

Leukocyte nicotinamide adenine dinucleotide phosphate-reduced oxidase is required for isocyanate-induced lung inflammation

Si-Yen Liu, PhD,^a Wei-Zhi Wang, BS,^b Chia-Liang Yen, MS,^a Ming-Yi Tsai, MS,^c Pei-Wen Yang, BS,^c Jiu-Yao Wang, MD, PhD,^{a,d} Chun-Yi Ho,^c and Chi-Chang Shieh, MD, PhD^{a,c,d} Tainan, Taiwan

Background: Isocyanates are low-molecular-weight compounds noted for inducing occupational and environmental asthma. Isocyanate-induced lung disease, an oxidant stress-dependent pulmonary inflammation, is the leading cause of occupational asthma.

Objectives: To address the role of leukocyte-produced oxidants in airway inflammation induced by toluene diisocyanate (TDI), and to elucidate the role of leukocyte nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase in pathogenesis by TDI.

Methods: Wild-type mice and NADPH oxidase-deficient mice (neutrophil cytosolic factor 1 mutant, *Ncf1*^{−/−}) were intranasally injected, challenged with inhalatory TDI, and then investigated for lung inflammation.

Results: Cell infiltration in lung tissue and leukocytes in bronchoalveolar lavage, airway reactivity to a methacholine challenge, and TDI-induced inflammatory cytokine expression and nuclear factor activation in the lung tissue were all markedly lower in *Ncf1*^{−/−} mice. Wild-type mice treated with blocking antibodies against CD4 and IL-17 showed markedly lower TDI-induced airway hyperresponsiveness.

Conclusion: Leukocyte NADPH oxidase is an essential regulator in TDI-induced airway inflammation through redox modification of immune responses. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: NADPH oxidase, redox regulation, toluene diisocyanate, leukocyte, lung inflammation

Isocyanate-induced lung disease is the leading cause of occupational asthma, which accounts for 9% to 15% of adult asthma in industrialized countries.^{1,2} Toluene diisocyanate (TDI), a highly reactive low-molecular-weight compound widely used in the

Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
DTNB:	5, 5'-Dithiobis-(2-nitrobenzoic acid)
H ₂ DCFDA:	2', 7'-Dichlorofluorescein diacetate
NAC:	N-acetyl-L-cysteine
NADPH:	Nicotinamide adenine dinucleotide phosphate-reduced
NF-κB:	Nuclear factor-κB
NOX2:	Leukocyte nicotinamide adenine dinucleotide phosphate-reduced oxidase
Nrf2:	Nuclear factor erythroid 2-related factor 2
OVA:	Ovalbumin
RL:	Resistance of lung
ROS:	Reactive oxygen species
TDI:	Toluene diisocyanate

manufacturing of various products including polyurethane foams, automobile paints, varnishes, and medical plastic casts, affects people through both cutaneous and respiratory contact and triggers airway symptoms in some individuals even with low-level exposure found in the workplace and environments.³⁻⁸ Inflammatory responses of the airway associated with the TDI exposure have been reported to include both T_H1 and T_H2 cytokines and chemokines, as well as the infiltration of various leukocytes into the bronchial tract and alveolar tissue.^{6,9-13} Among the genes reported to be associated with the development of TDI-induced asthma, the detoxification enzyme *N*-acetyltransferase and the anti-oxidant enzyme glutathione-S-transferase are closely related to cellular responses to oxidant stress.¹⁴⁻¹⁷

Oxidant stress mediated by reactive oxygen species (ROS), including superoxide, singlet O₂, H₂O₂, and hydroxyl radical, has long been known to be important in the immunopathogenesis of the lung, which is exposed to a higher concentration of oxygen than is most other tissue.^{10,18-21} Whereas the antioxidant processes decrease the level of oxidant stress, the ROS-producing enzymes, which include the electron transport chain in the mitochondria, xanthine oxidase, and nicotinamide adenosine dinucleotide phosphate reduced oxidases (NADPH oxidases), increase the oxidant stress in the tissue.^{22,23} The leukocyte NADPH oxidase (NOX2), which is highly expressed in phagocytes such as neutrophils and monocytes, is usually the most active producer of ROS in inflammatory tissues.^{24,25} We therefore hypothesized that a redox imbalance caused by NOX2 is involved in the TDI-induced airway inflammation. In this study, we used a mouse model to test the role of NOX2 activity and redox imbalance in lung inflammation.

From ^athe Institute of Basic Medicine, ^bthe Institute of Microbiology and Immunology, ^cthe Institute of Clinical Medicine, and ^dthe Department of Pediatrics, National Cheng Kung University Medical College.

Supported by grants from the National Science Council, Taiwan. C.-C.S. was supported by grants from the National Science Council and National Health Research Institute, Taiwan.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication February 16, 2010; revised December 1, 2010; accepted for publication December 1, 2010.

Reprint requests: Chi-Chang Shieh, MD, PhD, Department of Pediatrics, National Cheng Kung University, 138 Sheng-Li Road, Tainan 70403, Taiwan. E-mail: cshieh@mail.ncku.edu.tw.

0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2010.12.008

METHODS

Animals

Female, B6 (Cg)–Ncf1<mi>mlJ>/J mice (stock number 004742) were obtained from Jackson Laboratory (Bar Harbor, Me). Female wild-type C57BL/6J mice were from the Laboratory Animal Center at National Cheng Kung University. The experimental protocol was approved by the Institutional Animal Care and Use Committee of National Cheng Kung University.

Reagents

Toluene diisocyanate isomers were purchased from Merck (Darmstadt, Germany). Ovalbumin (OVA), *N*-acetyl-L-cysteine (NAC), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and rabbit anti-dinitrophenyl serum were from Sigma-Aldrich Co (St Louis, Mo). Mouse antigitutathione antibody (clone D8) was purchased from Chemicon (Temecula, Calif); 2', 7'-dichlorofluorescein diacetate (H₂DCFDA) from Invitrogen (Carlsbad, Calif); anti-mouse CD4 antibody (clone GK1.5), anti-mouse IL-17 antibody (eBioMM17F3), and anti-mouse neutrophil (anti-Ly-6G clone RB68C5) from eBioscience (San Diego, Calif); rabbit anti-mouse nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor- κ B (NF- κ B) from Abcam (Cambridge, United Kingdom); and mouse cytokine ELISA kits from R&D Systems (Minneapolis, Minn).

Exposure to TDI and OVA

For the TDI exposure experiment, the mice were sensitized with an intranasal injection of vehicle (solvent with olive oil: ethyl acetate, 4:1) or 3% TDI while they were under light anesthesia (sodium pentobarbital, 30 mg/kg) on days 0, 7, and 14. On days 15 to 18, ultrasonic nebulization was used to challenge the mice with 3% TDI for 15 minutes to mimic high-level exposure in a poorly ventilated workplace.²⁶ For NAC pretreatment, the mice were exposed to nebulized NAC (5 mg/mL) for 15 minutes before each challenge. For OVA sensitization and challenge, mice were intraperitoneally injected with 200 μ L OVA-alum (0.2 mg OVA: 2 mg alum) or 200 μ L control alum. On days 15 to 18, the mice were challenged with nebulized 3% OVA through the airway for 15 minutes. On day 19, all the mice were overdosed with sodium pentobarbital (100 mg/kg intraperitoneally), and the lungs were harvested for experiments.

