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TLR-7 agonist attenuates airway reactivity and inflammation through Nrf2-mediated antioxidant protection in a murine model of allergic asthma

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Abbreviations: BAL, Bronchoalveolar lavage; DETC, Diethylthiocarbamate; iNOS, inducible nitric oxide synthase; i.n., Intranasal; MyD88-IP, MyD88 inhibitory peptide; Nrf2, Nuclear factor E2-related factor 2; NOX, NADPH oxidase; ROS, Reactive oxygen species; RSQ, Resiquimod; SOD, Superoxide dismutase; TLR, Toll-like receptor
Abstract

Toll-like receptors (TLRs) through innate immune system recognize pathogen associated molecular patterns and play an important role in host defense against bacteria, fungi and viruses. TLR-7 is responsible for sensing single stranded nucleic acids of viruses but its activation has been shown to be protective in mouse models of asthma. The NADPH oxidase (NOX) enzymes family mainly produces reactive oxygen species (ROS) in the lung and is involved in regulation of airway inflammation in response to TLRs activation. However, NOX-4 mediated signaling in response to TLR-7 activation in a mouse model of allergic asthma has not been explored previously. Therefore, this study investigated the role TLR-7 activation and downstream oxidant-antioxidant signaling in a murine model of asthma. Mice were sensitized with ovalbumin (OVA) intraperitoneally and treated with TLR-7 agonist, resiquimod (RSQ) intranasally before each OVA challenge from days 14 to 16. Mice were then assessed for airway reactivity, inflammation, and NOX-4 and nuclear factor E2-related factor 2 (Nrf2) related signaling [inducible nitric oxide synthase (iNOS), nitrotyrosine, lipid peroxides and copper/zinc superoxide dismutase (Cu/Zn SOD)]. Treatment with RSQ reduced allergen induced airway reactivity and inflammation. This was paralleled by a decrease in ROS which was due to induction of Nrf2 and Cu/Zn SOD in RSQ treated group. Inhibition of MyD88 reversed RSQ-mediated protective effects on airway reactivity/inflammation due to reduction in Nrf2 signaling. SOD inhibition produced effects similar to MyD88 inhibition. The current study suggests that TLR-7 agonist is beneficial and may be developed into a therapeutic option in allergic asthma.

Keywords: Airway inflammation, TLR-7 agonist, NADPH oxidase, Nrf2, Cu/Zn SOD
1. Introduction

Asthma is one of the most common airway diseases characterized by eosinophilic/neutrophilic inflammation and airway hyperreactivity. ROS signaling plays an important role in the initiation and perpetuation of asthma pathogenesis. In response to allergens, there is increased production of ROS from inflammatory and structural cells in the airways of asthmatics that is associated with airway inflammation/remodeling and mucus hypersecretion (Lee and Yang, 2012; Nadeem et al. 2003; Nadeem et al. 2014a; Zuo et al. 2013). Excess ROS production overpowers the normal antioxidative capacity of the lung thus leading to impairment in the function of different lung components such as epithelium, endothelium and airway smooth muscle. Upregulation of antioxidants may be required to counteract increased ROS production in asthma (Boueiz and Hassoun 2009; Nadeem et al. 2008; Nadeem et al. 2014a).

The main source of cellular ROS production is the family of NADPH oxidases (NOXs), which consists of seven different isoforms (Bedard and Krause, 2007; Katsuyama et al. 2011; Nadeem et al. 2014a). Different NOXs play important roles in the lung under both physiological and pathological conditions. For example, phagocytic NOX-2 is the main isoform responsible for the ROS generation under inflammatory conditions in the lung. Overproduction of ROS by NOX-2 been shown to be associated with allergen and virus-induced airway inflammation (Fink et al. 2008; Nadeem et al. 2014b; Vlahos et al. 2011). Another important isoform of NOX in the lung is NOX-4. NOX-4 has been shown to be upregulated in asthmatics as compared to control subjects (Sutcliffe et al. 2012). NOX-4 is also involved in pulmonary fibrosis as well as virus-/bacteria-mediated lung
Together, these findings suggest that these two NOX isoforms play an important role in the regulation of airway inflammation.

