

Toll like receptor-2 modulates both innate and adaptive immune responses during chronic fungal asthma in mice

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Abstract. *Objective and Design:* We investigated the effect of TLR2 gene deletion in a murine model of chronic fungal asthma.

Methods: TLR2 wildtype (TLR2^{+/+}) and TLR2 deficient (TLR2^{-/-}) mice were sensitized to soluble *A. fumigatus* antigens and challenged with live *A. fumigatus* conidia, and the extent of allergic airways disease was analyzed in both groups of mice at 3, 7, 14, and 30 days after conidia.

Results: At day 7 post-conidia, TLR2^{-/-} mice exhibited significantly lower airway hyperresponsiveness, airway inflammation, and whole lung Th2 cytokine levels compared with the TLR2^{+/+} group. TLR2 deletion also significantly reduced mucus cell metaplasia and peribronchial fibrosis at day 30 after conidia. However, fungal material persisted in the TLR2^{-/-} group, and at day 30 after conidia TLR2^{-/-} mice exhibited enhanced airway neutrophil recruitment and airway hyperresponsiveness.

Conclusion: Thus, during chronic fungal asthma in mice, TLR2 is a major contributor to the maintenance of the adaptive Th2-cytokine driven and anti-fungal innate responses.

Key Words: innate immunity – Fungal asthma – TLR2

Introduction

Airborne *A. fumigatus* conidia exacerbate allergic and asthmatic diseases [1–3]. The mechanism through which *A. fumigatus* exerts this effect is unknown, but one explanation lies in the immune response evoked by this pathogen. Experimental and clinical data show that the pulmonary immune response to *A. fumigatus* conidia is skewed toward Th2 immunity, rather than an anti-fungal Th1 immune response [4]. Directing the immune response toward Th1 immunity is a

therapeutically attractive approach to containing the pathological consequences of fungal exacerbation of allergy airways disease and/or asthma.

The immune responses required for the recognition and elimination of fungus such as *A. fumigatus* remain to be fully elucidated. Accordingly, the toll like receptors (TLRs) have been shown to provide important immune signals for the anti-fungal responses by immune cells such as macrophages, neutrophils, and dendritic cells [5, 6]. Several receptors mediate the recognition of fungus in the host, it is the TLRs that evoke unique immune activating properties [7]. For example, TLR2, working through the central adaptor protein MyD88 [8], has been shown to collaborate with recognition receptors such as dectin-1 to recognize fungal components associated with *A. fumigatus* conidia [9, 10], thereby contributing to the phagocytosis and containment of this pathogen. The relative importance of TLR2 or MyD88 in the anti-Aspergillus immune process remains controversial since its absence does not necessarily lead to impaired anti-fungal immune responses *in vivo* [6] or *in vitro* [11, 12]. However, neutrophil depleted TLR2 gene deficient (TLR2^{-/-}) mice exhibit increased susceptibility to invasive aspergillosis compared with wildtype [13]. Together, these data suggest that the role of TLR2 is complex, and requires further investigation.

In the present study, we addressed the role of TLR2 in the innate and adaptive immune responses elicited by *A. fumigatus* in a model of chronic fungal asthma. We have previously observed dynamic changes in TLR2 transcript expression during chronic fungal asthma [14, 15]. Herein, we show that TLR2 deficiency modulates the initiation and maintenance of features of Th2 immunopathology during fungal asthma, including the elaboration of Th2 cytokines, mucus cell metaplasia and peribronchial fibrosis. However, TLR2 expression was necessary for elimination of fungal components from the lungs of TLR2^{-/-} mice, and the persistence of fungus in these mice promoted chronic changes in airway physiology. Thus, TLR2 contributes to both innate and adaptive pulmonary immune responses to *A. fumigatus*.

Materials and methods

Mice

Male and female, homozygous TLR2 gene deficient (TLR2^{-/-}) fully backcrossed ten generations onto a C57BL/6 background and wildtype C57BL/6 (TLR2^{+/+}) mice at 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained in a specific pathogen free facility. Prior approval for mouse usage was obtained from the University Laboratory of Animal Medicine facility.

Chronic fungal asthma model

We have previously described a model of chronic allergic airway disease induced by *A. fumigatus* conidia [16]. TLR2^{+/+} and TLR2^{-/-} mice were similarly sensitized to a commercially available preparation of soluble *A. fumigatus* antigens (Greer Laboratories, Lenoir, NC) as previously described [16].

Assessment of airway physiology

Immediately prior to and at days 3, 7, 14, and 30 after an intratracheal *A. fumigatus* conidia challenge, bronchial hyperresponsiveness was assessed in a BuxcoTM plethysmograph (Buxco, Troy, NY) (16). After the assessment of airway hyperresponsiveness, approximately 500 µl of blood was removed from each mouse and centrifuged at 900 g for 10 min to yield serum. A bronchoalveolar lavage (BAL) was then performed using 1 ml of filter-sterilized normal saline. Finally, whole lungs were dissected from each mouse and snap frozen in liquid N₂ or fixed in 10 % formalin for histological analysis (see below).

