both gp91^{phox}^{-/-} and WT mice, which peaked at 3 hrs and remained elevated for up to 8 hrs, and then declined after 24 hrs. MPO mRNA levels also increased in other tissues, such as heart, liver, and kidney, albeit to a much lesser degree than in lung (Fig. 5B and C). Interestingly, TNF α -induced changes in MPO gene expression were indistinguishable between gp91^{phox}^{-/-}, p47^{phox}^{-/-}, and WT mice (Fig. 5A, B, and C).

Consistent with increased MPO message levels, MPO protein levels (Fig. 5D) and activity (Fig. 5E) in lung also were significantly increased 8 hrs after TNF α treatment, and indistinguishable between gp91^{phox}^{-/-} and WT mice. Furthermore, morphological analysis showed significantly increased numbers of neutrophils in the lungs of gp91^{phox}^{-/-} mice compared to WT mice ($P < 0.05$) (Fig. 6A, B, and E). TNF α treatment further markedly induced accumulation of abundant interstitial neutrophils in both gp91^{phox}^{-/-} and WT mice; however, there was no significant difference between these two groups of animals (Fig. 6C, D, and E). These data indicate that the lungs are the main target for activated PMNs, and genetic deficiency of phagocytic NADPH oxidase does not impair PMN chemotaxis during TNF α -induced acute inflammation.

DISCUSSION

Increased ROS production and oxidative stress contribute to cellular and tissue injury during the development of inflammatory diseases (1, 5, 10, 29, 30). Evidence also suggests that ROS modulate a wide variety of cell-signaling processes (1, 5, 6). Yet, the relationship between oxidative stress and the pathology of inflammatory diseases is not fully understood, largely because of limited understanding of the mechanisms by which ROS function as

signals in physiological or pathophysiological settings. Recent studies, though still controversial, have suggested that NOX-ROS contribute to oxidative stress and may serve as cell signaling molecules enhancing acute inflammatory responses and tissue injury (1, 3, 4, 14–21). However, the role of NOX-ROS in TNF α -induced cell signaling and acute inflammatory responses *in vivo* is less clear.

In the present study, we found that genetic deficiency of gp91^{phox} or p47^{phox} significantly attenuated TNF α -induced NF κ B activation, upregulation of inflammatory gene expression, and acute tissue injury in the lungs but not other tissues of mice. These effects were associated with higher levels of leukocyte infiltration into the lungs compared to other tissues. Therefore, our data provide new evidence that both NOX-ROS dependent and independent pathways are involved in TNF α signaling, and especially phagocytic NOX-ROS play an important role in TNF α -induced acute inflammatory responses in the lungs.

It is now widely accepted that increased ROS production during acute lung inflammation largely originates from phagocytic NOX2-expressing cells within the respiratory tract, including alveolar macrophages and other resident or infiltrating inflammatory-immune cells, including neutrophils, eosinophils, and lymphocytes. However, direct evidence that NOX2 activation contributes to respiratory disease pathology is sparse. Although NOX2 in phagocytic cells primarily functions in host defense by generating antimicrobial ROS, activation of NOX2 in neutrophils was also found to regulate intracellular signaling pathways that modulate neutrophil (29) and endothelial (14) inflammatory gene expression via NF κ B activation. Similarly, stimulation of macrophage NOX2, which produces markedly less ROS compared to neutrophils, has been linked to regulation of NF κ B and AP-1 signaling and production of pro-inflammatory cytokines (30). In fact, accumulation of infiltrating neutrophils is a key event in the onset and development of inflammation and resultant host tissue injury in the lungs.

Our data showed that treatment of animals with TNF α strongly increased gene expression, protein levels, and enzymatic activity of MPO, a biomarker of neutrophils, in the lungs but not other tissues, such as heart, liver, and kidney. Furthermore, TNF α -induced increases of lung MPO and accumulation of interstitial neutrophils in the lungs were indistinguishable between NADPH oxidase knockout mice and WT mice. However, deficiency of NOX2 or p47^{phox} significantly limited TNF α -induced activation of NF κ B and expression of inflammatory genes. These data indicate that lung is the main target tissue for activated neutrophils during the acute phase of inflammation, and phagocytic NADPH oxidase critically contributes to TNF α -induced inflammatory responses in the lungs. These findings are consistent with previous studies showing that neutrophil NADPH oxidase plays a significant role in the mechanism of TNF α -induced NF κ B activation and ICAM-1 expression in the lungs of p47^{phox}–/– and WT mice (14).