Measuring total sulfhydryl levels

Mouse lung tissue (100 mg) was mechanically homogenized in 0.2 mL degassed PBS with a protease inhibitor cocktail. The protein concentration of the supernatant after the tissue had been centrifuged was determined by using the Bradford method. The sulfhydryl levels were then measured by using DTNB, as previously reported.²⁷

Examining lung histology

To assess lung histology, 5 randomly selected areas of the lower lobes of each lung were digitally photographed at low magnification ($\times 40$), and the cell infiltration and hemorrhage areas were outlined. The number of pixels contained in the designated areas and the number of pixels contained in the image of the entire lung field were determined by histogram function (Image J Software; <http://rsbweb.nih.gov/ij/index.html>). The total number of pixels outlined as cellular infiltrate and hemorrhage were divided by the total number of pixels in the entire lung field and multiplied by 100 to generate a percentage of area for each mouse. The tissue histology was examined and scored independently by 3 of the authors (S.-Y.L., P.-W.Y., and C.-C.S.) in a blind manner.

Measuring airway responsiveness

Airway responsiveness was assessed by using an intratracheal system (FinePointe) to measure airway resistance directly (Buxco Electronics, Inc, Wilmington, NC). Mice were exposed to increasing aerosol concentrations (0–25 mg/mL) of nebulized methacholine for 20 seconds, and the bronchopulmonary resistance was recorded as resistance of lung (RL) after each methacholine inhalation.

Preparing TDI-BSA conjugate and detecting antibodies

Toluene diisocyanate (100 mg/mL) and BSA (100 mg/mL) were incubated in a degassed 10-mmol/L sodium phosphate buffer for 1 hour at room temperature. Unbound TDI was removed by using dialysis against PBS. To detect TDI antibodies, 96-well plates were coated with 100 μ L 1 mg/mL TDI-BSA in coating buffer (0.15 mol/L NaCl, 0.8 mmol/L EDTA, 0.05 mol/L TRIS-HCl) overnight and washed with TRIS-buffered saline with Tween buffer. The test sera were added and incubated at room temperature for 1 hour. The bound antibodies were then detected by using antimouse IgE or antimouse IgG₁ and then horseradish peroxidase-coupled anti-immunoglobulin.

Preparing nuclear extracts

The mouse lungs were mechanically homogenized in degassed PBS. After centrifugation at 200g for 15 minutes, cells were resuspended in hypotonic buffer (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 0.5 mmol/L dithiothreitol) and homogenized by using a syringe with a 25-gauge needle. After they had been centrifuged, the nuclei were lysed in hypotonic buffer, and the soluble nuclear proteins were extracted by adding buffer with graded concentrations of KCl (20 mmol/L HEPES, 25% glycerol, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.02–1.6 mol/L KCl [pH 7.9]). Immunoblotting was then used to analyze the extracted nuclear proteins for Nrf2 and nuclear factor- κ B.

Statistical analysis

The data were obtained from 3 to 8 independent experiments and analyzed with the Student *t* test. Data are means $\pm$ SDs. Significance was set at *P* < .05.

RESULTS

TDI exposure and leukocyte NADPH oxidase activity induce oxidant stress in the lung

We first examined whether TDI exposure stimulates the generation of ROS in the airway. Wild-type mice were sensitized, then challenged with TDI and killed 24 hours after the last challenge. The bronchoalveolar lavage (BAL) cells were collected and incubated with H₂DCFDA to detect the production of intracellular H₂O₂. We found that H₂O₂ production significantly increased in BAL cells from wild-type mice treated with TDI compared with equal numbers of BAL cells from the mice without TDI treatment (Fig 1, A; compare the *first and fourth black columns*). The wild-type mice pretreated with the antioxidant NAC had lower H₂O₂ generation than the group without NAC pretreatment (Fig 1, A; *P* = .041). BAL cells from *Ncf1*^{−/−} mice generated a small amount of H₂O₂, and the level remained unchanged after TDI treatment (Fig 1, A, *white columns*). Different from TDI stimulation, OVA treatment induced no increase in ROS production in BAL cells from all groups, although pretreatment with NAC induced a significant and comparable decrease in both wild-type and *Ncf1*^{−/−} mice groups (Fig 1, B). The total sulfhydryl level in the lung tissue protein from TDI-treated wild-type mice markedly decreased, whereas the sulfhydryl levels in tissue from *Ncf1*^{−/−} mice did not significantly change after the TDI exposure (Fig 1, C). NAC pretreatment significantly increased the total sulfhydryl in TDI-treated wild-type mice to a level close to the level of the group treated with solvent (*P* < .001). OVA treatment did not decrease the sulfhydryl levels in wild-type and *Ncf1*^{−/−} mice (data not shown). To detect oxidative protein modification as markers for recent oxidant stress,²⁸ we examined protein carbonylation and *S*-glutathionylation in the lung tissue. We found that wild-type mice had a higher level of protein