Whole lung RNA isolation and TAQMAN analysis

Total RNA was isolated from homogenized mouse lungs using Trizol reagent (Invitrogen/ Life Technologies, Carlsbad, CA) as previously described in detail [17]. Purified RNA was treated with DNase and reverse transcribed into cDNA using TAQMAN Reverse Transcription Reagents (Foster City, CA). Pre-developed TAQMAN Gene Expression Assays were used to quantify TLR2, TLR4, TGF-β, and procollagen III transcripts as per manufacturer's (Applied Biosystems) instructions. The fold changes in transcript expression were calculated via the comparison of gene expression in naïve whole lung samples, which were assigned a value of 1 to that in whole lung samples from naïve mice.

Serum immunoglobulin analysis

Serum levels of IgE and IgG2a at days 7, 14, and 30 after conidia in the TLR2^{+/+} and TLR2^{-/-} groups were analyzed using complementary capture and detection antibody pairs for IgE and IgG2a (PharMingen, San Diego, CA). Immunoglobulin ELISAs were performed according to the manufacturer's directions.

Whole lung proteomic analysis

Murine TNF-α, IL-12, MIP-2, IL-4, IFN-γ, IL-5, IL-10, CCL17, and KC were determined in 50-µl samples from whole lung homogenates using a standardized sandwich ELISA technique previously described in detail [18]. The cytokine and chemokine levels in each sample were normalized to total protein levels measured using the Bradford assay.

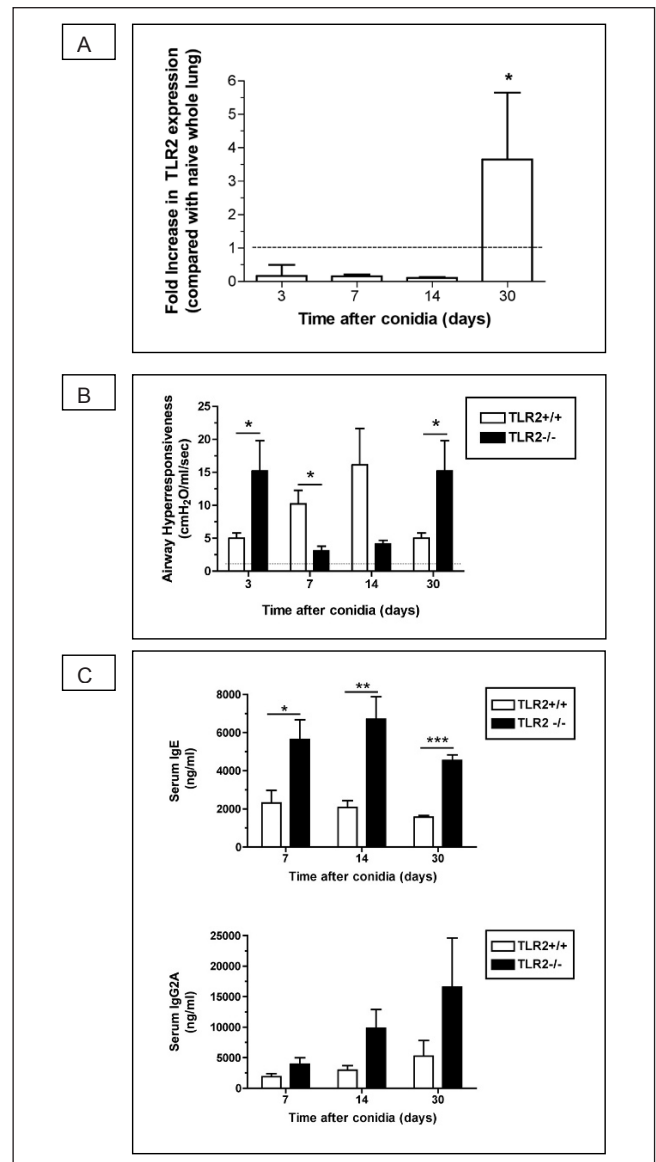


Fig. 1. A) Fold increase in whole lung TLR2 transcript levels at days 3, 7, 14, and 30 after *A. fumigatus* conidia intratracheal injection into *A. fumigatus*-sensitized TLR2 wildtype (^{+/+}) mice. RNA was isolated from whole lung samples as described in the Materials and Methods section, and quantitative TAQMAN was used to quantify TLR2 transcript levels. TLR2 transcript levels were measured in whole lung samples from non-sensitized TLR2^{+/+} mice, gene expression in these mice was normalized to an arbitrary value of 1 (dashed line), and fold changes in transcript expression in the fungal asthma groups were calculated by dividing transcript levels in this group by 1. Data are mean ± SEM of n = 3–5/group. B) Airway hyperresponsiveness in *A. fumigatus*-sensitized TLR2-wildtype (^{+/+}) and TLR2-deficient (^{-/-}) mice at various times after an *A. fumigatus* conidia challenge. Airway resistance (units = cm H₂O/ml/sec) was calculated at each time point prior to (dashed line) and after methacholine (420 µg/kg; i.v.). Values are expressed as mean ± SE; n = 5–10/group/time point. * P < 0.05, demonstrates a significant difference in airway hyperresponsiveness between TLR2^{+/+} and TLR2^{-/-} mice. C) Serum total IgE and IgG2A levels in *A. fumigatus*-sensitized TLR2 wildtype (^{+/+}) and TLR2 deficient (^{-/-}) mice at various times after *A. fumigatus* conidia challenge. Total IgE and IgG2a were measured using a specific ELISA as described in the Materials and Methods section. Data are expressed as mean ± SEM; n = 5/group/time point. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with values measured in the TLR2^{+/+} groups at the same time after the conidia challenge.