Acute lung injury is characterized clinically by derangements in lung vascular permeability, which is a cardinal feature of inflammation and the initial cause of the profound physiological abnormalities observed in patients with acute lung injury (31). In the present study we found in our murine models that deficiency of NOX2 significantly attenuated the TNF α -induced increase in total albumin concentration in BAL fluid, which reflects leakage of protein into the alveolar space across both the vascular and epithelial barriers. The cumulative effects of NOX2 knock-out on TNF α -induced lung injury could account for both a decrease in vascular leakage and a reduction in inflammation characterized by reduced lung NF κ B activation and inflammatory gene expression, despite the fact that lung interstitial neutrophil counts were similar between TNF α -treated NOX2 knock-out and WT mice. These data indicate that neutrophils lacking a functional NADPH oxidase have normal chemotactic activity but a reduced ability to mount an inflammatory response to TNF α in the

lungs, suggesting that phagocytic NADPH oxidase critically contributes to TNF α -induced acute lung injury.

Although it is known that ROS affect a number of signaling pathways by activating or inactivating specific protein kinases and phosphatases, respectively (1), the signaling molecules specifically targeted by ROS in the NF κ B pathway have not been unequivocally identified. While some studies have found that ROS act as second messengers and are involved in TNF α signaling and NF κ B activation (7–12, 14), others have not found such evidence (32, 34) or even inhibition of NF κ B activation by hydrogen peroxide (33). It is likely that ROS-mediated NF κ B activation is restricted to certain cell types and incubation conditions, such as the types and concentrations of ROS or pro-inflammatory cytokines used. In addition, most of the evidence for a role of ROS in NF κ B activation is derived from *in vitro* study, and *in vivo* evidence is scant. Our *in vivo* data suggest that TNF α -induced NF κ B activation in lung is indeed enhanced by NOX-ROS (Scheme 1), as it was significantly diminished in gp91^{phox}–/– and p47^{phox}–/– mice compared to WT mice. In contrast, TNF α -induced activation of AP-1, another critical transcription factor for inflammatory gene expression, was not affected by NADPH oxidase deficiency.

Our data further suggest that in non-lung tissues, NOX-ROS do not play a significant role in TNF α -mediated NF κ B activation and inflammatory gene expression. In this NOX-ROS independent pathway, TNF α binding to the TNF receptor 1 (TNF-R1) triggers a series of intracellular signals causing activation of I κ B kinase (IKK), which results in phosphorylation and subsequent ubiquitylation and degradation of I κ B and translocation of NF κ B to the nucleus (Scheme 1). *In vitro* evidence suggests that TNF-R1 stimulation also induces NADPH oxidase activity via PKC-mediated phosphorylation of p47^{phox} and activation of Rac1, thereby increasing ROS production and enhancing IKK and NF κ B activation (35, 36). However, our data suggest that this mechanism does not play a significant role *in vivo*, unless extensive neutrophil infiltration occurs and ROS are generated in large quantities by NOX2, such as in the lungs of TNF α -exposed mice. It is possible that these PMN-derived ROS, in particular hydrogen peroxide which is relatively long-lived and membrane-permeable are transmitted into resident tissue cells and serve as signaling molecules activating IKK and NF κ B (Scheme 1). These exogenous ROS generated by NOX2 of infiltrating PMNs seem to be more critical in mediating TNF α -induced inflammatory responses *in vivo* than ROS signals generated endogenously by NOX in resident macrophages or non-phagocytic cells (14). Nevertheless, we cannot exclude the possibility that ROS generated from other cellular sources, such as xanthine oxidase, mitochondria, or cytochrome P450, may have played a role in TNF α -induced cell signaling and inflammation in non-lung tissues (5, 37).