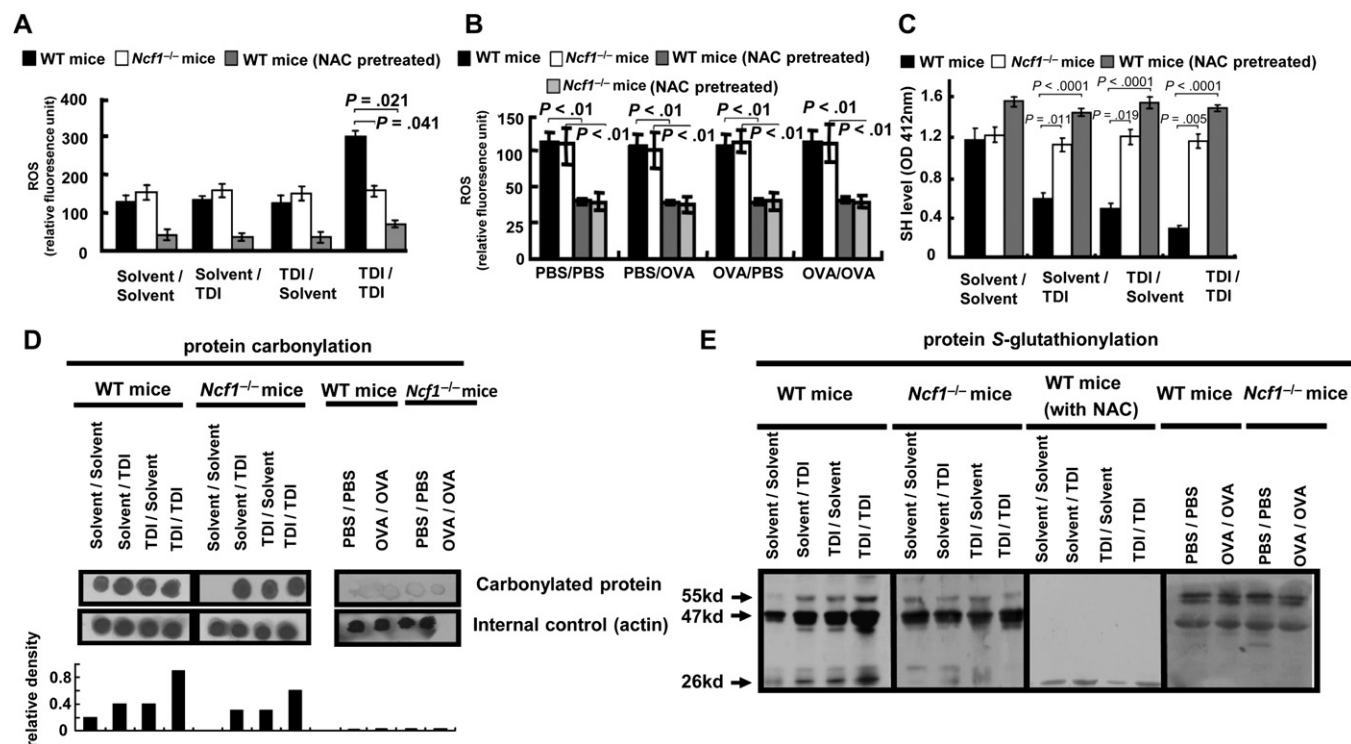


FIG 1. TDI exposure and leukocyte NADPH oxidase activity induced oxidant stress in the lung. After TDI or OVA exposure, the mice lung BAL cells were harvested, and lung tissue was mechanically homogenized. **A** and **B**, The ROS generation was examined by H₂DCFDA. **C** and **D**, The sulfhydryl level of lung homogenates was detected with DTNB. **E**, The carbonylation and glutathionylation level of lung protein extract were examined by immunoblotting with anti-DNP or antilutathione mAbs. Experimental results were expressed as means $\pm$ SDs (*n* = 5 mice per group). The experiments were repeated 5 times with similar results. SH, Sulfhydryl; WT, Wild-type.

carbonylation than *Ncf1*^{-/-} mice after solvent-alone exposure. TDI exposure significantly increased protein carbonylation in the lung tissue in both wild-type and *Ncf1*^{-/-} mice, whereas OVA induced little carbonylation (Fig 1, D, quantified as histogram). Lack of *Ncf1* therefore did not markedly affect the oxidizing effects leading to carbonylation by TDI. We went on to compare the S-glutathionylation in the lung tissue from the groups with different treatment. By using Western blotting to detect S-glutathionylation on the PAGE-resolved total lung tissue lysates containing equal amounts of protein, we found that several major bands with the apparent molecular weight of 26 kd, 47 kd, and 55 kd were visualized. The intensity of these bands increased with TDI treatment in the wild-type group. In contrast, *Ncf1*-deficient mice treated with TDI did not have increased levels of protein S-glutathionylated. The lung lysates from mice pretreated with NAC showed very weak S-glutathionylated signals. The OVA treatment group, however, did not have significantly increased S-glutathionylation (Fig 1, E). These results suggest that although TDI treatment leads to protein carbonylation, NADPH oxidase plays an important role in the oxidant stress that mediates the protein S-glutathionylation in the lung.

TDI exposure induces airway hyperresponsiveness in wild-type but not *Ncf1*^{-/-} mice

To address the role of leukocyte NADPH oxidase activity in the TDI-induced airway hyperresponsiveness (AHR), we tested the effects of TDI on allergic airway inflammation in wild-type and

Ncf1^{-/-} mice. The experimental scheme is shown in Fig 2, A. In wild-type mice with TDI treatment, the RL values significantly increased in response to methacholine stimulation. In contrast, TDI-treated *Ncf1*^{-/-} mice showed only a slight increase in RL value in response to methacholine stimulation (Fig 2, B, upper panel). A significant difference was noted between TDI-treated *Ncf1*^{-/-} and wild-type mice at the methacholine aerosol concentrations of 10 mg/mL and 25 mg/mL (Fig 2, B, upper panel). In OVA-treated groups, a similar increase in RL values in response to methacholine stimulation was observed. No significant difference was noted between wild-type and *Ncf1*^{-/-} mice (Fig 2, B, bottom panel). Pretreatment with NAC reduced the AHR in TDI-treated wild-type mice (Fig 2, C). These results indicate that NADPH oxidase-produced ROSs play an important role in the TDI-induced pulmonary hyperresponsiveness.

TDI exposure induces inflammatory cell infiltration in the lung

We then measured the change in the cellular constituents from the BAL fluid in TDI-treated mice by performing total cell counting and differential counting of the BAL cells. The total cell number in the BAL fluid from TDI-sensitized and TDI-challenged wild-type mice was significantly higher than that in the BAL fluid from *Ncf1*^{-/-} mice with the same treatment (Fig 3, A, top panel; *P* = .017). With differential counting, we found that, although BAL fluid from wild-type mice sensitized and challenged with TDI contained markedly increased lymphocytes,