Whole lung histologic analysis

Whole lungs from *A. fumigatus*-sensitized TLR2^{+/+} and TLR2^{-/-} mice prior to and at various times after *A. fumigatus* conidia challenge were fully inflated with 10% formalin, dissected, and placed in fresh 10% formalin for 24h. Routine histological techniques were used to paraffin-embed the entire lung, and 5- μ m sections of whole lung were stained with hematoxylin/eosin or periodic acid-Schiff reagent (PAS). Morphologic evaluations of inflammatory infiltrates and structural alterations such as goblet cell metaplasia were determined around blood vessels and airways using light microscopy at a magnification of 200 or 400x as previously described in detail [19].

Statistical analysis

All results are expressed as mean $\pm$ standard error of the mean (SEM). A Student's T test or Analysis of variance and a Student-Newman-Keuls Multiple Comparison test were used to determine statistical significance between TLR2^{+/+} and TLR2^{-/-} mice at various times after the conidia challenge; $P < 0.05$ was considered statistically significant.

Results

Biphasic alteration in TLR2 transcript expression in TLR2^{+/+} mice during fungal asthma

Whole lung samples from TLR2^{+/+} mice on a C57BL/6 background were analyzed by TAQMAN and the results of this analysis are shown in **Figure 1A**. At days 3, 7, and 14 after conidia challenge in *A. fumigatus*-sensitized TLR2^{+/+} mice, TLR2 transcript expression was not induced relative to levels in naïve TLR2^{+/+} mice (**Fig. 1A**). In contrast, TLR2 transcript expression in the fungal asthma group at day 30 after conidia was significantly increased approximately 24-fold relative to the other groups at days 3, 7, and 14 after conidia (**Fig. 1A**). Thus, these data indicate that TLR2 transcript expression was altered during the course of chronic fungal asthma.

Time-dependent changes in airway hyperresponsiveness in TLR2^{-/-} mice with chronic fungal asthma

Persistent methacholine-induced airway hyperresponsiveness is a prominent feature in this model of fungal asthma [16]. At all times examined after conidia both groups exhibited marked increases in airway hyperresponsiveness following a 420mg/kg intravenous methacholine challenge (indicated by the open and closed bars in **Figure 1B**) above the baseline airway resistance observed in both groups of asthmatic mice (shown by the dashed line in **Figure 1B**). No difference in baseline airway resistance was observed between the two groups of mice (not shown). At day 3 after conidia, the airway hyperresponsiveness measured in TLR2^{-/-} group was significantly greater than that measured in the TLR2^{+/+} group (**Fig. 1B**). At day 7 and 14 after conidia, methacholine-induced airway hyperresponsiveness was lower in the TLR2^{-/-} group relative to the wildtype group, and this difference reached statistical significance at the day 7 time point. At day 30 after conidia, the TLR2^{-/-} group again exhibited significantly greater airway hyperresponsiveness compared with the wildtype group at the same time after conidia (**Fig. 1B**).