It should be noted that the results from previous studies for a role of NOX-ROS in lung inflammation *in vivo* are still controversial. It has been shown that LPS or *Pseudomonas aeruginosa*-induced NF κ B activation in lung tissue was impaired in p47^{phox}–/– and gp91^{phox}–/– mice (3, 4, 16), and LPS-induced lung inflammation in Nrf2–/– mice was significantly reduced by genetic ablation of gp91^{phox} (38), supporting an important role of NOX-ROS in promoting inflammation. However, other investigators found no differences between p47^{phox}–/– and WT mice in LPS-induced lung injury, alveolar damage, and infiltration of neutrophils and monocytes (17), and no amelioration of pulmonary damage in *Pneumocystis*-infected gp91^{phox}–/– mice compared to WT mice (18). In fact, more recent studies found that genetic deficiency of NADPH oxidase was in many cases associated with enhanced lung inflammation and injury (19–21, 39–44), indicating that NADPH oxidase has beneficial functions in limiting inflammatory-immune responses or enhancing their resolution. For example, infection of gp91^{phox}–/– mice with the yeast *C. neoformans* or the influenza virus was found to result in increased Th1-skewed inflammation and granuloma

formation (42, 43). Deficiency of gp91^{phox} was also reported to markedly increase pulmonary cytokine and chemokine expression and neutrophil recruitment in a mouse model of pneumococcal pneumonia (20), and lung levels of pro-inflammatory mediators, such as keratinocyte chemoattractant, IL-6, MCP-1, and TNF α , were significantly increased in both gp91^{phox} and p47^{phox}-deficient mice in response to cigarette smoke exposure (19). Consistent with these data, we have recently shown that genetic deficiency of NADPH oxidase does not diminish, but rather enhances, LPS-induced acute lung inflammatory responses (21). These results might also be related to clinical observations in patients with the X-linked form of chronic granulomatous disease (gp91^{phox} deficiency), who are more prone to infection and present with hyper-inflammatory conditions (45–47).

However, in the current study we showed that reduced phagocytic NOX-ROS diminished TNF α -induced acute lung inflammatory responses and lung injury, which is opposite to our observations in mice exposed to LPS instead of TNF α (21). These apparently discrepant results highlight the above notion that the role of ROS in cell signaling and inflammatory gene expression can vary widely depending on, *e.g.*, the pro-inflammatory cytokine or bacterial agent used and the affected tissue or cell type. More studies are needed to increase our understanding of NOX-ROS-mediated cell signaling, in particular the cellular location of NOX and its association with target cells or proteins. In conclusion, our data suggest that phagocytic NOX-ROS signaling plays a critical role in promoting TNF α -induced, NF κ B-dependent acute inflammatory responses and tissue injury in the lungs, whereas NOX-ROS independent pathways play a dominant role in non-lung tissues.

Acknowledgments

This publication was made possible by Grant Number P01 AT002034 (BF/WJZ) from the National Center for Complementary and Alternative Medicine (NCCAM). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCCAM or the National Institutes of Health.

LIST OF ABBREVIATIONS

AP-1	activator protein-1
BAL	Bronchoalveolar lavage
b.w.	body weight
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ICAM-1	intercellular adhesion molecule-1
IKK	I κ B kinase
IL	interleukin
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MPO	myeloperoxidase
NFκB	nuclear factor κ B
NOX-ROS	NADPH oxidase-derived ROS
PMNs	polymorphonuclear neutrophils
Redox	reduction-oxidation
ROS	reactive oxygen species

sICAM-1	soluble intercellular adhesion molecule-1
sVCAM-1	soluble vascular cell adhesion molecule-1
TNFα	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule-1
WT	wild-type