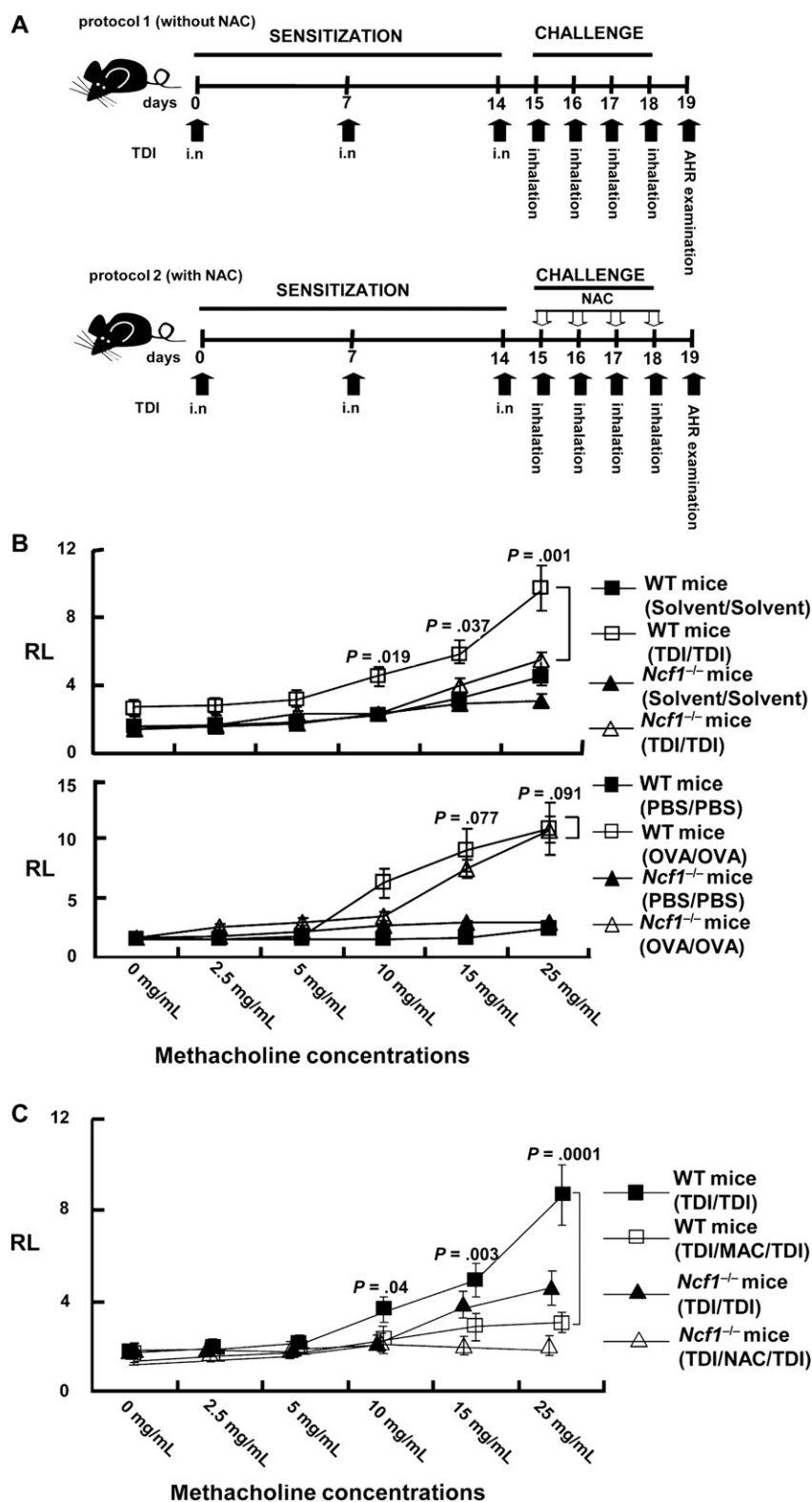


FIG 2. TDI exposure induced AHR in wild-type (WT) but not $Ncf1^{-/-}$ mice. **A**, Experimental scheme. **B**, Measurements of airway resistance of TDI-sensitized and TDI-challenged or OVA-sensitized and OVA-challenged WT and $Ncf1^{-/-}$ mice in response to increasing doses of methacholine. **C**, Measurements of airway responsiveness of TDI-treated WT and $Ncf1^{-/-}$ mice with or without NAC (5 mg/mL) pretreated are compared. The P values refer to the statistical significance in comparison between TDI-sensitized and TDI-challenged WT and $Ncf1^{-/-}$ mice. The results were expressed as means $\pm$ SDs ($n = 8$ mice per group). The experiments were repeated 2 times with similar results. *i.n.*, Intranasal.

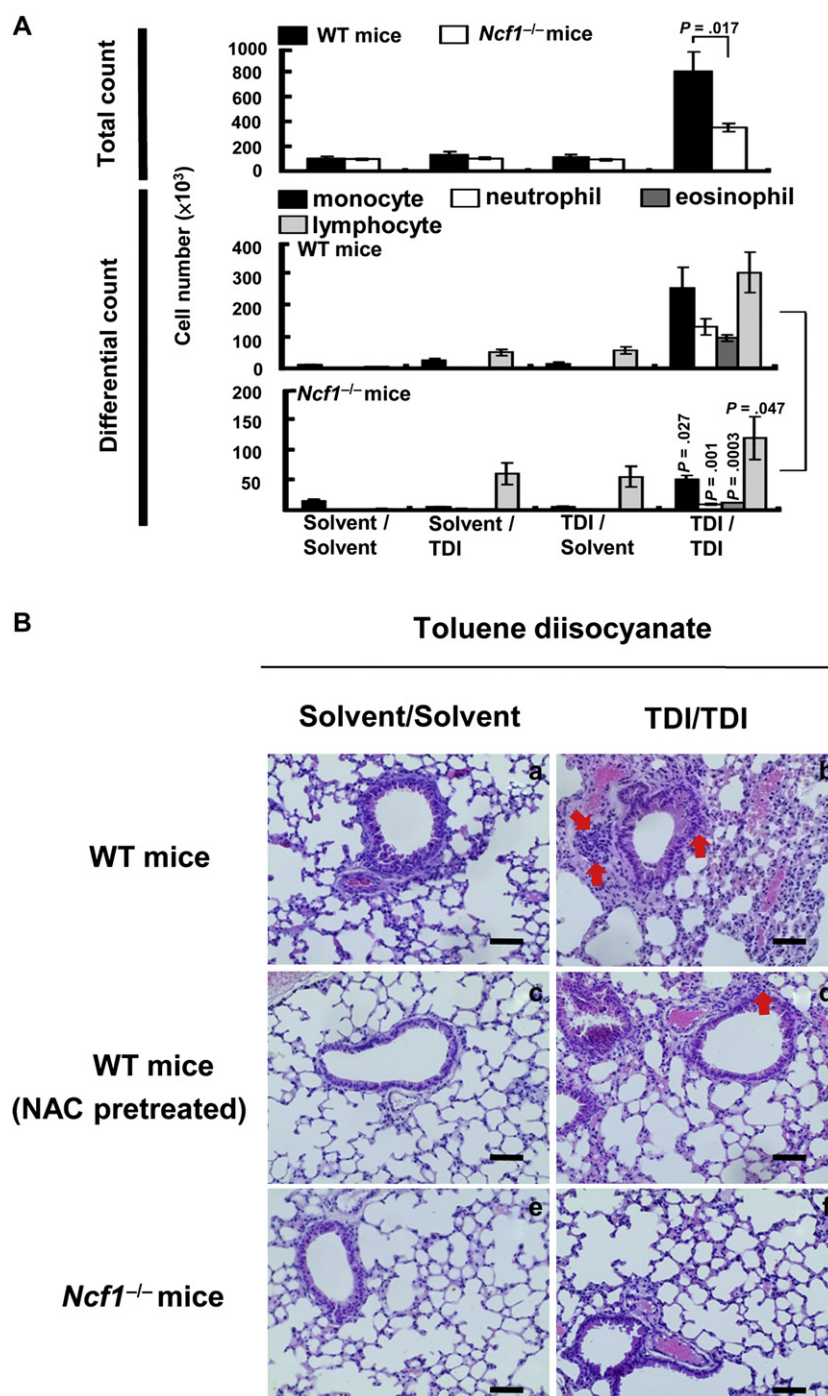


FIG 3. TDI exposure induced inflammatory cell infiltration in the lung. Lungs were harvested at 24 hours after the last 3% TDI or OVA challenge. The BAL cells were collected and counted. **A**, Total BAL fluid cells counts (*top group*) and cell differential counts in wild-type (WT) mice (*middle group*) and *Ncf1*^{-/-} mice (*bottom group*; *n* = 8 in each group). **B**, TDI-treated WT (*a and b*) and *Ncf1*^{-/-} mice (*e and f*) or NAC-pretreated TDI-treated WT (*c and d*) mouse lungs. The arrows indicate leukocyte infiltration. The reference scale bars stand for 0.1 mm. Original magnification $\times 100$. The mice lung histology photographs represent 1 of 8 mice in each group.

monocytes, neutrophils, and eosinophils, *Ncf1*^{-/-} mice exposed to TDI had a milder increase in leukocytes in BAL fluid. The most significant differences were observed in the numbers of eosinophils and neutrophils (Fig 3, A, middle and bottom panels). We then examined the histologic changes in the lung tissue from

different groups. The severity of inflammation was evaluated with light microscopy and digital image analysis. In solvent-treated, wild-type groups, there was very mild leukocyte infiltration around the bronchovascular bundle complex (Fig 3, B, panel a). However, in the TDI-sensitized and TDI-challenged wild-type

group, a marked increase of inflammatory cells in the bronchovascular bundle was noted (Fig 3, B, panel b). In the NAC-pretreated, TDI-treated group (Fig 3, B, panels c and d), the proportion of cell infiltration in the TDI-sensitized and TDI-challenged wild-type mice noticeably decreased (Fig 3, B, panel d). Different from the strong tissue inflammation found in wild-type mice, the lung tissue from TDI-sensitized and TDI-challenged *Ncf1*^{-/-} mice showed milder cell infiltration in the lung tissue (Fig 3, B, panel f).