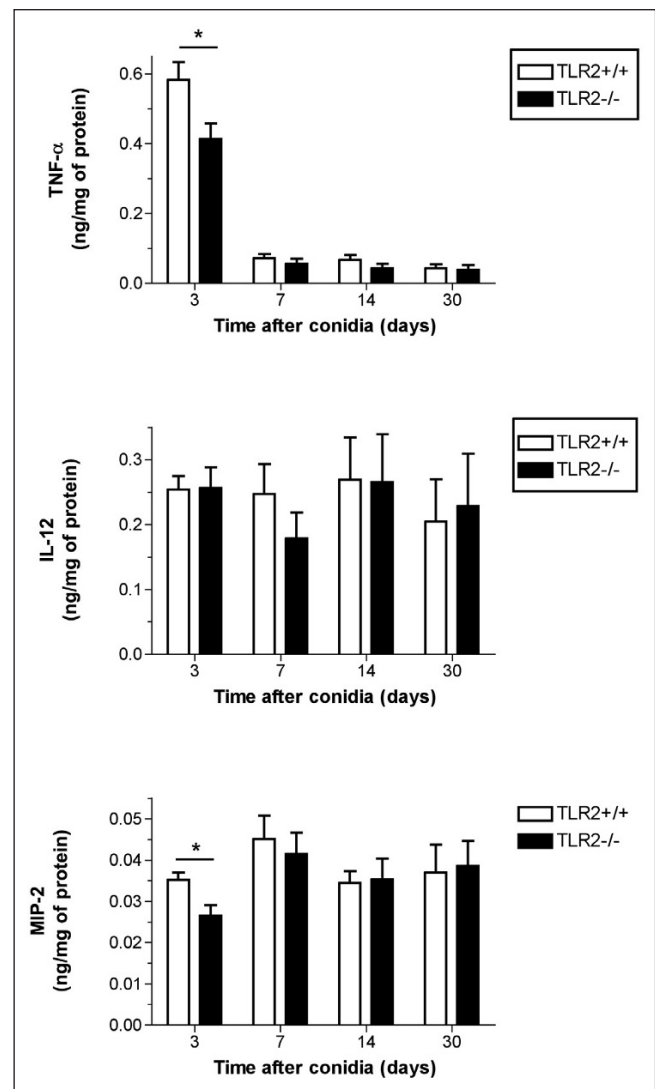


Fig. 2. Whole lung TNF- α , IL-12, and MIP-2 in *A. fumigatus*-sensitized TLR2 wildtype (^{+/+}) and TLR2 deficient (^{-/-}) mice at various times after *A. fumigatus* conidia challenge. Whole lung samples were processed as described in the Materials and Methods section, and ELISA was used to determine specific cytokine levels in cell free supernatants. Data are mean $\pm$ SEM of $n = 5-10$ /group. * $P \leq 0.05$ compared with chemokine and cytokine levels detected in whole lung samples removed from the TLR2^{+/+} group at the same time after conidia challenge.

Together, these data indicated that the absence of TLR2 had a dynamic, temporal-dependent effect on the responsiveness of the airways to a broncho-constrictive stimulus.

TLR2^{-/-} mice exhibit enhanced IgE and IgG2a at all times after conidia challenge

The sensitization and challenge of mice with *A. fumigatus* antigens and live conidia leads to the persistence of elevated immunoglobulins including IgE and IgG2a [16]. Similar serum levels of IgG and IgG2a were present in TLR2^{+/+} and TLR2^{-/-} mice immediately prior to the introduction of conidia (data not shown). Total serum IgE levels were present in both groups at days 7, 14, and 30 after conidia but the levels of