TLRs present on innate immune cells recognize pathogen associated molecular patterns and lead to a variety of responses which serve to protect the host from invading pathogens. Emerging evidence suggests that NOX-derived ROS contribute to diverse signaling processes, including TLR-induced inflammation (Amatore et al. 2015; Nadeem et al. 2015; Yang et al. 2013). For example, it has been demonstrated that NOX-2 plays an important role in TLR-2 as well as TLR-3 dependent inflammatory responses and antimicrobial activity against pathogens. Other TLRs involved in sensing the presence of pathogens in the airways also play an important role in airway inflammation through different isoforms of NOXs (Lee et al. 2008; Ryu et al. 2013; Yang et al. 2013). However, role of NOX-2/NOX-4 in response to TLR-7 activation has not been explored in a mouse model of allergic asthma previously.

TLR-7 signaling has been shown to be protective in murine models of allergic asthma. Several mechanisms have been postulated to contribute to TLR-7 mediated anti-inflammatory effect. For example, one study has shown suppression of Th2 cytokines whereas others have shown anti-inflammatory effect through suppression of both Th1 and Th2 cytokines in response to TLR-7 activation (Grela et al. 2011; Camateros et al. 2007; Moisan et al. 2006). However, no study so far has investigated the effect of TLR-7 activation on nuclear factor E2-related factor 2 (Nrf2) signaling in murine model of asthma. This pathway may be one of the mechanisms by which TLR-7 activation contributes to anti-inflammatory effects in the lung.
Nrf2 is a transcription factor that is activated by ROS and responsible for detoxification through induction of various antioxidant genes (Lee and Yang, 2012; Liu et al. 2015; Spiess et al. 2013; Yao et al. 2007). One of the antioxidants that may be induced through Nrf2 is superoxide dismutase (SOD). SOD may provide protection against excess ROS generated from inflammatory cells in asthmatic lung (Eggler et al. 2008; Nadeem et al. 2014a; Zhu et al. 2005). These observations are also supported by studies that show exacerbation of pulmonary inflammation in Nrf2 or SOD knockout mice (Cho et al. 2013; Kwon et al. 2012; Rangasamy et al. 2005).

In this study, we tested the hypothesis that TLR-7 agonist triggers activation of antioxidant pathways that prevent excessive airway inflammation in a murine model of allergic asthma. Our study shows that treatment of allergen sensitized and challenged mice with TLR-7 agonist, resiquimod leads to Nrf2-mediated antioxidant protection against excessive ROS production.
2. Materials and methods

2.1. Animals

Male Balb/c mice, 8 to 10 weeks of age (20-25 g), free of specific pathogens, were used in the experiments. The animals were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were kept under standard laboratory conditions of 12-h light-dark cycle and 24-26ºC ambient temperature. All experimental animals used in this study were under a protocol approved by Animal Care and Research Committee of College of Pharmacy, King Saud University.

2.2. Mice Sensitization and Challenge

Sensitization was performed according to the protocol described earlier (Nadeem et al. 2014b; Rievaj et al. 2011). Mice were sensitized on days 1 and 6 with intraperitoneal injections of 10 µg ovalbumin (grade V), adsorbed to 4 mg alum. Non-sensitized control animals received only alum with the same volumes. Two weeks after 1st sensitization, the mice were challenged intranasally (i.n.) under light anesthesia with 100 µg ovalbumin once only on days 14, 15, and 16. To assess the role of TLR-7 during allergic responses, a synthetic TLR-7 ligand, resiquimod (RSQ; Tocris, UK) was administered i.n before each allergen challenge at a concentration of 20 µg/mouse. MyD88 inhibitory peptide (MyD88-IP; InvivoGen, USA) or diethylthiocarbamate (DETC) was administered i.n at a concentration of 100 µg/mouse and 5 mg/mouse before RSQ to block MyD88 and SOD mediated effects respectively in allergic/control groups.
Mice were divided into following groups: Control group (CON): mice received only vehicles for sensitization and challenge; Control group administered RSQ or MyD88-IP (CON+RSQ or CON+MyD88-IP) respectively: mice received only vehicles for sensitization and challenge, and RSQ or MyD88-IP was administered i.n. on days 14, 15 and 16; Sensitized and challenged group (SEN^{CHAL}): mice were sensitized and challenged with ovalbumin using the same protocol described above; Sensitized and challenged group administered RSQ (SEN^{CHAL}+RSQ): mice were sensitized and challenged with ovalbumin using the same protocol described above and RSQ was administered before each allergen challenge; Sensitized and challenged group administered MyD88-IP before RSQ (SEN^{CHAL}+MyD88-IP+RSQ): mice were sensitized and challenged with ovalbumin using the same protocol described above and MyD88-IP was administered before every RSQ treatment; Sensitized and challenged group administered DETC before RSQ (SEN^{CHAL}+DETC+RSQ): mice were sensitized and challenged with ovalbumin using the same protocol described above and DETC was administered every RSQ treatment.