References

1. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev.* 2007; 87:245–313. [PubMed: 17237347]
2. Pollock JD, Williams DA, Gifford MA, Li LL, Du X, Fisherman J, Orkin SH, Doerschuk CM, Dinanuer MC. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet.* 1995; 9:202–9. [PubMed: 7719350]
3. Gao XP, Standiford TJ, Rahman A, Newstead M, Holland SM, Dinanuer MC, Liu QH, Malik AB. Role of NADPH oxidase in the mechanism of lung neutrophil sequestration and microvessel injury induced by Gram-negative sepsis: studies in $p47^{phox-/-}$ and $gp91^{phox-/-}$ mice. *J Immunol.* 2002; 168:3974–3982. [PubMed: 11937554]
4. Koay MA, Christman JW, Segal BH, Venkatakrishnan A, Blackwell TR, Holland SM, Blackwell TS. Impaired pulmonary NF-kappaB activation in response to lipopolysaccharide in NADPH oxidase-deficient mice. *Infect Immun.* 2001; 69:5991–5996. [PubMed: 11553535]
5. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev.* 2002; 82:47–95. [PubMed: 11773609]
6. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol.* 2000; 279:L1005–1028. [PubMed: 11076791]
7. Janssen-Heininger YM, Poynter ME, Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radic Biol Med.* 2000; 28:1317–1327. [PubMed: 10924851]
8. Liu H, Colavitti R, Rovira II, Finkel T. Redox-dependent transcriptional regulation. *Circ Res.* 2005; 97:967–74. [PubMed: 16284189]
9. Pahl H. Activators and target genes of Rel/NF-kB transcription factors. *Oncogene.* 1999; 18:6853–6866. [PubMed: 10602461]
10. Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol.* 2006; 290:L622–L645. [PubMed: 16531564]
11. Li N, Karin M. Is NF-kB the sensor of oxidative stress? *FASEB J.* 1999; 13:1137–1143. [PubMed: 10385605]
12. Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kB activation. *Free Radical Biol Med.* 1997; 22:1115–1126. [PubMed: 9034250]
13. Park HS, Jung HY, Park EY, Kim J, Lee WJ, Bae YS. Cutting edge: Direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kappaB. *J Immunol.* 2004; 173:3589–3593. [PubMed: 15356101]
14. Fan J, Frey RS, Rahman A, Malik AB. Role of neutrophil NADPH oxidase in the mechanism of tumor necrosis factor-alpha -induced NF-kappa B activation and intercellular adhesion molecule-1 expression in endothelial cells. *J Biol Chem.* 2002; 277:3404–3411. [PubMed: 11729200]
15. Kono H, Rusyn I, Yin M, Gabele E, Yamashina S, Dikalova A, Kadiiska MB, Connor HD, Mason RP, Segal BH, Bradford BU, Holland SM, Thurman RG. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *J Clin Invest.* 2000; 106:867–872. [PubMed: 11018074]
16. Sadikot RT, Zeng H, Yull FE, Li B, Cheng D-s, Kernodle DS, Jansen ED, Contag CH, Segal BH, Holland SM, Blackwell TS, Christman JW. $p47^{phox}$ deficiency impairs NF-kappa-B activation