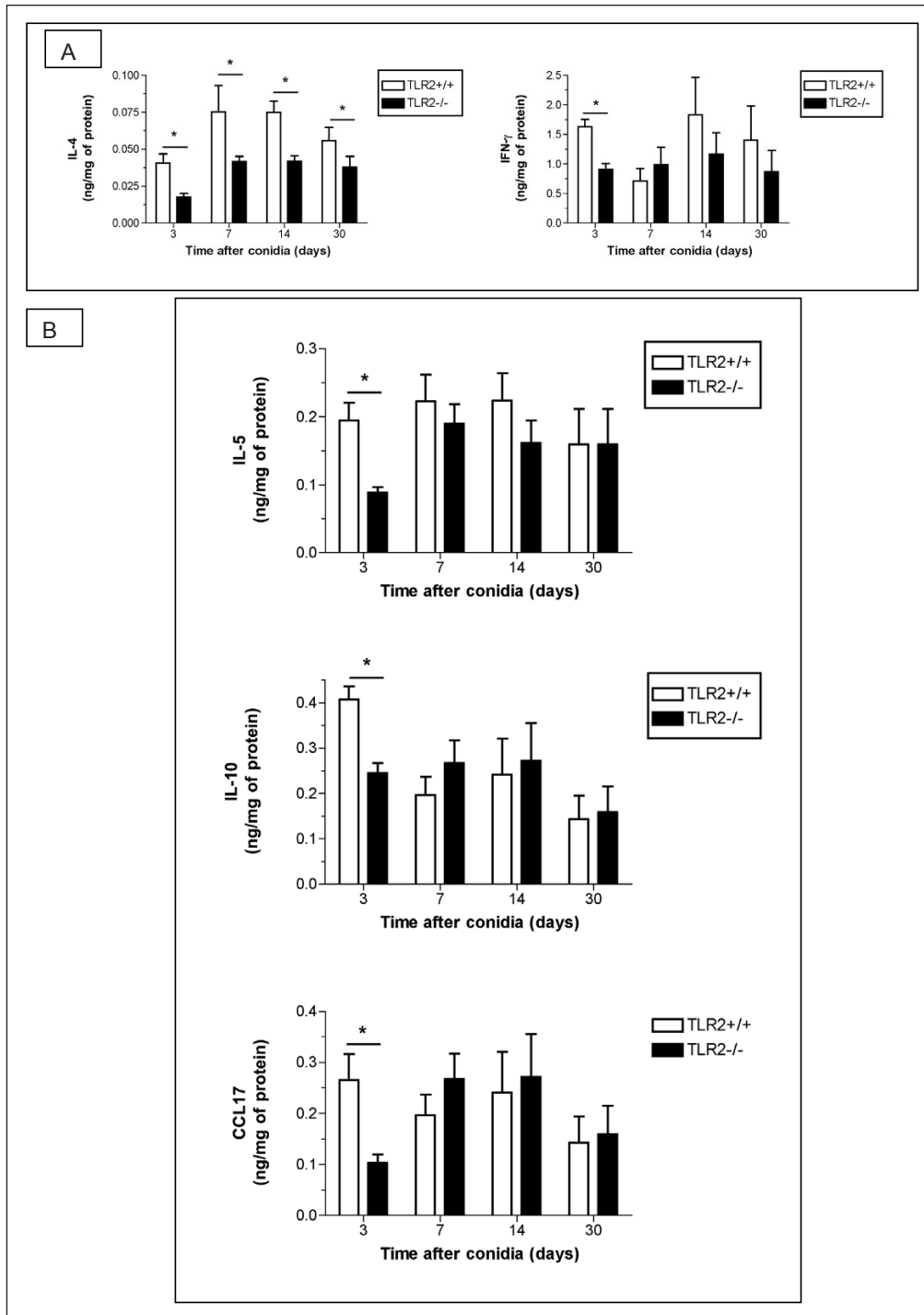


Fig. 3. A) Whole lung IL-4 and IFN- γ in *A. fumigatus*-sensitized TLR2 wildtype (^{+/+}) and TLR2 deficient (^{-/-}) mice at various times after *A. fumigatus* conidia challenge. Whole lung samples were processed as described in the Materials and Methods section, and ELISA was used to determine specific cytokine levels in cell free supernatants. Data are mean $\pm$ SEM of $n = 5-10$ /group. * $P \leq 0.05$ compared with chemokine and cytokine levels detected in whole lung samples removed from the TLR2^{+/+} group at the same time after conidia challenge. **B) Whole lung IL-5, IL-10, and CCL17 in *A. fumigatus*-sensitized TLR2 wildtype (^{+/+}) and TLR2 deficient (^{-/-}) mice at various times after *A. fumigatus* conidia challenge.** Whole lung samples were processed as described in the Materials and Methods section, and ELISA was used to determine specific cytokine levels in cell free supernatants. Data are mean $\pm$ SEM of $n = 5-10$ /group. * $P \leq 0.05$ compared with chemokine and cytokine levels detected in whole lung samples removed from the TLR2^{+/+} group at the same time after conidia challenge.

this Ig were significantly greater in the TLR2^{-/-} group compared with the TLR2^{+/+} group (**Fig. 1C**, top panel). Similarly, total IgG2a levels were present in both groups at all times examined after conidia but the levels in the TLR2 deficient group showed a trend toward higher expression compared than those measured in the wildtype group (**Fig. 1C**, bottom panel). Thus, *A. fumigatus*-sensitized TLR2^{-/-} mice exhibited enhanced Ig responses following a live conidia challenge suggesting that the humoral immune response to this fungus was intact and not subject to the same regulation as similarly sensitized and challenged TLR2^{+/+} mice.

Whole lung TNF- α and MIP-2 levels were significantly lower in TLR2^{-/-} mice compared with TLR2^{+/+} at day 3 after conidia

Balloy et al. [13] demonstrated that TLR2 was involved in the defense of mice from *A. fumigatus* infection, particularly when TLR2^{-/-} mice were depleted of their neutrophils. These authors showed that whole lung levels of TNF- α , IL-12, and MIP-2 were significantly lower in susceptible TLR2^{-/-} mice [13]. TNF- α [20] and MIP-2 [21] have been shown to have major roles in the activation of pulmonary resident macro-

