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DUOX2 promotes the elimination of the *Klebsiella pneumoniae* strain K5 from T24 cells through the reactive oxygen species pathway

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Abstract. Dual oxidase 2 (DUOX2) plays a major role in host defense in intestinal and airway epithelial cells through the reactive oxygen species (ROS) pathway. *Klebsiella pneumoniae* is a uropathogen that causes urinary tract infections. It is not known whether DUOX2 plays a role in host defense in bladder cancer epithelial cells. It is also not known whether *Klebsiella pneumoniae* invades T24 human bladder carcinoma cells and whether DUOX2 plays a role in eliminating the *Klebsiella pneumoniae* strain K5 through the ROS pathway in T24 cells. Thus, in the present study, we aimed to investigate the infectious capability of the *Klebsiella pneumoniae* K5 strain and the immunity-promoting capability of DUOX2 in T24 cells. We quantified the number of viable intracellular bacteria using the plate count method. DUOX2 expression was evaluated by western blot analysis and reverse transcription-quantitative PCR (RT-qPCR) follwoing treatment with or without multiple cytokines, phorbol 12-myristate 13-acetate (PMA), muramyl dipeptide (MDP), N-acetylmuramyl-D-alanyl-D-isoglutamine (MDP-DD), H_2O_2 inhibitor, catalase (CAT), the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase inhibitor, diphenyleneiodonium (DPI), or siRNA targeting DUOX2 (siDUOX2). The levels of ROS in the T24 cells infected with the K5 strain were examined following treatment with DPI, CAT or siDUOX2. Our results revealed that DUOX2 expression increased and the number of viable intracellular bacteria decreased in the T24 cells following infection with the K4 bacteria. Treatment with the cytokines and MDP and PMA also induced DUOX2 expression and decreased the number of viable intracellular bacteria. The levels of ROS also increased following treatment with the cytokines and MDP and PMA. However, when the cells were treated with the inhibitors (DPI or CAT), these effects were all reversed. Our data demonstrated that DUOX2 played an important role in innate immunity against bacterial cytoinvasion through the ROS pathway in T24 cells. Our findings also provide insight into the protection of uroepithelial cells from *Klebsiella pneumoniae* K5 bacterial cytoinvasion, and thus lay the foundation for the development of novel therapies for urinary tract infections.

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Introduction

The control of host-microbe homeostasis in the mucosal epithelium is essential for preventing microorganism-triggered inflammatory diseases. A number of common pathogenic bacteria are able to invade epithelial cells. Intestinal pathogens, such as *Salmonella*, *Shigella*, *Yersinia* and *Escherichia coli* are able to invade intestinal epithelial cells (1). Respiratory pathogens, such as *Pseudomonas aeruginosa* (2), *Haemophilus influenzae* (3), *Streptococcus pneumonia* (4) and *Klebsiella pneumoniae* (5) can enter airway epithelial cells. Uropathogens, such as *Escherichia coli* and *Klebsiella pneumoniae* (6) and *Stenotrophomonas maltophilia* (7) invade the host urothelium. Studies have demonstrated that some host cells have the ability to remove intracellular bacteria (8,9). We discovered that *Klebsiella pneumoniae* is capable of invading human T24 cells and that these cells, in turn, are capable of eliminating the infection.

Innate immunity plays an important role in the removal of invasive intracellular bacteria from epithelial cells. As regards invertebrates, the *Drosophila melanogaster* intestinal immune response relies mainly on 2 types of complementary and synergistic molecular effectors that restrict the proliferation of microorganisms: antimicrobial peptides and reactive oxygen species (ROS) (10). Oxidative defense mechanisms involving ROS play important roles in innate immunity (11). ROS mainly derive from mitochondrial metabolism or from regulated nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase (NOX) activity (12). The NOX family includes NOX1-5,

dual oxidase (DUOX)1 and DUOX2 (13), all of which function as components of the mucosal immune response (14). In fact, the T helper (Th)1 and Th2 cytokines promote host defense and pro-inflammatory responses by regulating the expression of DUOX1 and DUOX2 in human airways (11). Moreover, as previously demonstrated, DUOX2/dual oxidase maturation factor 2 (DUOXA2) co-transfected human thyroid cells treated with phorbol 12-myristate 13-acetate (PMA) exhibit increased H_2O_2 generation, which is associated with PMA-mediated DUOX2 phosphorylation (15).

ROS generation is necessary to initiate an innate immune response in normal human nasal epithelial cells (16), and this response mediates epithelial cell H_2O_2 production to counteract the replication of the respiratory syncytial virus (17) and influenza A virus (18) and prevent gastric *Helicobacter felis* colonization and the inflammatory response (10). DUOX2 activity is a possible source of ROS in all of these immune responses. Additionally, in the *Listeria monocytogenes* infection model, the simultaneous overexpression of nucleotide-binding oligomerization domain containing 2 (NOD2) and DUOX2 was found to result in cooperative protection against bacterial cytoinvasion (19). Therefore, as the main NOX, DUOX2 plays a major role in host defense in epithelial cells, including airway and intestinal epithelial cells, and acts as a host defense mechanism in phagocytes through the generation of ROS.