Different TDI-induced cytokine and specific antibody responses in wild-type and *Ncf1*^{-/-} mice

To determine the effects of TDI on the induction of cytokine production, lung homogenates were collected from different treatment groups and analyzed for their protein levels with ELISA. When comparing the lung samples from wild-type mice with TDI-sensitized and TDI-challenged treatment with the samples from the wild-type group without TDI treatment, we found that the levels of IFN- γ , TNF- α , IL-17, and IL-10 markedly increased in the TDI-treated group. When comparing the level of cytokines in lung tissue from wild-type and *Ncf1*^{-/-} mice, we found that the levels of IFN- γ , TNF- α , and IL-17 in TDI-sensitized and TDI-challenged wild-type mice lung were significantly higher than in TDI-sensitized and TDI-challenged *Ncf1*^{-/-} mice. No significant difference was found in the levels of IL-10 in wild-type and *Ncf1*^{-/-} mice after the TDI sensitization and challenge treatment (Fig 4, A). To detect the specific TDI antibodies, the TDI-conjugated BSA was prepared, and the resulting protein conjugate was resolved on native polyacrylamide gel electrophoresis (Fig 4, B, inset box). We used the TDI-conjugated BSA to perform ELISA tests for TDI-specific antibodies. The titers of TDI-specific IgE and IgG₁ in serum samples from TDI-sensitized and TDI-challenged mice significantly increased compared with mice without TDI exposure. The *Ncf1*^{-/-} mice had lower specific IgE titers but higher specific IgG₁ titers compared with wild-type mice (Fig 4, B, top panel). ELISA competition assays were performed to verify the specificity of TDI-specific antibody examination. We found that both IgE and IgG₁ titers were suppressed to baseline levels by soluble TDI-conjugated BSA but not by soluble native BSA in wild-type and *Ncf1*^{-/-} mice (Fig 4, B, middle and bottom panels).

TDI exposure induces redox-sensitive transcription factor translocation to nuclei

Nrf2 expression, an indicator of tissue response to oxidant stress, was examined in different groups of mice with or without TDI exposure. In wild-type mice, TDI treatment induced Nrf2 expression and translocation into nuclei compared with the control group without TDI stimulation (Fig 5, A). In contrast with wild-type mice, TDI induced less cellular expression and nucleus translocation of Nrf2 in *Ncf1*^{-/-} mice (Fig 5, A). In parallel with immunohistochemical analysis, nuclear extract of lung tissue was analyzed with immunoblotting to confirm the nucleus translocation of Nrf2 protein (Fig 5, B). These findings indicate that exposure of TDI causes Nrf2-initiated oxidant stress response in the airway of wild-type mice, but to a much less extent in *Ncf1*^{-/-} mice. The expression of Nrf2 also was examined in OVA-stimulated lung tissues. Different from TDI exposure, OVA treatment did not induce changes of Nrf2 expression or

intracellular location in wild-type mice and *Ncf1*^{-/-} mice (Fig 5, A). Next, we compared the activation of NF- κ B in wild-type and *Ncf1*^{-/-} lung tissue after TDI sensitization and challenge. Similar to the results of Nrf2 response, the immunohistologic staining and immunoblotting of nuclear extract of the lung showed that the nucleus translocation of NF- κ B was markedly higher in wild-type mice than *Ncf1*^{-/-} mice after TDI treatment (Fig 5, C and D).

CD4⁺ T cells and IL-17 are involved in TDI-induced lung inflammation

On the basis of our results showing the marked increase in neutrophils and IL-17 levels in the BAL fluid and lung homogenate from TDI-treated wild-type mice, we hypothesized that T_H17 cells may be important for the TDI-induced lung inflammation. We therefore performed *in vivo* cell depletion experiments to evaluate the roles of these factors in TDI-induced pulmonary inflammation. The animals were injected with 100 μ g anti-IL-17 mAb or anti-CD4 mAb, once before TDI sensitization and another time before TDI challenge. The cell depletion treatment lowered the CD4⁺ cells in the blood to <0.2%. Our results showed that the RL was significantly lower in wild-type mice depleted of CD4⁺ cells and IL-17 compared with mice treated with isotype-matched control antibody (Fig 6). These results prove that CD4-expressing cells and IL-17 are involved in TDI-induced lung inflammation.

DISCUSSION

By inducing lung inflammation with TDI, a chemical that causes oxidant stress in a mouse model with or without deficient leukocyte NADPH oxidase, we showed that leukocyte NADPH oxidase plays a crucial role in TDI-induced lung damage.

Although the evidence for the role of oxidant stress in lung inflammation has been accumulating in the past decade, the relationship between exposure to different allergens and redox-mediated leukocyte activation remains elusive.^{29,30} Airway inflammation in asthma is a complex process characterized by AHR, inflammation-mediated tissue destruction, and remodeling involving innate and acquired immune responses.^{31,32} The inflammatory process may induce the resident cells in the lung to produce ROS and lead to oxidant stress in the respiratory tract.³³⁻³⁵ To add to this complexity, macrophage-derived, neutrophil-derived, and eosinophil-derived ROSs, which are mainly produced by a highly regulated NOX2, contribute to oxidant stress in tissue and substantially affect the severity of inflammation in different disease models.^{36,37}

Mutations of phagocyte NADPH oxidase cause the primary immunodeficiency disease termed *chronic granulomatous disease*.³⁸ Many patients with chronic granulomatous disease are susceptible to various pathological conditions because they lack adequate ROS production and require treatment to restore the defense functions of leukocytes.^{24,39,40} NOX2 gene polymorphism, which may affect the production of ROS by leukocytes, is associated with different human diseases, including asthma, arthritis, stroke, and coronary artery disease.⁴¹⁻⁴⁴ It was recently reported that lack of NADPH oxidase activity markedly increased the severity of arthritis in a mouse model of arthritis.⁴⁵ How leukocyte NADPH oxidase contributes to tissue oxidant stress and regulates or facilitates inflammation in different tissue types is,