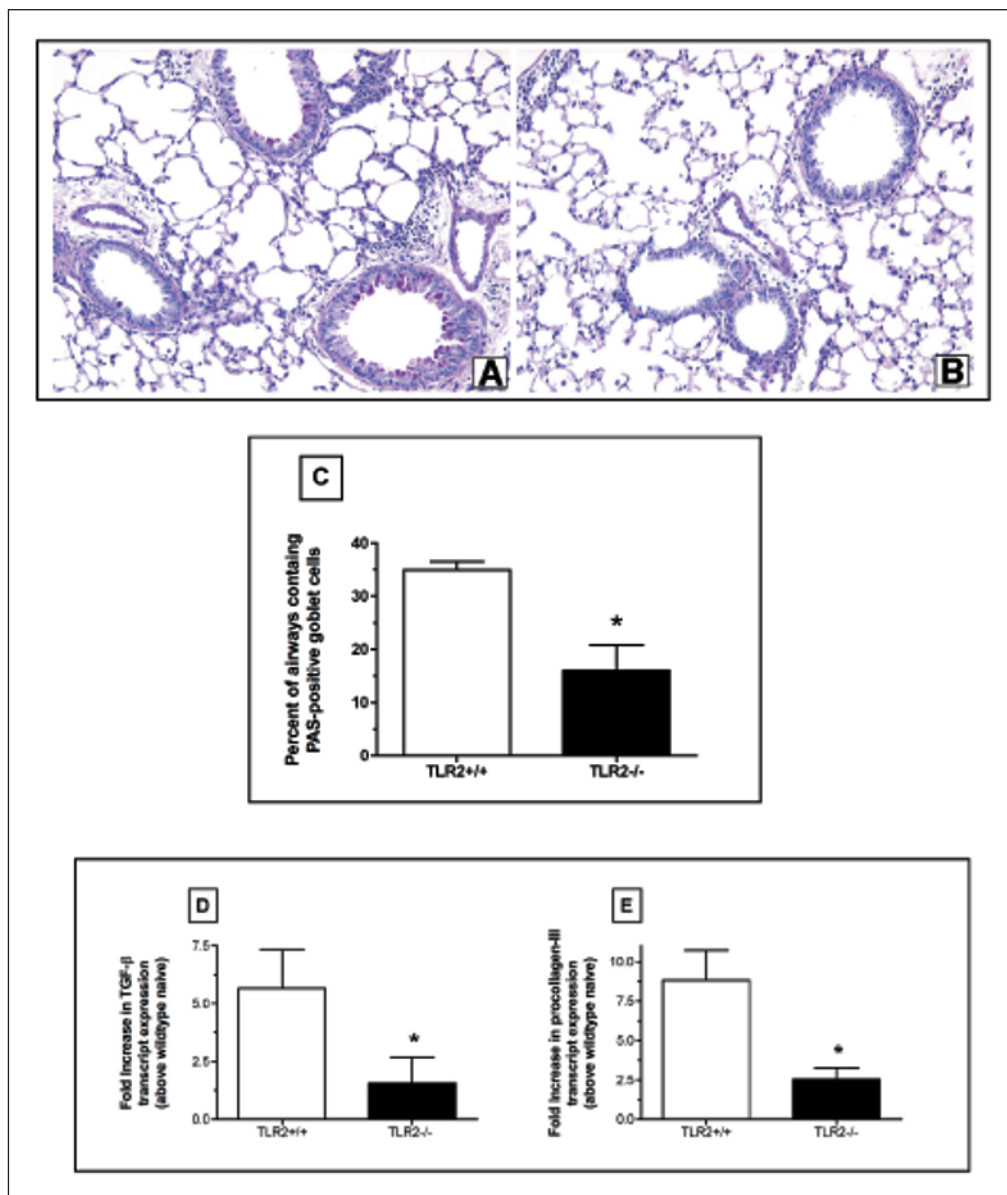


Fig. 4. Representative photomicrographs of PAS-stained whole lung sections from *A. fumigatus*-sensitized TLR2 wildtype (^{+/+}) (A) and TLR2-deficient (^{-/-}) (B) mice at day 30 after a live *A. fumigatus* conidia challenge. Goblet cells (stained dark magenta) were prominent in the airways of TLR2^{+/+} mice at this time after the conidia challenge. In contrast, goblet cells were rarely detected in the airways of TLR2^{-/-} mice at day 30 after conidia. Original magnification was 200x for each photomicrograph. (C) Quantitative analysis confirmed that significantly fewer PAS-positive goblet cells were detected in whole lung samples from TLR2^{-/-} mice compared with TLR2^{+/+} mice. Data are mean $\pm$ SEM of $n = 3$ /group. * $P \leq 0.05$ compared with PAS-positive cells detected in whole lung samples removed from the TLR2^{+/+} group at day 30 after conidia challenge. (D) and (E) Quantitative TAQMAN analysis confirmed that significantly less induction of TGF- β and procollagen III transcripts was evident in whole lung samples from TLR2^{-/-} mice compared with TLR2^{+/+} mice. Data are mean $\pm$ SEM of $n = 3$ /group. * $P \leq 0.05$ compared with transcript levels detected in whole lung samples removed from the TLR2^{+/+} group at day 30 after conidia challenge.

phages during *A. fumigatus* challenge in neutrophil-depleted mice. In the present study, whole lung levels of TNF- α and MIP-2, but not IL-12, were significantly lower in the knockout group compared with the wildtype group at day 3 after conidia (Fig. 2). Thus, these data suggested that the sensitization and challenge of TLR2^{-/-} mice with *A. fumigatus* had an immunomodulatory effect on the respiratory system of these mice, as revealed by transitory significant decreases in anti-fungal cytokines.

Th2-type cytokines and chemokines are significantly decreased in TLR2^{-/-} mice during chronic fungal asthma

The presence and function of Th2-type cytokines [22–24] and chemokines [17, 25] have major roles in the initiation and maintenance of chronic fungal asthma in mice. The

present study demonstrated that whole lung levels of IL-4, IL-5, IL-10, and CCL17 were all significantly lower in the TLR2^{-/-} group compared with the TLR2^{+/+} group at day 3 after conidia (Fig. 3A&B). Whole lung IL-4 levels were persistently lower in the knockout group relative to the wildtype group at all times after conidia challenge (Fig. 3A). It is of note that whole lung IL-13 levels were also persistently lower in the TLR2^{-/-} group compared with the TLR2^{+/+} group at all times after conidia challenge (data not shown). Whole lung IFN- γ levels were also lower in the TLR2^{-/-} group versus the TLR2^{+/+} group at day 3 after conidia, but no differences in IFN- γ levels were observed in these groups at later times after conidia (Fig. 3A). Taken together, these data showed that the presence of TLR2 was necessary, in part, for the full development of the Th2 immune response during the course of chronic fungal asthma.