Klebsiella pneumonae is a common uropathogen found in diabetic patients and hospital settings and comprises up to 5% of urinary tract infections (20,21). While the antimicrobial substances in urine (such as urea) and the mucin of urinary tract mucosal surfaces provide a physiochemical barrier, intrinsic antibacterial activity may play an important role in maintaining bladder sterility.

Although the activation of DUOX1 has been shown to protect mouse urothelial cells from pathogens through the production of H_2O_2 (22), to the best of our knowledge, DUOX2 expression in bladder cancer epithelial cells has not been reported to date. Furthermore, whether *Klebsiella pneumonae* invades human T24 cells and whether DUOX2 plays a role in eliminating *Klebsiella pneumonae* through ROS signaling in T24 cells remains unknown. Thus, the aim of this study was to investigate DUOX2 expression in T24 cells and to determine whether DUOX2 contributes to immune host defense and the elimination of the *Klebsiella pneumonae* K5 strain in T24 cells through ROS production.

Materials and methods

Cell culture and treatment. T24 human bladder carcinoma cells (maintained in our laboratory) were cultured in RPMI-1640 medium (HyClone Laboratories, Inc., Logan, UT, USA) containing 10% newborn calf serum (Lanzhou Minhai Bioengineering Co., Ltd., Gansu, China) and grown at 37˚C in a humidified atmosphere with 5% CO_2. The experiments were performed using cells in the exponential growth phase.

The pro-inflammatory cytokines, interferon-γ (IFN-γ), tumor necrosis factor (TNF-)α, interleukin (IL)-1β and IL-17 (0-20 ng/ml); the NOX activator, PMA (0-1 nM); the NOD2 cognate ligand, muramyl dipeptide (MDP; 0-1 μg/ml); N-acetylmuramyl-D-alanyl-D-isoglutamine (MDP-DD; an MDP negative control; 0-1 μg/ml); the NOX inhibitor, diphenyleneiodonium (DPI ; 0-50 nM/ml) and the antioxidant, catalase (CAT; 0-1,000 U/ml), were purchased from Sigma (St. Louis, MO, USA). The cytokines were dissolved in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). DPI and PMA were dissolved in dimethyl sulfoxide (DMSO). MDP and MDP-DD were dissolved in distilled water. CAT was dissolved in RPMI-1640 medium. Untreated cells were used as controls.

Knockdown of DUOX2 using small interfering RNA (siRNA). The T24 cells were transfected with 20 nM/l siRNA targeting DUOX2 (DUOX2 siRNA; sense, 5'-GGCAGGAGACUAU GUUCUATT-3' and antisense, 5'-UAGAACAUAGUCUCCU GCCTT-3') according to the manufacturer's instructions. In brief, the T24 cells were seeded in 6- or 24-well culture plates 24 h prior to transfection. Lipofectamine 2000 (5 μl; Invitrogen Life Technologies, Carlsbad, CA, USA) and DUOX2 siRNA (10 μl; Shanghai GenePharma Co., Ltd., Shanghai, China) were added to serum-free cell culture medium prior to use and were then mixed. The mixture was incubated for 20 min before being added to the T24 cells (~80% confluent) which were grown for an additional 48 h prior to use.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions, and the RNA concentration was measured at an absorbance of 260 nm using a spectrophotometer (ULTROSPEC 2100; Biochrom Ltd., Cambridge, UK). The quality of the isolated RNA was confirmed on a 1% agarose gel. qPCR was performed using SYBR Premix Ex Taq (Takara Bio, Inc., Shiga, Japan) and a Bio-Rad C1000 real-time system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The primers for DUOX2 were sense, 5'-AACCTAAGCAGCTCA CAACT-3' and antisense, 5'-CAAGAGCAATGATGGTGAT-3'), as described in a previous study (7). The primers for GAPDH were sense, 5'-GGTCTCCTCTGACTTCAACA-3' and antisense, 5'-AGCC AAATTCGTTGTCATAC-3'. For each PCR reaction, cDNA was synthesized from 3 mg of RNA. The thermal cycling conditions involved an initial step consisting of denaturation at 95˚C for 30 sec, and then 40 cycles of the following 3 steps were performed: denaturation at 94˚C for 45 sec, annealing at 55˚C for 45 sec and elongation at 72˚C for 1 min. The results of RT-qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. The T24 cells were treated with siRNA, the cytokines (IFN-γ, TNF-α, IL-1β and IL-17), PMA, MDP or MDP-DD for 24 h and lysed in 2X lysis buffer (250 mM Tris-Cl, pH 6.5, 2% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue and 10% glycerol). The cell lysate (30 mg protein) was electrophoresed in sample buffer containing 10% SDS, transferred onto PVDF membranes that were blocked for 3 h in Tris-buffered saline (TBS; 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) containing 0.1% Tween-20 and 3% BSA, and subsequently incubated overnight at 4˚C with a primary antibody against DUOX2 (ab170308; Abcam Plc, Cambridge, UK) at a ratio of 1:500. The membranes were then washed 3 times with Tween-10/Tris-buffered saline (TTBS; 0.5% Tween-20 in TBS) and incubated with an appropriate horseradish perox-