- and host defense in *Pseudomonas pneumonia*. *J Immunol*. 2004; 172:1801–1808. [PubMed: 14734763]
17. Sato K, Kadiiska MB, Ghio AJ, Corbett J, Fann YC, Holland SM, Thurman RG, Mason RP. In vivo lipid-derived free radical formation by NADPH oxidase in acute lung injury induced by lipopolysaccharide: a model for ARDS. *FASEB J*. 2002; 16:1713–20. [PubMed: 12409313]
 18. Swain SD, Wright TW, Degel PM, Gigliotti F, Harmsen AG. Neither neutrophils nor reactive oxygen species contribute to tissue damage during *Pneumocystis pneumonia* in mice. *Infect Immun*. 2004; 72:5722–32. [PubMed: 15385471]
 19. Yao H, Edirisinghe I, Yang SR, Rajendrasozhan S, Kode A, Caito S, Adenuga D, Rahman I. Genetic ablation of NADPH oxidase enhances susceptibility to cigarette smoke-induced lung inflammation and emphysema in mice. *Am J Pathol*. 2008; 172:1222–1237. [PubMed: 18403597]
 20. Marriott HM, Jackson LE, Wilkinson TS, Simpson AJ, Mitchell TJ, Buttle DJ, Cross SS, Ince PG, Hellewell PG, Whyte MK, Dockrell DH. Reactive oxygen species regulate neutrophil recruitment and survival in pneumococcal pneumonia. *Am J Respir Crit Care Med*. 2008; 177:887–895. [PubMed: 18202350]
 21. Zhang WJ, Wei H, Frei B. Genetic deficiency of NADPH oxidase does not diminish, but rather enhances, LPS-induced acute inflammatory responses in vivo. *Free Radic Biol Med*. 2009; 46:791–798. [PubMed: 19124074]
 22. Beutler B, Cerami A. The history, properties, and biological effects of cachectin. *Biochemistry*. 1988; 27:7575–7582. [PubMed: 3061461]
 23. Vilcek J, Lee TH. Tumor necrosis factor: new insights into the molecular mechanisms of its multiple actions. *J Biol Chem*. 1991; 266:7313–7316. [PubMed: 1850405]
 24. Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science*. 2002; 296:1634–1635. [PubMed: 12040173]
 25. Hashimoto SY, Gon Y, Matsumoto K, Takeshita I, Horie T. N-acetylcysteine attenuates TNF- α -induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells. *Br J Pharmacol*. 2001; 132:270–276. [PubMed: 11156586]
 26. Rahman A, Kefer J, Bando M, Niles WD, Malik AB. E-selectin expression in human endothelial cells by TNF- α -induced oxidant generation and NF- κ B activation. *Am J Physiol (Lung Cell Mol Physiol)*. 1998; 275:L-533–L-544.
 27. Li JM, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: role of p47phox phosphorylation and binding to TRAF4. *Mol Cell Biol*. 2005; 25:2320–30. [PubMed: 15743827]
 28. Gu Y, Xu YC, Wu RF, Souza RF, Nwariaku FE, Terada LS. TNF α activates c-Jun amino terminal kinase through p47(phox). *Exp Cell Res*. 2002; 272:62–74. [PubMed: 11740866]
 29. Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic Biol Med*. 2007; 42:153–164. [PubMed: 17189821]
 30. Forman HJ, Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am J Respir Crit Care Med*. 2002; 166:S4–S8. [PubMed: 12471082]
 31. Matthay MA. Severe sepsis a new treatment with both anticoagulant and antiinflammatory properties. *N Engl J Med*. 2001; 344:759–762. [PubMed: 11236781]
 32. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K. Evidence that reactive oxygen species do not mediate NF-B activation. *EMBO J*. 2003; 22:3356–3366. [PubMed: 12839997]
 33. Korn SH, Wouters EF, Vos N, Janssen-Heininger YM. Cytokine-induced activation of nuclear factor- κ B is inhibited by hydrogen peroxide through oxidative inactivation of I B kinase. *J Biol Chem*. 2001; 276:35693–35700. [PubMed: 11479295]
 34. Bowie A, O'Neill LAJ. Oxidative stress and nuclear factor- κ B activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol*. 2000; 59:13–23. [PubMed: 10605930]
 35. Chen X-L, Zhang Q, Zhao R, Ding X, Tummala PE, Medford RM. Rac1 and superoxide are required for the expression of cell adhesion molecules induced by tumor necrosis factor- α in endothelial cells. *J Pharmacol Exp Ther*. 2003; 305:573–580. [PubMed: 12606638]