2.3. Measurement of airway reactivity in vivo

Six hours after final allergen challenge, airway reactivity to methacholine in conscious, unrestrained mice were assessed by a whole-body noninvasive plethysmograph (Buxco Electronics Inc.) as described earlier (Nadeem et al. 2014b; Nadeem et al. 2015). Baseline Penh was determined by exposing mice to nebulized saline. The mice were then exposed to increasing concentrations of aerosolized methacholine dissolved in saline (0-32 mg/ml) to obtain a dose response and Penh values were recorded at each dose.

2.4. Bronchoalveolar lavage (BAL)
The mice were sacrificed by isoflurane anesthesia and the trachea was cannulated to perform BAL one day after final allergen challenge; phosphate-buffered saline was introduced into the lungs via the tracheal cannula and the total cells were counted manually in a hemocytometer chamber followed by spinning of cells onto glass slides for differential count. A differential count of at least 300 cells was made according to standard morphologic criteria on cytocentrifuged Diff-Quik stained slides. The number of cells recovered per mouse was calculated and expressed as mean ± SE per ml for each group. The level of total protein concentration as a measure of lung permeability/injury in BAL fluid (BALF) was determined using a commercial kit (Bio-Rad, Hercules, CA) using the Bradford method.

2.5. Real-time PCR

Total RNA was isolated by TRIzol reagent (Invitrogen, USA) from the tracheas/lungs of different groups as described previously (Nadeem et al. 2014b) and checked for purity by Nanodrop 1000 (Thermo Scientific, USA). This was followed by conversion of 0.5 μg of total RNA into cDNA using High Capacity cDNA archive kit (Applied Biosystems, USA) according to the manufacturer’s instructions as described earlier (Nadeem et al. 2014b; Nadeem et al. 2015). Real-time PCR was performed on an ABI PRISM 7500 Detection System (Applied Biosystems) using Taqman Universal Mastermix (Applied Biosystems, USA), cDNA, and FAM-labeled Taqman gene expression kit. For the real-time PCR of NOX-2, NOX-4, Cu/Zn SOD, Nrf2, and iNOS, the Taqman assays-on-demand gene expression kits were purchased from Applied Biosystems. 18S rRNA (Ribosomal RNA) was used as an endogenous control. The fold difference in expression of target cDNA was determined using the comparative C_T method. The fold difference in
gene expression of the target was calculated as described earlier (Livak and Schmittgen, 2001).

2.6. Western immunoblotting

Aliquots of the tracheal supernatants isolated from different groups (30 µg protein/well) were separated on 10% SDS-PAGE as described earlier (Nadeem et al. 2015). Proteins were transferred to nitrocellulose membranes and then probed either with polyclonal goat NOX-4, or polyclonal goat NOX-2, or polyclonal rabbit Nrf2, or polyclonal rabbit nitrotyrosine, or polyclonal rabbit Cu/Zn SOD antibodies (Santa Cruz Biotechnology, USA) at a dilution of 1:500-1000, or GAPDH rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:5000. This was followed by the incubation with the secondary horseradish peroxidase-conjugated antibodies (anti-goat and anti-rabbit IgGs; Santa Cruz Biotechnology, USA) for 1 h at room temperature. For detection of bands, the membranes were treated with enhanced chemiluminescence reagent (Amersham ECL, GE Healthcare) followed by exposure to ECL Hyperfilm. The relative expression of the protein bands was quantified by densitometric analysis. Western blot values are expressed in percentage after normalization to GAPDH levels.

2.7. Reactive oxygen species (ROS) assay: For reactive oxygen species generation, the harvested trachea were incubated with 100 µM 6-carboxy-2’,7’-dichlorofluorescin diacetate (DCFH-DA) for 30 min at 37°C. DCFH-DA forms a fluorescent product, DCF (dichlorofluorescein) upon oxidation with ROS. Fluorescence caused by DCF in each well was measured and recorded for 30 min at 485 nm (excitation) and 530 nm (emission) by the method of Wang and Joseph (2006) using a multi-mode fluorescent microplate reader (FLUOstar Omega, BMG LabTech, USA) with temperature maintained
at 37°C as described earlier by us (Nadeem et al. 2014b; Nadeem et al. 2015). The background fluorescence caused by buffer and DCF were subtracted from the total fluorescence in each well caused by the trachea in the presence of DCF. Fluorescence intensity was expressed as ROS generation (% control).