idase-conjugated secondary antibody (5127s; Cell Signaling Technology, Beverly, MA, USA). Protein bands were visualized using ECL reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Removal of intracellular bacteria. The K5 bacterial strain was first grown in Luria-Bertani (LB) medium for 4-5 h at 37˚C while being shaken, and then further subcultured for 16-18 h. A total of 100 ml of bacteria grown to the exponential growth phase (OD650 nm, 0.4-0.6) was centrifuged at 10,000 x g at room temperature for 2 min and resuspended in PBS. The T24 cells were incubated with the bacteria and resuspended in RPMI-1640 medium at 37˚C for 2 h. The extracellular bacteria were then removed by washing 3 times with PBS supplemented with 100 mg/ml gentamicin to kill the extracellular bacteria. The cell monolayer was then washed twice with PBS and solubilized in 200 μl 0.5% Triton X-100 in PBS at 37˚C for 15 min for cell lysis. The solute was serially diluted in LB and plated on LB agar plates followed by incubation at 37˚C, and on the following day the colonies were counted.

Measurement of ROS levels. The T24 cells were incubated with 10 μM 2',7'-dichlorofluorescin diacetate (DCFDA; Molecular Probes, Eugene, USA) for 30 min and washed with 1 ml of PBS solution at least 5 times to remove the extracellular ROS. The levels of DCFDA fluorescence were measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a microplate reader (Thermo Fisher Scientific). The values were averaged in order to obtain the mean relative fluorescence intensity.

Statistical analysis. Statistical analysis was performed using the t-test with SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). A p-value <0.05 was considered to indicate a statistically significant difference.

Results

DUOX2 expression induced by infection with Klebsiella pneumoniae strain K5 suppresses the persistence of K5 in T24 cells. To observe the intracellular destiny of the K5 bacterial strain in the T24 cells, the intracellular bacterial colonization level in the lysates was counted at different time points following the bacterial infection of the T24 cells by RT-qPCR. At the same time, we examined whether DUOX2 activity affected the persistence of the K5 strain in the T24 cells. The number of viable intracellular bacteria peaked at 12 h, with a 52% increase being observed at 12 h compared to the 2-h time point (p<0.01); however, this number significantly decreased at 24, 48 and 72 h post-infection of the T24 cells with the K5 bacterial strain when compared with the number observed at 12 h (Fig. 1A; all p<0.01). This suggests that the intracellular bacterial growth was effectively restricted in the T24 cells. The expression levels of DUOX2 following infection of the T24 cells with the K5 bacterial strain were significantly elevated at 12, 24, 48 and 72 h, reaching peak levels at 48 h (Fig. 1B and C).

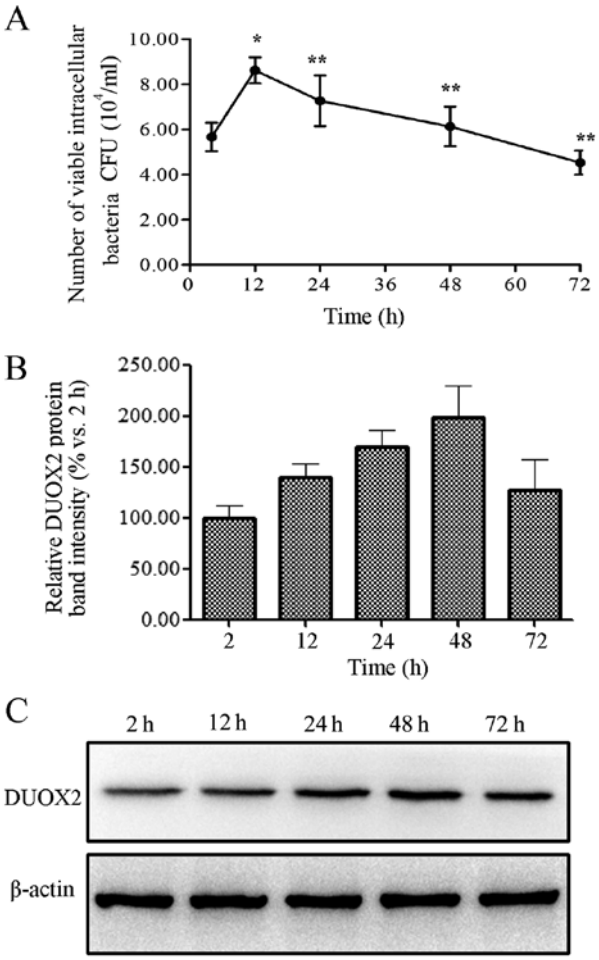

Figure 1. Infection with the *Klebsiella pneumoniae* K5 bacterial strain induces dual oxidase 2 (DUOX2) expression in T24 cells. Bacteria and T24 cells were incubated with the K5 bacteria for 2 h, and the extracellular bacteria were removed with gentamicin (100 mg/ml). The number of viable intracellular bacterial was calculated using the plate count method, and DUOX2 expression was measured by western blot analysis at 2, 12, 24, 48 and 72 h post-infection. (A) Number of viable intracellular bacteria. (B) Relative DUOX2 protein band intensity. (C) DUOX2 protein expression measured by western blot analysis. Data are representative results of at least 3 independent experiments, each performed in triplicate. *,**p<0.01, compared to the number of viable bacteria at 2 and 12 h, respectively. CFU, colony forming units.