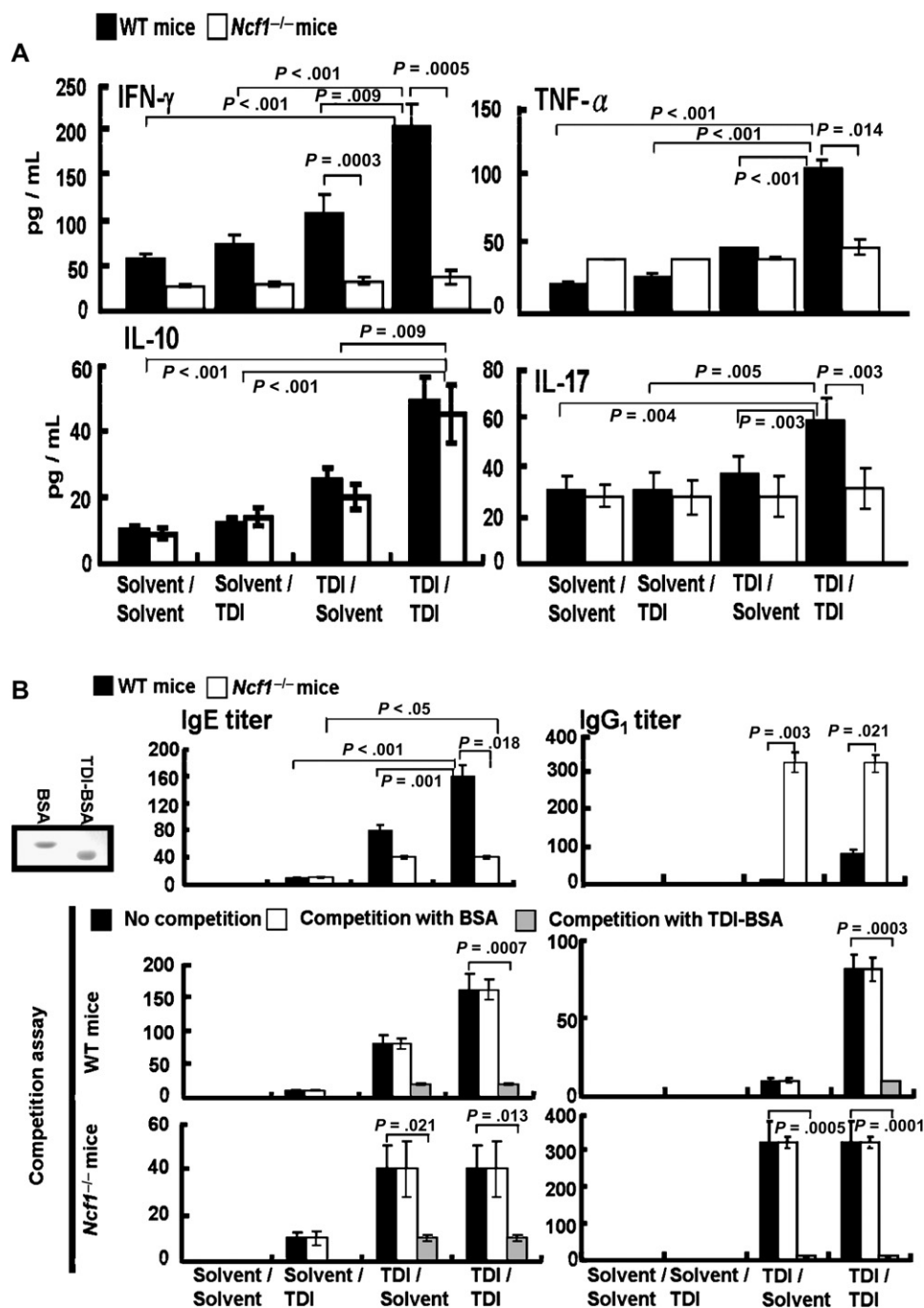


FIG 4. Different TDI-induced cytokine and specific antibody responses in wild-type (WT) and *Ncf1*^{-/-} mice. **A**, Pooled mice lung homogenates were determined for TNF-α, IFN-γ, IL-17, and IL-10 by ELISA. **B**, Specific antibody titers against TDI were determined by ELISA against TDI-BSA and BSA (inset box). The IgE (left) and IgG (right) titers are shown in the top group. The ELISA-based competition assays for WT and *Ncf1*^{-/-} mice are shown in the middle and bottom panels. Pooled mice sera were from 8 mice in each experimental group. Experimental results were expressed as means ± SDs (n = 8 per group). The experiments were repeated 3 times with similar results.

therefore, an important question to ask if we are to elucidate the pathogenesis of these diseases.

Toluene diisocyanate, one of the most important allergens to induce occupational asthma,^{1,46} is an irritant for respiratory tract cells and causes cell damage, which directly leads to cell dysfunction.⁴⁷ Spraying or spilling of TDI in a poorly ventilated

workplace may lead to exposure up to 900 ppb, which is above the odor threshold of human beings.²⁶ TDI and other isocyanates are oxidizing agents that react rapidly with cellular proteins and lead to molecular changes.^{48,49} It has been reported that both immunologic and nonimmunologic effects of isocyanates may induce pathogenic mechanisms distinct from other airway

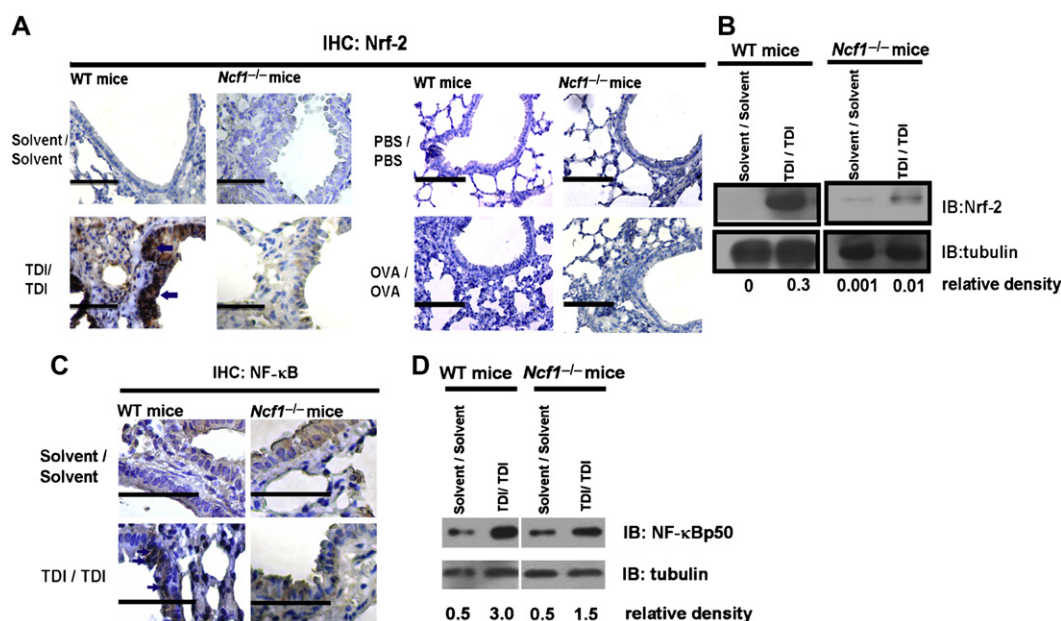


FIG 5. TDI exposure induced redox-sensitive transcription factor expression and translocation into nucleus in the lung. Detection of nuclear factors Nrf2 and NF- κ B in the lung tissue with or without TDI treatment was performed with immunohistochemistry (IHC) staining and biochemical analysis on nuclear extract from lung tissue. **A**, IHC staining with Nrf2 in lung tissue. **B**, Immunoblotting staining with Nrf2 in mice lung nuclear extract. **C**, IHC staining with NF- κ B in lung tissue. **D**, Immunoblotting staining with NF- κ B in lung tissue. The figure represents 1 of 8 mice in each group. The arrows indicate positive nuclear staining. The reference scales stand for 100 μ m. Original magnification $\times 400$. WT, Wild-type.

inflammatory conditions.⁴⁶ Consistent with the strong oxidant activity of TDI, we found that after wild-type and *Ncf1*^{-/-} mice were repeatedly exposed to TDI, there was a marked increase in carbonylation, an irreversible oxidative change on proteins, in their lung tissue. In contrast, only wild-type mice with intact leukocyte NADPH oxidase had a significant change in glutathionylation and a decrease in sulfhydryl levels after TDI treatment. We previously reported that ROSs produced by NADPH oxidase and peptide glutathionylation are crucial in redox regulation of integrin-mediated adhesion function of very late antigen-4, which may be pivotal in directing leukocyte trafficking to inflammatory sites.^{18,19} Glutathione has recently been shown to be present in respiratory lining fluid at levels comparable to intracellular concentrations and to be crucial for dynamic redox homeostasis in the airway.^{35,50-52} It is therefore reasonable to infer that ROS production by activated leukocytes and the subsequent protein modification, including glutathionylation, may provide the necessary reversible signals required for leukocyte trafficking to induce immune responses for lung inflammation.¹⁸

The redox modification of cell surface and cytoplasmic molecules have been shown to be key determinants in pulmonary inflammation.⁵³ The Nrf2-signaling pathway mediates the cellular response to low-level oxidants and leads to transcription of genes related to antioxidant, anti-inflammation, and detoxification.⁵⁴ NF- κ B, also a redox-sensitive nuclear factor, is a key molecule in response to oxidant stress that starts inflammatory responses including cytokine expression.^{55,56} Our results thus suggest that the oxidant stress mediated by leukocyte NADPH oxidase is required for lung cells to reach the adequate stress level to start the cellular response for both antioxidant and inflammation responses after repeated exposure to TDI. This redox-modulated cell response then leads to a distinct allergic reaction in the lung.