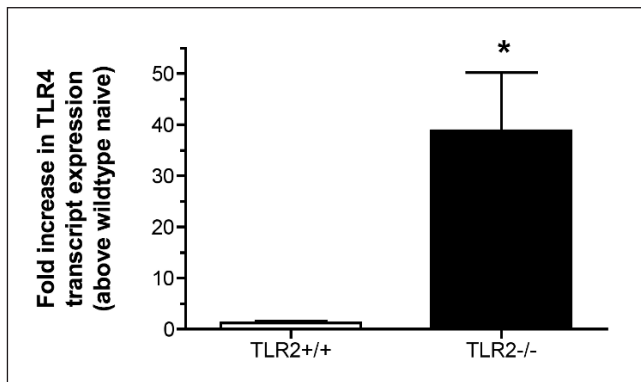


Fig. 5. Fold increase in whole lung TLR4 transcript levels at day 3 after *A. fumigatus* conidia intratracheal injection into *A. fumigatus*-sensitized TLR2 wildtype (^{+/+}) and TLR2 deficient (^{-/-}) mice. RNA was isolated from whole lung samples as described in the Materials and Methods section, and quantitative TAQMAN was used to quantify TLR4 transcript levels. TLR4 transcript levels were measured in whole lung samples from non-sensitized TLR2^{+/+} mice, gene expression in these mice was normalized to an arbitrary value of 1 (dashed line), and fold changes in transcript expression in the fungal asthma groups were calculated by dividing transcript levels in this group by 1. Data are mean $\pm$ SEM of $n = 3$ /group. * $P \leq 0.05$ compared with transcript levels detected in whole lung samples removed from the TLR2^{+/+} group at day 30 after conidia challenge.

Decreased mucus cell metaplasia and peribronchial fibrosis in TLR2^{-/-} mice

Major features of the Th2 immune response during chronic fungal asthma are the persistence of mucus cell metaplasia and peribronchial fibrosis [16, 17, 23]. As shown in **Figure 4A**, mucus cells were prominently stained a bright magenta color with PAS at day 30 after conidia challenge. Conversely, mucus cells were much less prominent or absent in many of variously sized airways in TLR2^{-/-} mice at the same time after conidia challenge (**Fig. 4B**). Quantitative analysis of goblet cells in airways revealed that approximately 35 % of the airways in TLR2^{+/+} mice contained PAS-positive goblet cells whereas approximately 15 % of airways exhibited PAS-positive cells in TLR2^{-/-} mice (**Fig. 4C**). Quantitative confirmation of a decreased peribronchial fibrotic response in TLR2^{-/-} mice is provided in panels D and E of **Figure 4**. Specifically, quantitative transcript analysis revealed that both whole lung levels of TGF- β (**Fig. 4D**) and procollagen III (**Fig. 4E**) were significantly less increased in TLR2^{-/-} mice compared with their wildtype counterparts at day 30 after conidia. Thus, the magnitude of Th2-type cytokine mediated airway remodeling as evidenced by goblet cell metaplasia and peribronchial fibrosis was significantly decreased with the gene deletion of TLR2.

Putative mechanisms driving the enhanced airway hyperresponsiveness in TLR2^{-/-} mice: enhanced TLR4 at day 3 and fungal persistence at day 30 after the conidia challenge

An explanation for the increased airway resistance is not immediately evident but further analysis of whole lung samples revealed the following. First, TLR4 transcript levels were

dramatically and significantly increased in TLR2^{-/-} mice at day 3 after conidia (**Fig. 5**). This finding was agreement with a previous observation that TLR4 mRNA was increased in TLR2^{-/-} mice challenged with *H. influenzae* [26] and that TLR4 contributes to experimentally induced asthma [27, 28]. Second, markedly greater fungal material was present in whole lung samples from the TLR2^{-/-} group at all times after conidia. This was highlighted by the increased presence of GMS-stained material in whole lung samples from the TLR2^{-/-} group compared with the TLR2^{+/+} group (**Fig. 6A**, TLR2^{+/+} versus **Fig. 6B**, TLR2^{-/-}). The persistence of fungal material in the TLR2^{-/-} group was also associated with an increase in neutrophils, but not eosinophils (not shown), in the BAL (**Fig. 6C**) and increased whole lung levels of KC, a neutrophil chemoattractant (**Fig. 6D**). Thus, TLR2 plays a major role in the modulation of the innate immune response during fungal asthma.

Discussion

Allergic responses to *A. fumigatus* complicate allergy and asthma necessitating the need to understand the immune mechanisms that drive these responses. TLR2 has garnered interest as this receptor appears to be involved, to varying degrees, in the activation of macrophages, neutrophils, and dendritic cells that encounter various *A. fumigatus* morphotypes [5, 8, 29–31]. TLR2 also works with dectin-1 to facilitate the phagocytosis of conidia by murine macrophages [9, 10], and the TLR2^{-/-} mouse has been found to be susceptible to invasive aspergillosis when depleted of neutrophils [13]. Herein, we examined the role of TLR2 in a model of chronic fungal asthma. The absence of TLR2 during chronic fungal asthma had two major effects on the initiation and maintenance of several features of this model: 1) a marked decrease in Th2-type cytokine immunity and the corresponding airway remodeling and; 2) the persistence of fungal material.