Treatment with IFN-γ, TNF-α, IL-1β, PMA or MDP induces DUOX2 expression in T24 cells infected with the K5 bacterial strain. We verified that the T24 cells expressed DUOX2 (Fig. 1 B and C). The T24 cells were then treated with the cytokines, TNF-α, INF-γ, IL-1β, IL-17, or with PMA or MDP, and all treatments decreased the number of viable intracellular bacteria when compared with the control group (p<0.05; Fig. 2B). The DUOX2 expression levels in the T24 cells were significantly increased by all treatments, with INF-γ (4.17-fold) and MDP (3.88-fold) having the greatest effect on DUOX2 expression when compared with the control group (p<0.05; Fig. 2A, C and D).

DUOX2 knockdown leads to an increase in the number of viable K5 bacteria. The T24 cells were transfected with DUOX2 siRNA (siDUOX2) before being infected with the K5 bacterial strain. The extracellular bacteria were removed, and this was followed by treatment with IFN-γ, TNF-α, IL-1β, IL-17, PMA, MDP or MDP-DD. Transfection of the cells with

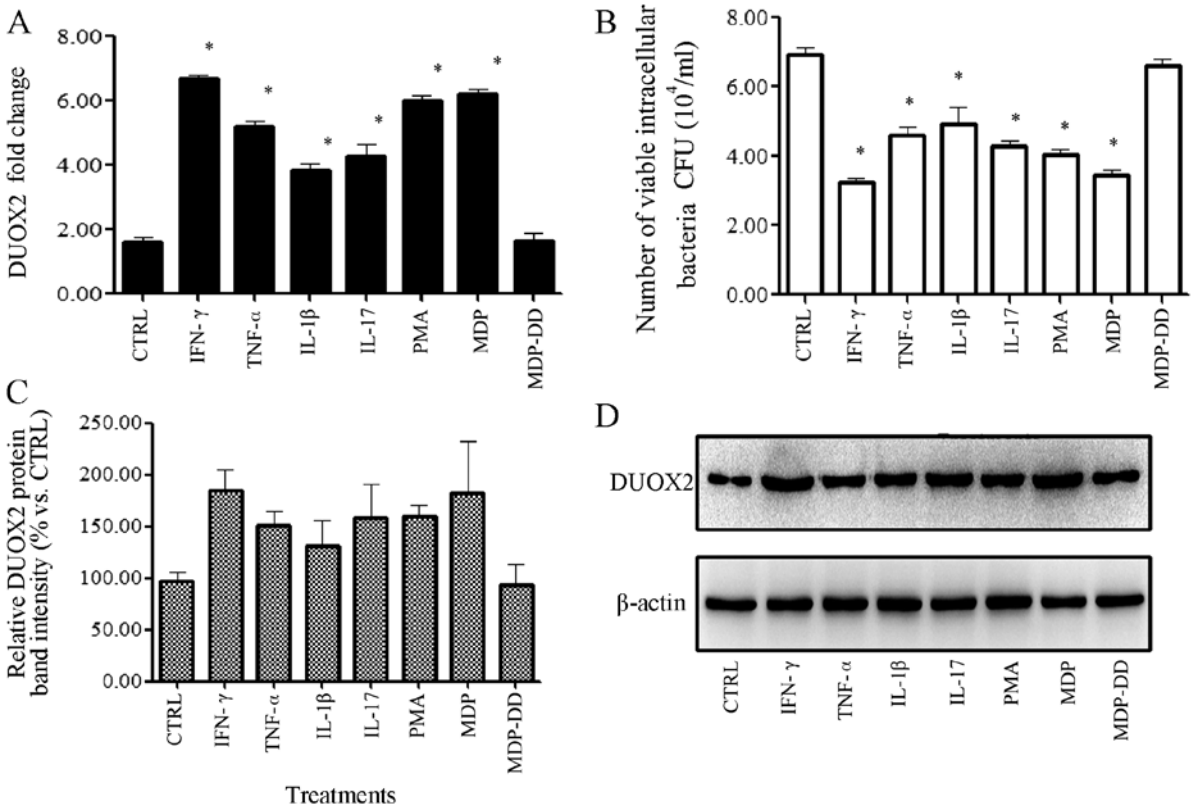

Figure 2. Chemical factors induce dual oxidase 2 (DUOX2) expression in T24 cells infected with the *Klebsiella pneumoniae* K5 bacterial strain 24 h post-infection. T24 cells were incubated with the K5 bacterial strain for 2 h, followed by treatment with various cytokines, phorbol 12-myristate 13-acetate (PMA), muramyl dipeptide (MDP) and N-acetylmuramyl-D-alanyl-D-isoglutamine (MDP-DD) for 2 h. The extracellular bacteria were removed with gentamicin (100 mg/ml). The number of viable intracellular bacteria was calculated using the plate count method, and DUOX2 expression was measured by qPCR and, western blot analysis. (A) DUOX2 was upregulated following treatment with PMA, MDP and cytokines. Treatment with MDP-DD had no effect. (B) The number of viable intracellular bacteria was decreased following treatment with the reagentst. (C) Relative DUOX2 protein band intensity. (D) DUOX2 protein expressino was upregulated following treatment. Data are representative of at least 3 independent experiments, performed in triplicate. CFU, colony forming units; CTRL, control; IFN γ, interferon-γ; TNF-α tumor necrosis factor; IL, interleukin. *p<0.05, compared to the control group.

siDUOX2 reduced DUOX2 expression which was induced by IFN-γ, TNF-α, IL-1β, IL-17, PMA and MDP (Fig. 3A).