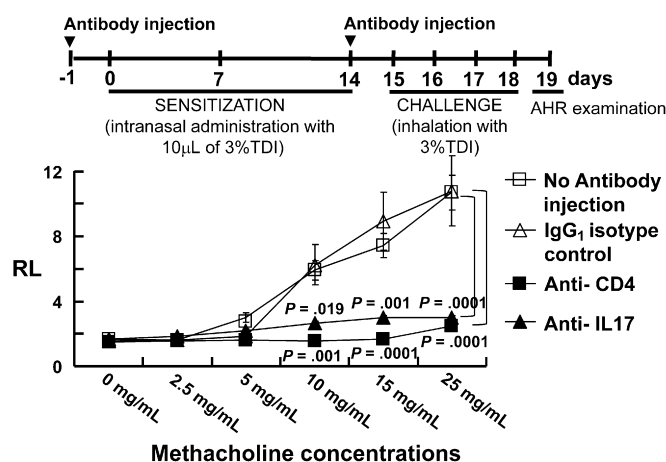


FIG 6. CD4⁺ T cells and IL-17 are involved in TDI-induced lung inflammation. Measurements of airway responsiveness of 3% TDI-sensitized and TDI-challenged CD4 cell-depleted (black square), IL-17-depleted (black triangle), and wild-type mice to increasing doses of methacholine are shown. Experimental results were expressed as means $\pm$ SDs ($n = 8$ mice in each group). The experiments were repeated 2 times with similar results.

On the basis of the current evidence supporting the importance of leukocyte NADPH oxidase and redox regulation by neutrophils, we postulate that the IL-17-neutrophil axis in the pathogenesis of lung inflammation may be more important in oxidant stress-dependent allergen (eg, TDI) than in other allergens (eg, OVA). Because oxidant stress is relatively mild in some animal models using other allergens, as clearly shown in this study, the role of redox regulation and oxidant stress may tend not to be evident in these models.^{57,58} Because the importance of redox

regulation in allergic inflammation in human beings has become more and more evident, animal models with different levels of oxidant stress that more closely mimic human lung inflammatory conditions induced by a variety of allergens may prove to be important experimental approaches to understand airway inflammation in asthma.⁵⁹⁻⁶¹

In conclusion, our findings indicate that leukocyte NADPH oxidase is critical in TDI-induced lung inflammation. Change of redox imbalance by antioxidants or defective leukocyte oxidase may change the lung inflammation in terms of early innate immune cell recruitment and the subsequent specific immune responses.

We thank Prof S. H. Chen and Prof David Wang for help in experiments.

Clinical implications: Therapies targeting leukocyte or tissue oxidant production and antioxidant mechanisms may change the pathogenic redox imbalance in the lung.

REFERENCES

- Maestrelli P, Boschetto P, Fabbri LM, Mapp CE. Mechanisms of occupational asthma. *J Allergy Clin Immunol* 2009;123:531-42.
- Balmes J, Becklake M, Blanc P, Henneberger P, Kreiss K, Mapp C, et al. American Thoracic Society statement: occupational contribution to the burden of airway disease. *Am J Respir Crit Care Med* 2003;167:787-97.
- Jan RL, Chen SH, Chang HY, Yeh HJ, Shieh CC, Wang JY. Asthma-like syndrome in school children after accidental exposure to xylene and methylene diphenyl diisocyanate. *J Microbiol Immunol Infect* 2008;41:337-41.
- Donnelly R, Buick JB, Macmahon J. Occupational asthma after exposure to plaster casts containing methylene diphenyl diisocyanate. *Occup Med (Lond)* 2004;54:432-4.
- Lemiere C, Romeo P, Chabouille S, Tremblay C, Malo JL. Airway inflammation and functional changes after exposure to different concentrations of isocyanates. *J Allergy Clin Immunol* 2002;110:641-6.
- Wisniewski AV, Redlich CA. Recent developments in diisocyanate asthma. *Curr Opin Allergy Clin Immunol* 2001;1:169-75.
- Baur X, Marek W, Ammon J, Czuppon AB, Marczyński B, Raulf-Heimsoth M, et al. Respiratory and other hazards of isocyanates. *Int Arch Occup Environ Health* 1994;66:141-52.
- Dragos M, Jones M, Malo JL, Ghezzi H, Gautrin D. Specific antibodies to diisocyanate and work-related respiratory symptoms in apprentice car-painters. *Occup Environ Med* 2009;66:227-34.
- Matheson JM, Johnson VJ, Vallyathan V, Luster MI. Exposure and immunological determinants in a murine model for toluene diisocyanate (TDI) asthma. *Toxicol Sci* 2005;84:88-98.
- Vanoirbeek JA, Tarkowski M, Ceuppens JL, Verbeken EK, Nemery B, Hoet PH. Respiratory response to toluene diisocyanate depends on prior frequency and concentration of dermal sensitization in mice. *Toxicol Sci* 2004;80:310-21.
- Sumi Y, Kyi M, Miyazaki Y, Ohtani Y, Miyake S, Yoshizawa Y. Cytokine mRNA expression in isocyanate-induced hypersensitivity pneumonitis. *Respiration* 2003;70:284-91.
- Matheson JM, Lemus R, Lange RW, Karol MH, Luster MI. Role of tumor necrosis factor in toluene diisocyanate asthma. *Am J Respir Cell Mol Biol* 2002;27:396-405.
- Saetta M, Di SA, Maestrelli P, De MN, Milani GF, Pivrotto F, et al. Airway mucosal inflammation in occupational asthma induced by toluene diisocyanate. *Am Rev Respir Dis* 1992;145:160-8.
- Minelli C, Granell R, Newson R, Rose-Zerilli MJ, Torrent M, Ring SM, et al. Glutathione-S-transferase genes and asthma phenotypes: a Human Genome Epidemiology (HuGE) systematic review and meta-analysis including unpublished data. *Int J Epidemiol* 2010;39:539-62.
- Mapp CE, Fryer AA, De MN, Pozzato V, Padoan M, Boschetto P, et al. Glutathione S-transferase GSTP1 is a susceptibility gene for occupational asthma induced by isocyanates. *J Allergy Clin Immunol* 2002;109:867-72.
- Wikman H, Piirila P, Rosenberg C, Luukkonen R, Kaaria K, Nordman H, et al. N-acetyltransferase genotypes as modifiers of diisocyanate exposure-associated asthma risk. *Pharmacogenetics* 2002;12:227-33.
- Sheehan D, Meade G, Foley VM, Dowd CA. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J* 2001;360:1-16.
- Liu SY, Tsai MY, Chuang KP, Huang YF, Shieh CC. Ligand binding of leukocyte integrin very late antigen-4 involves exposure of sulfhydryl groups and is subject to redox modulation. *Eur J Immunol* 2008;38:410-23.
- Chuang KP, Huang YF, Hsu YL, Liu HS, Chen HC, Shieh CC. Ligation of lymphocyte function-associated antigen-1 on monocytes decreases very late antigen-4-mediated adhesion through a reactive oxygen species-dependent pathway. *Blood* 2004;104:4046-53.
- Klatt P, Lamas S. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 2000;267:4928-44.
- Bowler RP, Crapo JD. Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol* 2002;110:349-56.
- Sumimoto H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *FEBS J* 2008;275:3249-77.
- Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 2004;55:373-99.
- Babior BM. NADPH oxidase. *Curr Opin Immunol* 2004;16:42-7.
- Heyworth PG, Cross AR, Curnutte JT. Chronic granulomatous disease. *Curr Opin Immunol* 2003;15:578-84.
- Ott MG, Diller WF, Jolly AT. Respiratory effects of toluene diisocyanate in the workplace: a discussion of exposure-response relationships. *Crit Rev Toxicol* 2003;33:1-59.
- Loretz B, Thaler M, Bernkop-Schnurch A. Role of sulfhydryl groups in transfection? A case study with chitosan-NAC nanoparticles. *Bioconjug Chem* 2007;18:1028-35.
- Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med* 2006;10:389-406.
- Park HS, Kim SR, Lee YC. Impact of oxidative stress on lung diseases. *Respirology* 2009;14:27-38.
- Emelyanov A, Fedoseev G, Abulimity A, Rudinski K, Fedoulov A, Karabanov A, et al. Elevated concentrations of exhaled hydrogen peroxide in asthmatic patients. *Chest* 2001;120:1136-9.
- Boyce JA, Broide D, Matsumoto K, Bochner BS. Advances in mechanisms of asthma, allergy, and immunology in 2008. *J Allergy Clin Immunol* 2009;123:569-74.
- Barrett NA, Austen KF. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* 2009;31:425-37.
- Rahman I, Biswas SK, Kode A. Oxidant and antioxidant balance in the airways and airway diseases. *Eur J Pharmacol* 2006;533:222-39.
- Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. *Am J Respir Crit Care Med* 2003;167:1600-19.
- Fitzpatrick AM, Teague WG, Holguin F, Yeh M, Brown LA. Airway glutathione homeostasis is altered in children with severe asthma: evidence for oxidant stress. *J Allergy Clin Immunol* 2009;123:146-52.
- Yang Z, Sharma AK, Marshall M, Kron IL, Laubach VE. NADPH oxidase in bone marrow-derived cells mediates pulmonary ischemia-reperfusion injury. *Am J Respir Cell Mol Biol* 2009;40:375-81.
- Olofsson P, Holmberg J, Tordsson J, Lu S, Akerstrom B, Holmdahl R. Positional identification of *Ncf-1* as a gene that regulates arthritis severity in rats. *Nat Genet* 2003;33:25-32.
- Holland SM. Chronic granulomatous disease. *Clin Rev Allergy Immunol* 2010;38:3-10.
- Huang YF, Liu SY, Yen CL, Yang PW, Shieh CC. Thapsigargin and flavin adenine dinucleotide ex vivo treatment rescues trafficking-defective gp91phox in chronic granulomatous disease leukocytes. *Free Radic Biol Med* 2009;47:932-40.
- Lin SJ, Huang YF, Chen JY, Heyworth PG, Noack D, Wang JY, et al. Molecular quality control machinery contributes to the leukocyte NADPH oxidase deficiency in chronic granulomatous disease. *Biochim Biophys Acta* 2002;1586:275-86.
- Izakovicova HL, Kankova K, Znojil V. Haplotype analysis of the NADPH oxidase p22 phox gene in patients with bronchial asthma. *Int Arch Allergy Immunol* 2009;148:73-80.
- San JG, Fortuno A, Belouqui O, Diez J, Zalba G. NADPH oxidase CYBA polymorphisms, oxidative stress and cardiovascular diseases. *Clin Sci (Lond)* 2008;114:173-82.
- Olsson LM, Lindqvist AK, Kallberg H, Padyukov L, Burkhardt H, Alfredsson L, et al. A case-control study of rheumatoid arthritis identifies an associated single nucleotide polymorphism in the *NCF4* gene, supporting a role for the NADPH-oxidase complex in autoimmunity. *Arthritis Res Ther* 2007;9:R98.
- Shimo-Nakanishi Y, Hasebe T, Suzuki A, Mochizuki H, Nomiya T, Tanaka Y, et al. Functional effects of NAD(P)H oxidase p22(phox) C242T mutation in human leukocytes and association with thrombotic cerebral infarction. *Atherosclerosis* 2004;175:109-15.