2.8. Lipid peroxides assay: Tissue lipid peroxides were measured as malondialdehyde (MDA)-thiobarbituric acid (TBA) adducts by the method of Jentzsch et al. (1996) as described earlier (Nadeem et al. 2003). Briefly, the sample was incubated with butylated hydroxyl toluene, ortho-phosphoric acid and TBA at 90°C for 45 min, followed by ice-cooling and extraction of MDA-TBA adducts in n-butanol. Absorption was read at 535 and 572 nm for baseline correction in a multititer plate reader. MDA-TBA adducts were calculated using the difference in absorption at the two wavelengths compared to the standard curve generated by the use of tetraethoxypropane. Results were expressed in nmol/mg protein.

2.9. Chemicals

Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (USA).

2.10. Statistical analysis

The data were expressed as mean±SEM. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed by Tukey’s multiple comparison tests. A ‘P’ value of less than 0.05 was considered significant for all statistical tests. All the statistical analyses were performed using Graph Pad Prism statistical package.
3. Results

3.1. Effect of TLR-7 agonist on allergen induced airway reactivity and inflammation

As shown in Fig.1A, allergen challenge led to significant increase in airway inflammation as reflected by an increase in total cell, eosinophil and neutrophil counts. Treatment with TLR-7 agonist, RSQ attenuated allergen induced airway inflammation (Fig 1A). Allergen challenge also led to significant increase in Penh, a measure of airway reactivity, which was also attenuated by TLR-7 agonist, RSQ (Fig 1B). Control group treated with RSQ did not show any significant effect either on airway inflammation (Fig. 1A) or airway reactivity (Fig. 1A). Total protein concentration in BAL fluid was significantly elevated in allergen sensitized and challenged group (157±16 µg/ml, n=6) when compared with control group (87±9 µg/ml, n=5); however RSQ treated allergic group (103±12 µg/ml, n=6) did not have any significant difference when compared with control group. These data suggest that RSQ is responsible for activating anti-inflammatory signaling in allergic mice.

3.2. Effect of TLR-7 agonist on allergen induced NOX-2/NOX-4 expression and ROS production

Since ROS signaling has been shown to be responsible for both airway reactivity and inflammation, we hypothesized that RSQ mediated protective effect could be through reduction in ROS in the lung. Allergen sensitized and challenged mice had higher tracheal ROS production as compared to control group. Treatment with RSQ led to attenuation of allergen induced tracheal ROS generation (Fig 2A). However, tracheal NOX-2 mRNA and protein expression was not affected by RSQ in sensitized and
challenged group. Moreover, tracheal NOX-4 mRNA and protein expression was upregulated only in allergic mice treated with RSQ as compared to other groups (Fig 2B-C). Lung NOX-2 and NOX-4 showed pattern of mRNA expression similar to trachea in all groups (Fig. 2D). These data suggest that reduction in ROS despite increase in NOX-2 expression may be due to induction in antioxidant signaling responsible for ROS scavenging.

3.3. Effect of TLR-7 agonist and allergen on Nrf2 and Cu/Zn SOD expression

Since SOD is mainly responsible for scavenging of ROS produced by NOXs, we reasoned that RSQ treated group could have increased SOD expression. Indeed, RSQ treated group had significantly increased Cu/Zn SOD mRNA and protein expression in trachea as compared to other groups (Fig. 3A-B). Next we examined, if there was any effect on Nrf2 expression as its activation is connected with induction of antioxidant genes. Our study showed that RSQ treated group had significantly increased tracheal Nrf2 mRNA and protein expression as compared to other groups (Fig. 3C-D). RSQ treatment to allergic mice also enhanced Cu/Zn SOD and Nrf2 mRNA expression in the lung (Fig. 3E).

Next, we examined the effect of TLR-7 agonist on other oxidative parameters such as iNOS, nitrotyrosine and lipid peroxides. Higher expression of Cu/Zn SOD in trachea led to lower nitrotyrosine protein expression and lipid peroxides levels without any effect on iNOS expression in RSQ treated allergic group compared to allergic group (Fig. 4A-C). This suggests that excessive ROS generation is scavenged by increased expression of Cu/Zn SOD in the trachea of RSQ treated mice. Therefore, reduction in ROS is mainly
due to Nrf2 mediated upregulation of Cu/Zn SOD and may be responsible for anti-inflammatory effects of RSQ in allergic mice.