The knockdown of DUOX2 using siRNA affected the antibacterial activity, and the number of viable intracellular bacteria increased by varying degrees (p>0.05) compared with the control group (Fig. 3B). The protein expression of DUOX2 was also suppressed to varying degrees following transfection of the cells with siDUOX2 (p<0.01; Fig. 3C and D).

Treatment with CAT or DPI suppresses the clearance of bacteria mediated by cytokine-, PMA- or MDP-induced DUOX2 expression in T24 cells infected with the K5 bacterial strain. To determine whether the NOX inhibitor, DPI, or the H_2O_2 inhibitor, CAT, affects the expression of DUOX2 in the T24 cells infected with the K5 bacterial strain, the T24 cells were incubated with the K5 bacterial strain for 2 h, the extracellular bacteria were removed by treatment with gentamicin (100 mg/ml) for 1 h, and the cells were then treated with CAT or DPI for 1 h. After 1 h, the inhibitor was replaced with IFN-γ, TNF-α, IL-1β, IL-17, PMA, MDP or MDP-DD for 24 h. Both CAT and DPI suppressed the induction of DUOX2 expression by all cytokines, PMA, and MDP (p<0.01; Fig. 4 A and C) compared with the control group. This led to no significant changes being observed in the viable intracellular bacterial numbers compared with the control group (P>0.05; Fig. 4B and D).

Treatment with cytokines, PMA or MDP increases intracellular ROS levels and this increase is reversed by CAT and DPI. To identify the mechanisms responsible for the generation of intracellular ROS induced by infection with the K5 bacterial strain, the T24 cells were incubated with the K5 bacterial strain for 2 h, treated with gentamicin for 2 h to remove extracellular bacteria, and then treated with the inhibitors (CAT or DPI) for 2 h. The T24 cells were then incubated with 10 μM DCFDA for 30 min, and the DCFDA fluorescence levels were then measured at 488/525 nm. All reagents (cytokines and PMA and MDP) were found to increase ROS generation (p<0.05; Fig. 5A compared to the control group; however, no changes in ROS levels were observed in the MDP-DD-treated cells. Treatment with siDUOX2, CAT or DPI inhibited the generation of intracellular ROS compared to the control group (p<0.05; Fig. 5A-C).

Discussion

It is known that DUOX1 is expressed in uroepithelial cells (22); however, to the best of our knowledge, whether DUOX2 plays a role in the removal of the K5 bacterial strain has not been reported to date. Moreover, whether DUOX2 inhibits bacterial cytoinvasion through the ROS signaling-mediated innate immune response in uroepithelial cells also remains unknown. The present study demonstrated that bladder epithelial cells