45. Hagenow K, Gelderman KA, Hultqvist M, Merky P, Backlund J, Frey O, et al. *Ncf1*-associated reduced oxidative burst promotes IL-33R+ T cell-mediated adjuvant-free arthritis in mice. *J Immunol* 2009;183:874-81.
46. Redlich CA, Karol MH. Diisocyanate asthma: clinical aspects and immunopathogenesis. *Int Immunopharmacol* 2002;2:213-24.
47. Piskorska E, Holynska-Iwan I, Kaczorowski P, Soczywko-Ciudzinska J, Wicinski M, Lampka M, et al. Toluene diisocyanate caused electrophysiological disturbances in the upper airways wall. *Int J Occup Med Environ Health* 2009;22:125-34.
48. Lantz RC, Lemus R, Lange RW, Karol MH. Rapid reduction of intracellular glutathione in human bronchial epithelial cells exposed to occupational levels of toluene diisocyanate. *Toxicol Sci* 2001;60:348-55.
49. Valstar DL, Schijf MA, Nijkamp FP, Bloksma N, Henricks PA. Glutathione-conjugated toluene diisocyanate causes airway inflammation in sensitised mice. *Arch Toxicol* 2004;78:533-9.
50. Brown LA, Ping XD, Harris FL, Gauthier TW. Glutathione availability modulates alveolar macrophage function in the chronic ethanol-fed rat. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L824-32.
51. Rahman I, Biswas SK, Jimenez LA, Torres M, Forman HJ. Glutathione, stress responses, and redox signaling in lung inflammation. *Antioxid Redox Signal* 2005;7:42-59.
52. Moriarty-Craige SE, Jones DP. Extracellular thiols and thiol/disulfide redox in metabolism. *Annu Rev Nutr* 2004;24:481-509.
53. Riedl MA, Nel AE. Importance of oxidative stress in the pathogenesis and treatment of asthma. *Curr Opin Allergy Clin Immunol* 2008;8:49-56.
54. Kaspar JW, Nitire SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radic Biol Med* 2009;47:1304-9.
55. Janssen-Heininger YM, Poynter ME, Aesif SW, Pantano C, Ather JL, Reynaert NL, et al. Nuclear factor kappaB, airway epithelium, and asthma: avenues for redox control. *Proc Am Thorac Soc* 2009;6:249-55.
56. Reynaert NL, van der Vliet A, Guala AS, McGovern T, Hristova M, Pantano C, et al. Dynamic redox control of NF-kappaB through glutaredoxin-regulated S-glutathionylation of inhibitory kappaB kinase beta. *Proc Natl Acad Sci U S A* 2006;103:13086-91.
57. Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, et al. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J Exp Med* 2005;202:47-59.
58. Reynaert NL, Aesif SW, McGovern T, Brown A, Wouters EF, Irvin CG, et al. Catalase overexpression fails to attenuate allergic airways disease in the mouse. *J Immunol* 2007;178:3814-21.
59. Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* 2008;1:213-20.
60. Paruchuri S, Tashimo H, Feng C, Maekawa A, Xing W, Jiang Y, et al. Leukotriene E4-induced pulmonary inflammation is mediated by the P2Y12 receptor. *J Exp Med* 2009;206:2543-55.
61. Pichavant M, Goya S, Meyer EH, Johnston RA, Kim HY, Matangkasombut P, et al. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* 2008;205:385-93.