3.4. Reversal of TLR7 agonist induced anti-inflammatory responses by MyD88 inhibitor peptide and SOD inhibitor

Since MyD88 signaling is responsible for TLR7 mediated effects, we examined if its inhibition led to reversal of TLR7 agonist induced protective responses. Also if SOD was involved in mediating any anti-inflammatory effects of TLR7 agonist, its inhibition is likely to produce effects similar to MyD88 inhibition. Allergic mice treated either with SOD inhibitor (DETC) or MyD88 inhibitor peptide (MyD88-IP) before RSQ had greater airway reactivity and inflammation as depicted by increased Penh and cell counts (both total and eosinophil counts) as compared to RSQ treated allergic mice (Fig. 5A-B). Control group treated with MyD88-IP did not show any significant effect on airway reactivity (Penh) when compared to control group (data not shown). Allergic mice treated either with DETC or MyD88-IP before RSQ also had greater ROS generation as compared to RSQ treated allergic mice (Fig. 6A). NOX-4 expression was unaffected by DETC treatment, whereas MyD88-IP treatment before RSQ treatment reduced its expression in allergic mice (Fig. 6B-C). NOX-2 expression was unaffected either by treatment with MyD88-IP or DETC (data not shown). The probable cause of increased ROS generation in DETC treated group was due to inhibition of SOD, whereas in MyD88-IP treated group, it was due to decreased Nrf2 and Cu/Zn SOD expression (Fig. 7A-B). These data show that inhibition of MyD88 signaling leads to reduction in Nrf2-
Cu/Zn SOD signaling. Hence, Nrf2-Cu/Zn SOD pathway could be mainly responsible for inhibitory effects of TLR-7 agonist on airway reactivity and inflammation in this study.

Next we examined, if there was reversal of oxidative stress also by inhibition of MyD88 or SOD signaling. Indeed, we found increased nitrotyrosine content along with lipid peroxides levels (Fig. 7E-F) without any effect on iNOS (data not shown) in trachea of allergic mice treated either with DETC or MyD88-IP before RSQ as compared to RSQ treated allergic mice. These data show that inhibition of MyD88 or SOD before RSQ treatment in allergic mice leads to induction of oxidative stress. Overall, the complete data set suggests that TLR-7 agonist shows anti-inflammatory activity in allergic mice through activation of Nrf2-Cu/Zn SOD pathway (Fig. 8).
4. Discussion

This study explores one of the mechanisms by which TLR-7 activation confers protective effect in a murine model of allergic asthma. TLR-7 agonist, RSQ before allergen challenge prevented ROS generation and subsequent AHR and airway inflammation. This was paralleled by activation of Nrf2 and Cu/Zn SOD signaling. This suggests that TLR-7 activation enhances antioxidant network in the lung for protection against ROS-mediated airway reactivity and inflammation.

Aeroallergens as well as viral/bacterial products cause release of several mediators via activation of TLRs in the lung. Among these mediators, ROS generation is also thought to be responsible for various aspects of airway hyperresponsiveness and inflammation (Nadeem et al. 2014b; Nadeem et al. 2015; Ryu et al. 2013). This has been confirmed by ROS scavengers which attenuate allergen induced airway hyperresponsiveness and inflammation in various animal models of asthma (Nadeem et al. 2014b; Peh et al. 2015). Furthermore, different isoforms of NOX have been shown to regulate airway inflammation and hyperreactivity in both animal and human studies (Sutcliffe et al. 2012; Nadeem et al. 2014b; Nadeem et al. 2015; Voraphani et al. 2014). However, to the best of our knowledge, no study has investigated the effect of TLR-7 activation on NOX-2/NOX-4 signaling during allergic responses in a murine model of asthma.

Activation of TLRs has been shown to be associated with both aggravation as well as amelioration of airway reactivity and inflammation. For example, TLR-3, TLR4 and TLR-9 ligands have been shown to exacerbate allergic airway inflammation in mice (Adner et al. 2013; Clarke et al. 2014; Reuter et al. 2012). On the other hand, TLR-7 activation has been found to prevent allergen-induced airway hyperresponsiveness,
eosinophilia and airway remodeling in murine models of allergic asthma (Moisan et al. 2006; Camateros et al. 2007; Grela et al. 2011).