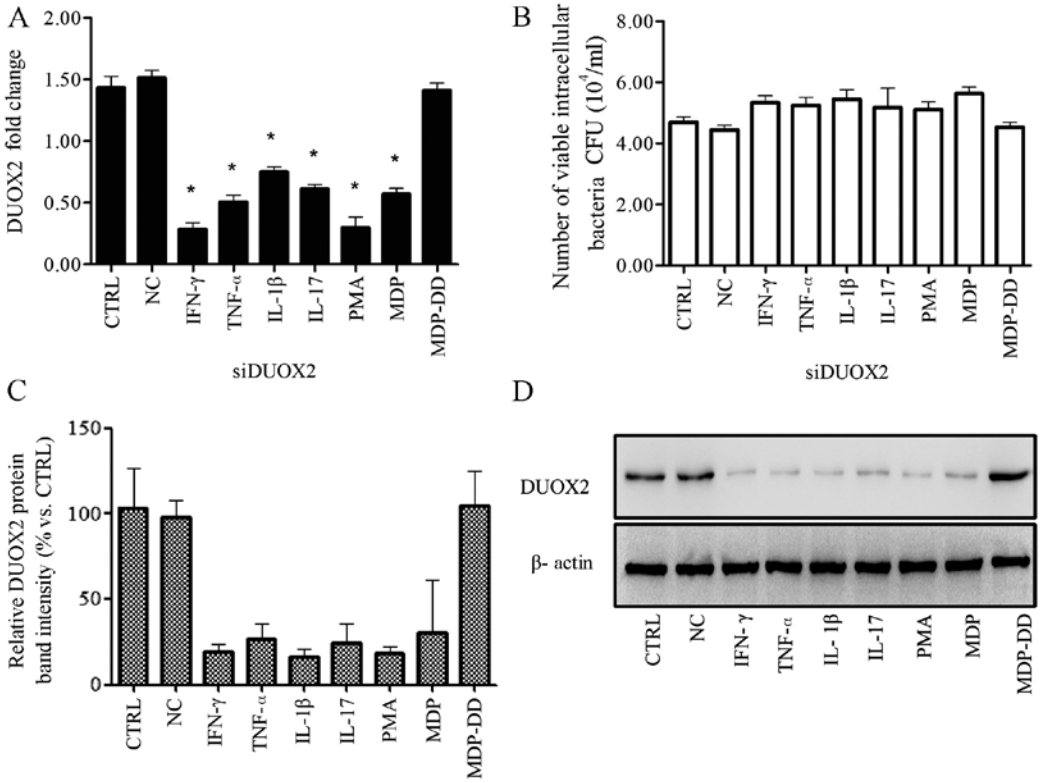

Figure 3. Knockdown of dual oxidase 2 (DUOX2) leads to increased cell colonization 24 h post-infection with the *Klebsiella pneumoniae* K5 bacterial strain. One day prior to the knockdown of DUOX2 by siRNA, the T24 cells were incubated with the K5 bacteria for 2 h, and this was followed by treatment with various cytokines, phorbol 12-myristate 13-acetate (PMA), muramyl dipeptide (MDP) or N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP-DD) for 2 h. The extracellular bacteria were removed with gentamicin (100 mg/ml). The number of viable intracellular bacteria was calculated using the plate count method, and DUOX2 expression was measured by qPCR and western blot analysis. (A) DUOX2 was downregulated following transfection with siRNA and treatment with cytokines, PMA, or MDP. (B) The number of viable intracellular bacteria increased following transfection with siRNA and treatment with the chemical reagents. (C) Relative DUOX2 intensity following transfection with siRNA, and treatment with the cytokines, PMA, or MDP. (D) DUOX2 protein expression was downregulated following transfection with siRNA and treatment with the cytokines, PMA, or MDP. Data are representative of at least 3 independent experiments, performed in triplicate. CFU, colony forming units; CTRL, control; IFN γ, interferon-γ; TNF-α tumor necrosis factor; IL, interleukin. $^{*}p<0.05$, compared to the control group.

have an innate antimicrobial mechanism that relies on ROS, and that DUOX2 plays an important role in the removal of the K5 bacterial strain through a ROS-mediated innate immune response which promotes the elimination of intracellular bacteria. The number of viable bacteria peaked at 12 h post-infection and significantly diminished thereafter, as DUOX2 expression increased (Fig. 1), indicating that infection with the K5 bacterial strain infection induced DUOX2 expression, which in turn led to the removal of the bacteria.

The role of DUOX2 in the removal of bacteria was confirmed by treatment with multiple cytokines (IFN-γ, TNF-α, IL-1β and IL-17), PMA and MDP. We observed that each treatment induced DUOX2 expression and also reduced the number of viable intracellular bacteria (Fig. 2). This observation was corroborated by the knockdown of DUOX2 expression with siRNA (Fig. 3). Following the knockdown of DUOX2 expression, treatment with the cytokines, PMA or MDP did not decrease the number of viable intracellular bacteria, which remained relatively unaltered compared with the control group (Fig. 3B). These findings are in line with the following findings from other studies: the knockdown of endogenous DUOX2 expression by RNA interference (RNAi) was shown to increase bacterial cytoinvasion and to abrogate the cytoprotective effects exerted by NOD2 in Caco-2 cells (23); *Drosophila* dual oxidase (dDuox) was shown to be essential for the maintenance of the intestinal redox system (24); and activated DUOX was shown to protect zebrafish larvae from infection with *Salmonella enterica* serovar Typhimurium (25).

In this study, we demonstrated that treatment with CAT (H_2O_2 inhibitor and DPI (NOX inhibitor) decreased the DUOX2 expresion levels induced by treatment with the cytokines, PMA, or MDP. As a result, following treatment with CAT and DPI, the numbers of viable bacterial numbers were relatively unaltered when compared to the control groups, even those which had been treated with the cytokines, PMA, or MDP (Fig. 4B and D). Additionally, treatment with all of the cytokines, PMA, or MDP increased the levels of ROS, while treatment with siDUOX2, CAT and DPI decreased the ROS levels, suggesting that DUOX2 promotes the elimination of bacterial from T24 cells through the ROS signaling pathway.

IFN-γ, TNF-α and IL-1β are well-known classic pro-inflammatory and anti-pathogenic cytokines. IFN-γ increases DUOX2 expression levels in the respiratory epithelium (26). TNF-α, in synergy with IFN-β, induces DUOX2 NOX expression (17). Signal transduction and inflammatory responses induced by IL-1β are related to the activity of NOX-1 in Caco-2