36. Li JM, Mullen AM, Yun S, Wientjes F, Brouns GY, Thrasher AJ, Shah AM. Essential role of the NAD(P)H oxidase subunit p47(phox) in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor- α . *Circ Res*. 2002; 90:143–50. [PubMed: 11834706]
37. Zhang DX, Gutterman DD. Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. *Am J Physiol Heart Circ Physiol*. 2007; 292:H2023–H2031. [PubMed: 17237240]
38. Kong X, Thimmulappa R, Kombairaju P, Biswal S. NADPH oxidase-dependent reactive oxygen species mediate amplified TLR4 signaling and sepsis-induced mortality in Nrf2-deficient mice. *J Immunol*. 2010; 185:569–77. [PubMed: 20511556]
39. Segal BH, Davidson BA, Hutson AD, Russo TA, Holm BA, Mullan B, Habitzruther M, Holland SM, Knight PR 3rd. Acid aspiration-induced lung inflammation and injury are exacerbated in NADPH oxidase-deficient mice. *Am J Physiol Lung Cell Mol Physiol*. 2007; 292:L760–768. [PubMed: 17114280]
40. Kassim SY, Fu X, Liles WC, Shapiro SD, Parks WC, Heinecke JW. NADPH oxidase restrains the matrix metalloproteinase activity of macrophages. *J Biol Chem*. 2005; 280:30201–30205. [PubMed: 15983040]
41. Morgenstern DE, Gifford MA, Li LL, Doerschuk CM, Dinauer MC. Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defense and inflammatory response to *Aspergillus fumigatus*. *J Exp Med*. 1997; 185:207–218. [PubMed: 9016870]
42. Snelgrove RJ, Edwards L, Williams AE, Rae AJ, Hussell T. In the absence of reactive oxygen species, T cells default to a Th1 phenotype and mediate protection against pulmonary *Cryptococcus neoformans* infection. *J Immunol*. 2006; 177:5509–5516. [PubMed: 17015737]
43. Snelgrove RJ, Edwards L, Rae AJ, Hussell T. An absence of reactive oxygen species improves the resolution of lung influenza infection. *Eur J Immunol*. 2006; 36:1364–1373. [PubMed: 16703568]
44. Segal BH, Han W, Bushey JJ, Joo M, Bhatti Z, Feminella J, Dennis CG, Vethanayagam RR, Yull FE, Capitano M, Wallace PK, Minderman H, Christman JW, Sporn MB, Chan J, Vinh DC, Holland SM, Romani LR, Gaffen SL, Freeman ML, Blackwell TS. NADPH oxidase limits innate immune responses in the lungs in mice. *PLoS One*. 2010; 5:e963.
45. Bylund J, MacDonald KL, Brown KL, Mydel P, Collins LV, Hancock RE, Speert DP. Enhanced inflammatory responses of chronic granulomatous disease leukocytes involve ROS-independent activation of NF- κ B. *Eur J Immunol*. 2007; 37:1087–1096. [PubMed: 17330823]
46. Kobayashi SD, Voyich JM, Braughton KR, Whitney AR, Nauseef WM, Malech HL, DeLeo FR. Gene expression profiling provides insight into the pathophysiology of chronic granulomatous disease. *J Immunol*. 2004; 172:636–643. [PubMed: 14688376]
47. Schappi MG, Jaquet V, Belli DC, Krause KH. Hyperinflammation in chronic granulomatous disease and anti-inflammatory role of the phagocyte NADPH oxidase. *Semin Immunopathol*. 2008; 30:255–271. [PubMed: 18509648]

Fig. 1. Time course of TNF α -induced changes in serum levels of sVCAM-1 (A), sICAM-1 (B), MCP-1 (C), and TNF α (D) in WT and gp91^{phox}^{-/-} mice

Wild-type (solid circles) and gp91^{phox}^{-/-} mice (open circles) were injected i.p. with HBSS (0-time control) or TNF α (50 μ g/kg b.w.) as described under Materials and Methods. At the indicated time points after TNF α injection, the animals were sacrificed and blood was collected. Serum sVCAM-1, sICAM-1, MCP-1, and TNF α were measured by ELISA. Data shown are mean values $\pm$ SEM of four to five animals. No statistically significant differences between WT and gp91^{phox}^{-/-} mice were observed.

Fig. 2. Time course of TNF α -induced changes in mRNA levels of inflammatory mediators in the lungs of WT and gp91^{phox}^{-/-} mice

Wild-type (solid circles) and gp91^{phox}^{-/-} mice (open circles) were injected i.p. with HBSS (0-time control) or TNF α (50 μ g/kg b.w.) as described under Materials and Methods. At the indicated time points after TNF α injection, the animals were sacrificed and total RNA was isolated from the lungs. Real-time quantitative PCR analysis was performed for VCAM-1, ICAM-1, E-selectin, TNF α , IL-1 β , IL-6, and GAPDH mRNA. Data are expressed as percentage of GAPDH used as the internal control gene and are presented as mean values $\pm$ SEM of four to five animals. * P <0.05 compared to WT mice.