Several mechanisms have been proposed for the protective effect of TLR-7 agonists in murine models of allergic asthma. For example, some studies have suggested that treatment with TLR-7 agonist causes a shift in immune response from allergic Th2 cytokines to non-allergic Th1 cytokines (Brugnolo et al. 2003; Grela et al. 2011; Xirakia et al. 2010), whereas other studies have shown anti-inflammatory effect of TLR-7 agonist through suppression of both Th2 and Th1 cytokines (Adner et al. 2013; Camateros et al. 2007). Furthermore, Moisan et al. (2006) have shown downregulation of only Th2 cytokines after TLR-7 activation without any effect on Th1 cytokines (Moisan et al. 2006). However, other mechanisms which may confer protection against airway inflammation after TLR7 activation have not been explored and need to be investigated.

RSQ is a member from the imidazoquinoline family of compounds and is known to ligate TLR-7 similar to single-stranded RNA molecules of viral origin. MyD88 is a crucial adapter molecule linking the TLRs with other signal transduction components such as Nrf2 (Kim et al. 2011). TLR7 signaling has also been shown to be dependent on MyD88 for signal transduction after activation with RSQ (Moisan et al. 2006). We demonstrate that in the context of allergic asthma, the anti-inflammatory effects of RSQ on airway inflammation and airway reactivity are dependent on MyD88 signaling and subsequent Nrf2 induction. This was confirmed by MyD88 inhibitor peptide which reversed protective effects of TLR-7 agonist on airway reactivity and inflammation. Other TLRs have also been shown to be associated with Nrf2 signaling previously (Lee et al. 2008; Vijayan et al. 2011; Yang et al. 2013).
RSQ treatment mainly affected eosinophilic airway inflammation in this model. It is well known that eosinophilic inflammation is one of the hallmarks of allergic asthma and OVA model is mainly dependent on eosinophilic inflammation. Consistent with this notion, reduction in eosinophilia by RSQ may be sufficient for overall reduction in airway inflammation. This is consistent with previous reports (Moisan et al. 2005; Grela et al. 2011). However, chronic studies on other animal models of asthma and clinical trials are required to shed more light on this aspect.

There are three methods namely airway resistance by invasive technique, contraction measurements on isolated tracheal preparations and unrestrained whole body plethysmography which are most commonly used to evaluate airway reactivity/hyperresponsiveness. It has been shown in the past that these three methods provide parallel and consistent results (Justice et al. 2001). Our past publications and unpublished observations also suggest that Penh most probably reflects the airway resistance obtained with invasive method as well as contraction measurements on isolated trachea (Assaduzzaman et al. 2015; Nadeem et al, 2015; Nadeem et al, 2014b). Use of Penh to measure AHR has been also supported by numerous publications in peer-reviewed international journals. However, airway resistance data obtained with invasive method may be required for confirmation of Penh and this is a limitation of our study.

It has also been shown recently that TLRs activation is connected with NOXs signaling via MyD88 (Lee et al. 2012; Zhao et al. 2014). For example, TLR-2 and TLR-4 signaling pathways have been shown to be associated with NOX-2 as well as NOX-4 via MyD88 (Lee et al. 2008; Zhao et al. 2014). Another study has shown association of TLR-2 as well as TLR-4 with dual oxidases (Ryu et al. 2013). However, no previous study has
shown association of TLR-7 signaling with NOX-4. Our study shows for the first time that TLR-7 activation upregulates NOX-4 via MyD88 signaling in a murine model of asthma. MyD88 inhibitor peptide corroborated this observation as it led to reduction in expression of NOX-4 without having any effect on NOX-2.

Upregulation of NOX-4 after TLR-7 activation seems to be a protective strategy in the lung because it leads to activation of Nrf2 signaling. Nrf2 has been shown to be protective in various inflammatory animal models (Cho et al. 2013; Liu et al. 2015; Rangasamy et al. 2005). Importance of Nrf2 is also strengthened by the fact is that its induction leads to transcription of many antioxidant genes which include hemeoxygenase 1, glutathione peroxidase and SOD (Eggler et al. 2008; Lee et al. 2008; Vijayan et al. 2011; Zhu et al. 2005). A recent study also suggests that NOX-4 is responsible for cardioprotective effects through upregulation of Nrf2 (Smyrnias et al. 2015).