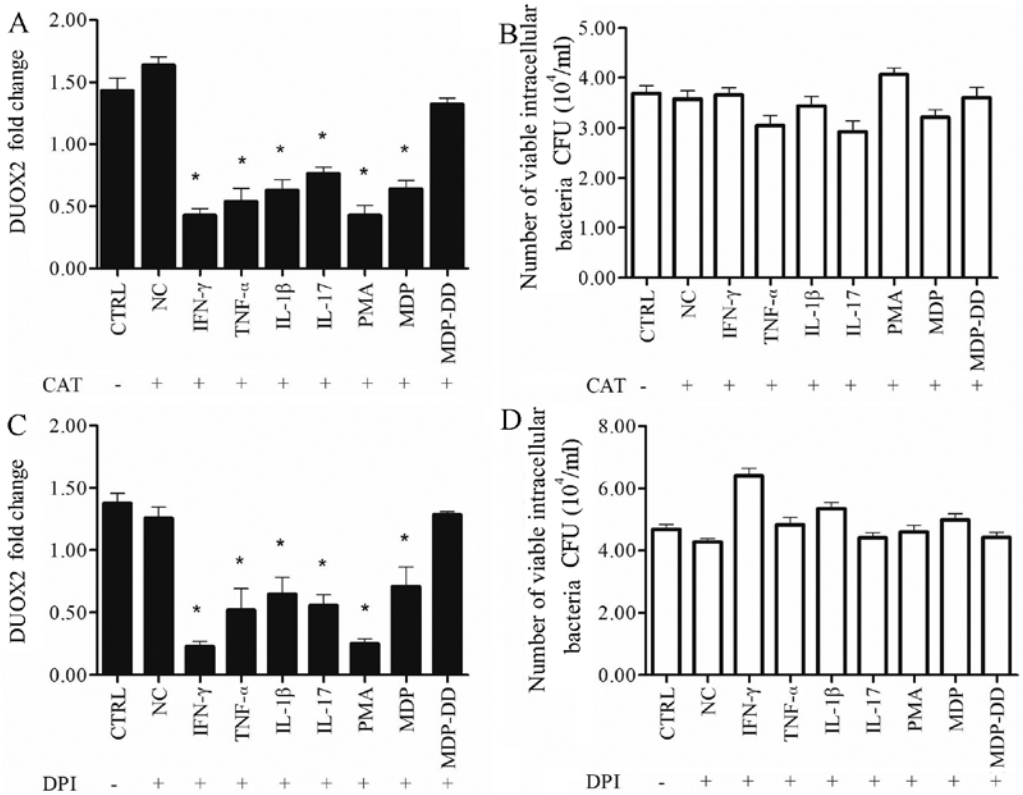

Figure 4. Treatment with catalase (CAT; H_2O_2 inhibitor) or diphenyleneiodonium (DPI; NOX inhibitor) decreases dual oxidase 2 (DUOX2) expression and suppresses the clearance of bacteria mediated by cytokine-, phorbol 12-myristate 13-acetate (PMA)- or muramyl dipeptide (MDP)-induced DUOX2 expression in T24 cells infected with the *Klebsiella pneumoniae* K5 bacterial strain. T24 cells were incubated with the K5 bacteria for 2 h and then treated with the inhibitors (CAT and DPI) for 2 h, and the extracellular bacteria were removed with gentamicin (100 mg/ml). The number of viable intracellular bacteria was calculated using the plate count method, and dual oxidase 2 (DUOX2) expression was measured by qPCR. The cells were treated with (A and C) CAT (0-1,000 U/ml) or (C and D) DPI (0-50 nM/ml). DUOX2 expression was downregulated and the number of viable intracellular bacteria was increased following treatment with the inhibitors. Data are representative result of at least 3 independent experiments, performed in triplicate. CFU, colony forming units; CTRL, control; IFN γ, interferon-γ; TNF-α tumor necrosis factor; IL, interleukin; MDP-DD, N-acetylmuramyl-D-alanyl-D-isoglutamine. *p<0.05, compared to the control group.

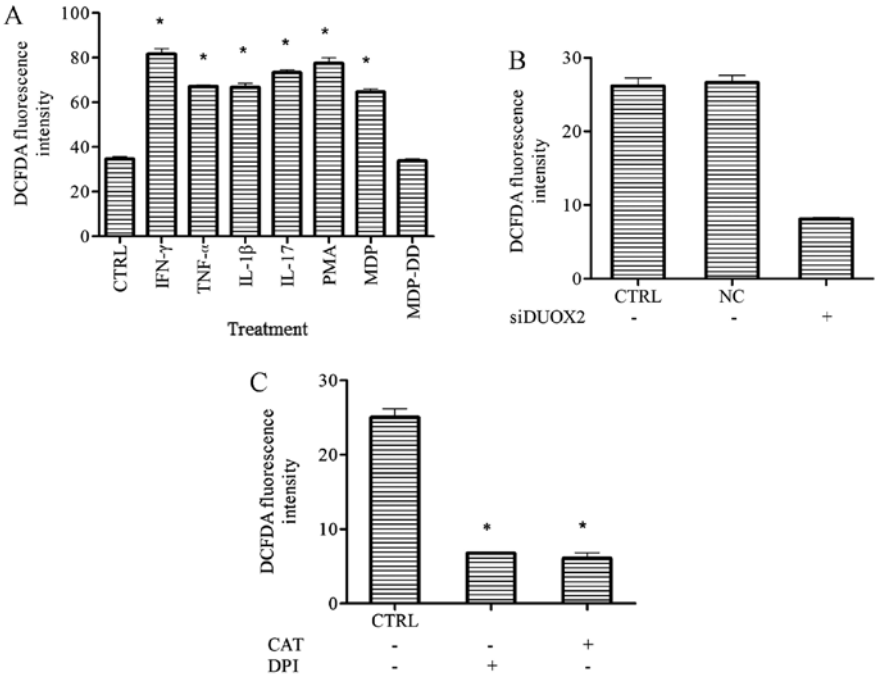

Figure 5. The generation o intracellular reactive oxygen species (ROS) is induced in T24 cells 6 h following infection with the *Klebsiella pneumoniae* K5 bacterial strain. The cells were either transfected or not or siRNA targeting dual oxidase 2 (siDUOX2) and then treated with the chemical inhibitors for 2 h; ROS levels were then measured by determining the relative fluorescence intensity. (A) ROS levels were increased following treatment with the cytokines, phorbol 12-myristate 13-acetate (PMA), muramyl dipeptide (MDP) or N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP-DD). (B) ROS levels were decreased following transfection with siDUOX2. (C) ROS levels were downregulated following treatment with the inhibitors catalase (CAT; H_2O_2 inhibitor) or diphenyleneiodonium (DPI; NOX inhibitor). All the experiments were repeated at least 3 times. CTRL, control; IFN γ, interferon-γ; TNF-α tumor necrosis factor; IL, interleukin. *p<0.05, compared to the control group.