Fig. 3. TNF α -induced changes in mRNA levels of inflammatory mediators in the lungs of WT and p47^{phox}^{-/-} mice

Wild-type (black bars) and p47^{phox}^{-/-} mice (grey bars) were injected i.p. with HBSS (control) or TNF α (50 μ g/kg b.w.) as described under Materials and Methods. Three hours after injection, the animals were sacrificed and total RNA was isolated from the lungs. Real-time quantitative PCR analysis was performed for E-selectin, VCAM-1, ICAM-1, MCP-1, TNF α , and GAPDH mRNA. Data shown are from TNF α -treated groups, expressed as percentage of GAPDH used as the internal control gene, and presented as mean values $\pm$ SEM of five animals. * P <0.05 compared to WT mice.

Fig. 4. TNF α -induced NF κ B activation in the lungs and other tissues of WT, gp91^{phox}^{-/-}, and p47^{phox}^{-/-} mice

Wild-type and gp91^{phox}^{-/-} or p47^{phox}^{-/-} mice were injected i.p. with HBSS (control) or TNF α (50 μ g/kg b.w.) as described under Materials and Methods. At the indicated time points (panel A), or three hours (panels B and C) after TNF α injection, the animals were sacrificed and nuclear extracts were isolated. NF κ B (p65)-DNA binding activity was quantified by ELISA. Data shown are from TNF α -treated groups and presented as mean values $\pm$ SEM of four to five animals. * P <0.05 compared to WT mice.

Fig. 5. TNF α -induced changes in myeloperoxidase mRNA and protein levels and enzyme activity in the lungs and other tissues of WT, gp91^{phox}^{-/-}, and p47^{phox}^{-/-} mice

Wild-type, gp91^{phox}^{-/-}, and p47^{phox}^{-/-} mice were injected i.p. with HBSS (0-time control) or TNF α (50 μ g/kg b.w.) as described under Materials and Methods. At the indicated time points (panel A) or three hours (panels B and C) after TNF α injection, the animals were sacrificed and total RNA was isolated from lung (panel A) and other tissues (panels B and C). Real-time quantitative PCR analysis was performed for MPO and GAPDH mRNA. After normalization to GAPDH, the results for the MPO gene were expressed as fold of HBSS-treated control. For panel D, eight hours after TNF α injection, the animals were sacrificed and lung homogenates were prepared. MPO protein was analyzed by Western blot as described under Material and Methods. Equal sample loading was determined by quantitation of protein as well as reprobing the membranes for α -tubulin. For panel E, eight hours after TNF α injection, the animals were sacrificed and lung homogenates were prepared and MPO activity was assayed as described under Materials and Methods. Data shown are mean values $\pm$ SEM of four to five animals.

Fig. 6. TNF α -induced neutrophil infiltration into the lungs of WT and gp91^{phox}-/- mice
Representative lung tissue sections obtained from WT mice treated with HBSS (A) or TNF α (C) and from gp91^{phox}-/- mice treated with HBSS (B) or TNF α (D) were stained with hematoxylin and eosin (20x magnification), and the infiltrated interstitial neutrophils were counted (E) as described under Materials and Methods. Data shown are mean values $\pm$ SEM of four to five animals. * P <0.05 compared to WT mice, # P <0.05 compared to HBSS-treated control mice.

Scheme 1. Diagram depicting the NOX-ROS dependent and NOX-ROS independent pathways leading to activation of NFκB

In the NOX-ROS independent pathway (left), TNFα binds to the TNF receptor 1 (TNF-R1) located on the plasma membrane of target cells, thus activating IκB kinase (IKK). Activated IKK phosphorylates IκB, leading to its ubiquitylation and subsequent degradation. This releases NFκB and allows its translocation to the nucleus, where it binds to DNA promoter regions and induces inflammatory gene transcription. TNFα binding to TNF-R1 may also induce phosphorylation of p47^{phox} and activation of Rac1, which activates NADPH oxidase, thus increasing intracellular production of ROS and enhancing NFκB activation. The results presented in this paper do not support the notion that NOX-ROS play a significant role in TNFα-induced NFκB activation in non-lung tissues *in vivo* (arrow marked "?"). In the NOX-ROS dependent pathway (right), TNFα binds to TNF-R1 located on the plasma membrane of PMNs, which in turn activates NOX2 and produces extracellular ROS. The ROS from PMNs, in particular hydrogen peroxide, diffuse into target cells and serve as signaling molecules for NFκB activation, thereby enhancing acute inflammation in the lungs of TNFα-exposed mice.