Our study also shows upregulation of Cu/Zn SOD after TLR-7 activation via MyD88-Nrf2 pathway. This was confirmed by using MyD88 inhibitor in RSQ treated allergic mice where it led to reduction in expression of both Nrf2 and Cu/Zn SOD. On the other hand, SOD inhibitor had no effect on Nrf2 expression. Therefore, increased Cu/Zn SOD expression after treatment with RSQ could probably be responsible for protection against the ROS-mediated airway reactivity and inflammation in this study. Deficiency of SOD has been shown to be associated with airway inflammation in both human and animal studies (Gosh et al. 2013; Kwon et al. 2012). Our study confirms the earlier observations about the involvement of SOD in amelioration of airway inflammation.
In conclusion, the present study shows that treatment with TLR-7 agonist, RSQ leads to Nrf2-mediated antioxidant protection against excessive ROS production through MyD88 signaling in allergic mice (Fig. 8). This leads to protection against allergen-induced airway inflammation in mice. Our findings show a different mechanism through which TLR-7 activation exerts its beneficial effect in allergic asthma.
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Disclosures

The authors declare no conflict of interest.
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Figures legends

**Fig. 1** Effect of TLR-7 agonist, RSQ on allergen induced airway reactivity and inflammation. A) Total leukocyte and differential counts, and B) Penh. Airway reactivity to methacholine was measured as Penh, 6 h after the final allergen challenge using a Buxco system for whole body plethysmography in which mice were exposed to increasing concentrations of methacholine (0-32 mg/mL). Airway inflammation in BAL was assessed 24 h after the final allergen challenge through total cell, eosinophil and neutrophil cell counts. Values are expressed as mean ± SE, n = 5-9/group. *P< 0.05, vs. SEN^CHAL group.

**Fig. 2** Effect of TLR-7 agonist, RSQ on NOX-2/NOX-4 expression and ROS generation in trachea and lung of allergic mice. A) Tracheal ROS generation, B) Tracheal NOX-2 and NOX-4 mRNA expression, C) Tracheal NOX-2 and NOX-4 protein expression, and D) Lung NOX-2 and NOX-4 mRNA expression. Expression of NOX-2/NOX-4 mRNA in all the groups was assessed by real time PCR. For mRNA expression by comparative C_T method using real time PCR, first column was made as the calibrator against which the other groups were compared. NOX-2/NOX-4 protein expression and ROS measurement were assessed by western blot and biochemical analyses respectively. Values are expressed as mean ± SE, n = 6-8/group. *P< 0.05, vs. SEN^CHAL group.

**Fig. 3** Effect of TLR-7 agonist, RSQ on Cu/Zn SOD and Nrf2 expression in trachea and lung of allergic mice. A) Tracheal Cu/Zn SOD mRNA expression, B) Tracheal Cu/Zn SOD protein, C) Tracheal Nrf2 mRNA expression, D) Tracheal Nrf2 protein expression, and E) Tracheal Cu/Zn SOD and Nrf2 mRNA expression. Expression of Cu/Zn SOD and
Nrf2 mRNA in all the groups was assessed by real time PCR. For mRNA expression by comparative C<sub>T</sub> method using real time PCR, first column was made as the calibrator against which the other groups were compared. Cu/Zn SOD and Nrf2 protein expression was assessed by western blot analysis. Values are expressed as mean ± SE, n = 6-8/group. *P< 0.05, vs. SEN<sup>CHAL</sup> group.

**Fig. 4** Effect of TLR-7 agonist, RSQ on parameters of oxidative stress in trachea of allergic mice. A) Nitrotyrosine expression, B) Lipid peroxide levels, and C) iNOS mRNA expression. Expression of iNOS mRNA in all the groups was assessed by real time PCR. For mRNA expression by comparative C<sub>T</sub> method using real time PCR, first column was made as the calibrator against which the other groups were compared. Nitrotyrosine levels were assessed by western blot, whereas lipid peroxides were assessed biochemically. Values are expressed as mean ± SE, n = 6-8/group. *P< 0.05, vs. SEN<sup>CHAL</sup> group.