cells (27,28). IL-17 is essential for host defense against a number of microbes, particularly extracellular bacteria and fungi (29), and it induces NOX production in human cartilage, chondrocytes and osteoarthritic human and mouse cartilage (30,31). As an immunoadjuvant and peptidoglycan (a structural subunit of the bacterial cell wall), MDP enhances the ability of phagocytes to engulf bacteria, increases natural killer (NK) cell lethality and promotes T cell activation in order to strengthen host resistance to pathogen infection. MDP is a well-known ligand for the intracellular NOD2 receptor (32).

NOX appears to be a particularly important enzyme for ROS generation in non-phagocytic cells, and it is a prominent generator of ROS in the airway epithelium (33,34). Mounting evidence indicates that intracellular ROS facilitates cellular damage or stress and contributes to innate immune activation (35). ROS enhance host immunity through the prevention of pathogen-induced pro-inflammatory cytokines (36-38).

Nuclear factor (NF)-κB is a critical signaling molecule in H_2O_2-induced inflammation and responses evoked by a variety of stimuli, including growth factors, lymphokines, ultraviolet irradiation, pharmacological agents and oxidative stress (39). A growing body of evidence indicates that endogenous ROS are characteristic of typical second messengers responsible for the activation of NF-κB in a variety of cell types, including neutrophils, lymphocytes, endothelial cells and epithelial cells (19). In particular, the NOX family member, DUOX2, is involved in NOD2-dependent ROS production (19). DUOX2 functions as a molecular switch that enhances the protective properties of NOD2 through both direct (generation of bactericidal ROS) and indirect mechanisms (by amplifying NF-κB signaling) (16). PMA, a protein kinase C activator, activates NAPDH protein molecules, induces DUOX2 phosphorylation to generate high levels of H_2O_2 (15,30) and promotes antibacterial effects exerted by the NOX family. The antibacterial role of PMA may be evidenced through the NF-κB signaling pathway. MDP is a peptidoglycan moiety derived from commensal and pathogenic bacteria and a ligand of its intracellular sensor NOD2. The *ex vivo* stimulation of human carotid plaques with MDP has been shown to lead to the enhanced activation of the inflammatory signaling pathways, p38 and MAPK, and the NF-κB-mediated release of pro-inflammatory cytokines (40). IFN-β and IL-1β can cooperate synergistically to upregulate DUOX2 expression, and the contribution of NF-κB signaling to DUOX2 expression cannot be excluded, since IL-1β signaling also activates NF-κB (22). TNF-α, IFN-γ, IL-1β and IL-17 are important cytokines that regulate host immune defense, and thus we hypothesized that they may also function through NF-κB signaling to increase DUOX2 expression in infected T24 cells.

Mechanistically, NOX-mediated ROS generation constitutes a well-known host defense mechanism in phagocytes, and DUOX1 and DUOX2 provide extracellular H_2O_2 to lactoperoxidase (LPO) to produce antimicrobial hypothiocyanite ions (11). DUOX-generated H_2O_2, in conjunction with LPO and thiocyanate (SCN^-), kills bacteria in the respiratory tract (41,42).The presence of H_2O_2 in the airway allows LPO to oxidize the airway surface liquid (ASL) component, SCN^- (27), thereby generating antimicrobial hypothiocyanite ($OSCN^-$) in order to support oxidant-mediated bacterial killing in the airways (42).In a previous study, *Pseudomonas aeruginosa* flagellin infected human airway epithelial cells to facilitate host invasion through the inactivation of the DUOX/LPO/SCN^- protective system (42). $OSCN^-$ is abundantly found in saliva and other mucosal secretions, acting as an effective microbicidal or microbiostatic agent, and the DUOX/LPO/SCN^- antimicrobial system is fully assembled only in the final stages of saliva formation, with H_2O_2 provided by DUOX2 (43). H_2O_2 generated by DUOX in the airway epithelium supports the production of bactericidal OSCN(-) in the presence of the airway surface liquid components, LPO, and SCN(-), and OSCN(-) eliminates *Staphylococcus aureus* and *Pseudomonas aeruginosa* on airway mucosal surfaces (11).DUOX1 also plays a role in the protection of urothelial cells, and alterations in ROS production in the urothelium may contribute to the development of bladder diseases (22).

In conclusion, the findings of the present study demonstrate that innate immunity may protect T24 bladder cancer cells from invasion by the K5 bacterial strain through the upregulation of DUOX2 expression, mediated through ROS signaling. Our findings provide insight into the mechanisms through which uroepithelial cells are protected from cytoinvasion by the K5 bacterial strain, and may thus lead to the development of novel therapeutic strategies for urinary tract infections.

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