Genetic deficiency of gp91^{phox} (A) or p47^{phox} (B) does not diminish TNF α -induced inflammatory gene expression in non-lung tissues

Wild-type, gp91^{phox}−/−, and p47^{phox}−/− mice were injected i.p. with HBSS (control) or TNF α (50 μ g/kg b.w.) as described under Materials and Methods. Three hours after injection, the animals were sacrificed and total RNA was isolated. Real-time quantitative PCR analysis was performed for E-selectin, VCAM-1, ICAM-1, IL-6, MCP-1, TNF α , and GAPDH mRNA. Data shown are only from TNF α -treated groups, expressed as percentage of GAPDH used as the internal control gene, and presented as mean values $\pm$ SEM of four to five animals.

Table 1A

		Heart	Liver	Kidney	Small Intestine	Spleen
E-selectin	WT	208 $\pm$ 27	75 $\pm$ 14	373 $\pm$ 106	384 $\pm$ 149	652 $\pm$ 209
	gp91 ^{−/−}	171 $\pm$ 31	80 $\pm$ 12	406 $\pm$ 56	303 $\pm$ 52	548 $\pm$ 68
VCAM-1	WT	122 $\pm$ 8	108 $\pm$ 5	87 $\pm$ 17	173 $\pm$ 31	140 $\pm$ 14
	gp91 ^{−/−}	110 $\pm$ 16	119 $\pm$ 8	92 $\pm$ 9	157 $\pm$ 25	139 $\pm$ 12
ICAM-1	WT	110 $\pm$ 6	84 $\pm$ 6	80 $\pm$ 23	225 $\pm$ 55	165 $\pm$ 32
	gp91 ^{−/−}	96 $\pm$ 11	97 $\pm$ 8	70 $\pm$ 8	187 $\pm$ 29	193 $\pm$ 15
IL-6	WT	252 $\pm$ 58	136 $\pm$ 16	41 $\pm$ 18	180 $\pm$ 48	212 $\pm$ 60
	gp91 ^{−/−}	228 $\pm$ 26	345 $\pm$ 43*	32 $\pm$ 9	191 $\pm$ 51	216 $\pm$ 29
MCP-1	WT	185 $\pm$ 25	90 $\pm$ 14	71 $\pm$ 22	315 $\pm$ 101	464 $\pm$ 127
	gp91 ^{−/−}	162 $\pm$ 29	129 $\pm$ 16	84 $\pm$ 9	386 $\pm$ 81	448 $\pm$ 56
TNFα	WT	170 $\pm$ 21	97 $\pm$ 10	71 $\pm$ 18	385 $\pm$ 173	223 $\pm$ 43
	gp91 ^{−/−}	152 $\pm$ 25	160 $\pm$ 13*	67 $\pm$ 8	128 $\pm$ 18	163 $\pm$ 21

Table 1B

		Heart	Aorta	Liver	Kidney
VCAM-1	WT	139 $\pm$ 21	131 $\pm$ 13	107 $\pm$ 6	99 $\pm$ 3
	p47 ^{−/−}	114 $\pm$ 22	122 $\pm$ 19	123 $\pm$ 11	103 $\pm$ 9
ICAM-1	WT	110 $\pm$ 16	105 $\pm$ 4	107 $\pm$ 8	113 $\pm$ 9
	p47 ^{−/−}	110 $\pm$ 17	86 $\pm$ 16	118 $\pm$ 15	143 $\pm$ 59
MCP-1	WT	162 $\pm$ 49	130 $\pm$ 14	109 $\pm$ 7	123 $\pm$ 8
	p47 ^{−/−}	151 $\pm$ 50	134 $\pm$ 23	141 $\pm$ 19	106 $\pm$ 12
TNFα	WT	206 $\pm$ 39	113 $\pm$ 17	125 $\pm$ 16	106 $\pm$ 5
	p47 ^{−/−}	201 $\pm$ 36	99 $\pm$ 21	200 $\pm$ 39	113 $\pm$ 7

* $P < 0.05$ compared to WT mice.