**Fig. 5** Reversal of protective effect of TLR-7 agonist, RSQ in allergic mice by SOD inhibitor (DETC) and MyD88 inhibitor peptide (MyD88-IP). A) Penh, and B) Total leukocyte and differential counts. Airway reactivity to methacholine was measured as Penh, 6 h after the final allergen challenge using a Buxco system for whole body plethysmography in which mice were exposed to increasing concentrations of methacholine (0-32 mg/mL). Airway inflammation in BAL was assessed 24 h after the final allergen challenge through total cell and eosinophil cell counts. Values are expressed as mean ± SE, n = 6-8/group. *P< 0.05, vs. SEN<sup>CHAL</sup>+RSQ group.
**Fig. 6** Effect of SOD inhibitor (DETC) and MyD88 inhibitor peptide (MyD88-IP) on ROS generation and NOX-4 expression in trachea of RSQ-treated allergic mice. A) ROS generation, B) NOX-4 mRNA expression, and C) NOX-4 protein expression. Expression of NOX-4 mRNA in all the groups was assessed by real time PCR. For mRNA expression by comparative C<sub>T</sub> method using real time PCR, first column was made as the calibrator against which the other groups were compared. NOX-4 protein expression and ROS measurement were assessed by western blot and biochemical analyses respectively. Values are expressed as mean ± SE, n = 6-8/group. *P< 0.05, vs. SEN<sub>CHAL</sub>+RSQ group.

**Fig. 7** Effect of SOD inhibitor (DETC) and MyD88 inhibitor peptide (MyD88-IP) on antioxidant and oxidant parameters in trachea of RSQ-treated allergic mice. A) Cu/Zn SOD mRNA expression, B) Cu/Zn SOD protein, C) Nrf2 mRNA expression, and D) Nrf2 protein expression, E) Nitrotyrosine expression, and F) Lipid peroxide levels. Expression of Cu/Zn SOD and Nrf2 mRNA in all the groups was assessed by real time PCR. For mRNA expression by comparative C<sub>T</sub> method using real time PCR, first column was made as the calibrator against which the other groups were compared. Cu/Zn SOD, Nrf2 and nitrotyrosine protein expression was assessed by western blot analysis, whereas lipid peroxides were assessed biochemically. Values are expressed as mean ± SE, n = 6-8/group. *P< 0.05, vs. SEN<sub>CHAL</sub>+RSQ group.

**Fig. 8** Proposed signaling mechanism by which TLR-7 activation may be linked to attenuation of airway inflammation/reactivity in a murine model of asthma.
Highlights

► RSQ attenuates allergen-induced airway reactivity/inflammation.
► RSQ up regulates Nrf2 and Cu/Zn SOD in allergic mice.
► RSQ attenuates oxidative stress in allergic mice.
► MyD88-IP reverses RSQ-mediated effects in allergic mice.
Figure(s)

(A)

(B)

Fig. 1
Fig. 2

(A) Bar chart showing ROS production (% control) for CON, SEN^{CHAL}, and SEN^{CHAL}+RSQ.

(B) Bar chart showing mRNA expression for NOX-2 and NOX-4.

(C) Bar chart showing protein expression for NOX-2 and NOX-4.

Images showing Western blots for NOX-2 (90 kDa), NOX-4 (70 kDa), and GAPDH.
Fig. 2 (D)
Fig. 3

(A) Cu/Zn SOD mRNA expression

(B) Cu/Zn SOD protein expression

(C) Nrf2 mRNA expression

(D) Nrf2 protein expression

Cu/Zn SOD (23 kDa)

Nrf2 (61 kDa)

GAPDH

CON
SENCAL
SENCAL+RSQ

* indicates statistical significance.
Fig. 3
Fig. 4

(A) Nitrotyrosine expression

(B) Lipid peroxides, nmol/mg protein

(C) iNOS mRNA expression
Fig. 5

(A) 

(B)
Fig. 6

(A) Graph showing ROS production (% control) for different conditions.

(B) Graph showing NOX-4 mRNA expression for different conditions.

(C) Graph showing NOX-4 protein expression for different conditions.

(D) Western blot analysis showing NOX-4 (70 kDa) and GAPDH.
**Fig. 7**

(A) Cu/Zn SOD mRNA expression

(B) Cu/Zn SOD protein expression

(C) Nrf2 mRNA expression

(D) Nrf2 protein expression

Cu/Zn SOD (23 kDa)

Nrf2 (61 kDa)

GAPDH
Fig. 7

(E) Nitrotyrosine expression

(F) Lipid peroxides, nmol/mg protein

SEN^{CHAL}+RSQ
SEN^{CHAL}+DETC+RSQ
SEN^{CHAL}+MyD88-IP+RSQ

Nitrotyrosine (70 kDa)
GAPDH
Allergen sensitization & challenge

Resiquimod, TLR-7 agonist

Murine Lung

NOX2 upregulation

TLR-7 activation

MyD88 signaling

NOX-4 upregulation

Nrf2 activation

Cu/Zn SOD upregulation

ROS

Oxidative stress

Dysregulated airway responses (Airway reactivity/inflammation)

× indicates blockade

Fig. 8