Airway hyperresponsiveness is a hallmark of many models of allergic and asthmatic airway disease, but this physiologic change persists during fungal asthma due to the retention of fungal material in the lungs of *A. fumigatus*-sensitized mice challenged with conidia [32–34]. Our histological analysis of whole lung samples at various days after conidia suggested that there was increased abundance of fungal material in the lungs of *A. fumigatus*-sensitized TLR2^{-/-} mice versus the wildtype group. mRNA transcript analysis of whole lung samples showed that TLR4 was dramatically enhanced in the TLR2^{-/-} group at day 3 after conidia. Thus the increased presence of TLR4 might have contributed to the enhanced airway hyperresponsiveness in the TLR2^{-/-} group. TLR4 has been shown to contribute to allergic airway disease due to OVA [27, 28] and it has also been shown to mediate immune responses to *A. fumigatus* in the mouse [6, 29–31]. At day 30 after conidia, markedly greater fungal material was present in the TLR2^{-/-} group compared with the TLR2^{+/+} group, and this persistence of fungal material might explain the increased magnitude of airway hyperresponsiveness in the former group compared with the latter. Thus, changes in airway resistance were dynamic in *A. fumigatus*-sensitized mice, appearing to occur in a manner that was dependent, in part, on the presence of fungal material in the lung.

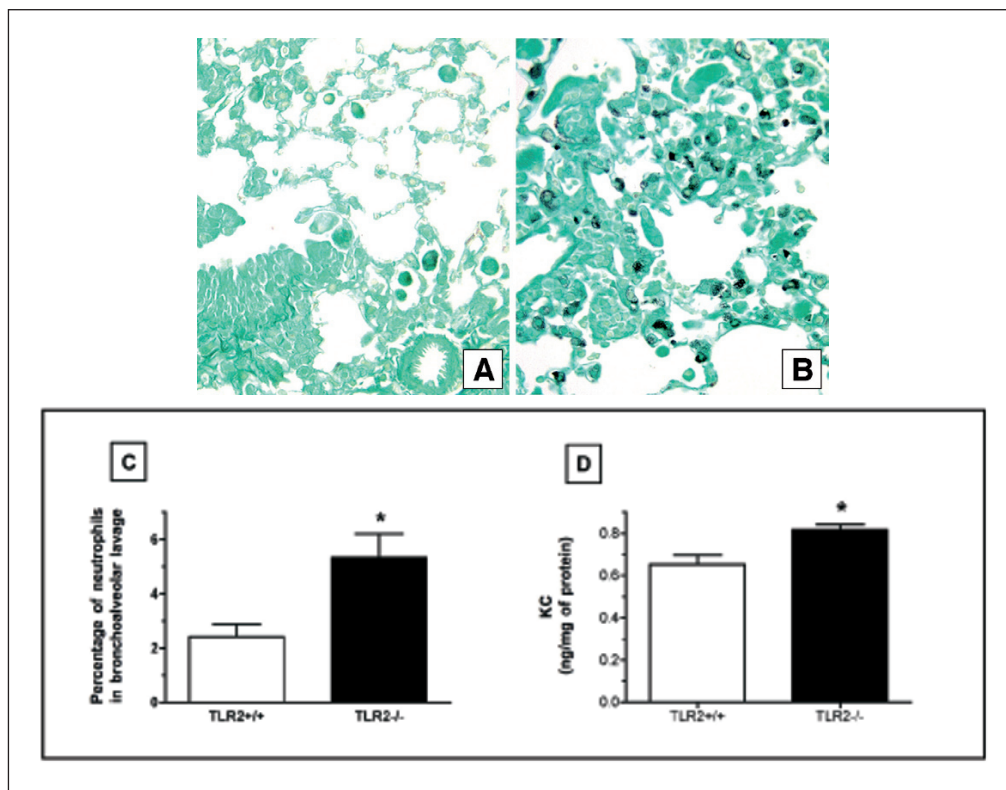


Fig. 6. Representative photomicrographs of Gomori methenamine silver (GMS)-stained whole lung sections from *A. fumigatus*-sensitized TLR2 wildtype ($^{+/+}$) (A) and TLR2-deficient ($^{-/-}$) (B) mice at day 30 after a live *A. fumigatus* conidia challenge. Original magnification was 2005 for each photomicrograph. Bronchoalveolar lavage neutrophil content (C) and KC chemokine levels (D) in whole lung samples from day 30 after *A. fumigatus* conidia intratracheal injection into *A. fumigatus*-sensitized TLR2 wildtype ($^{+/+}$) and TLR2 deficient ($^{-/-}$) mice. Neutrophils were quantified in BAL samples from both groups of mice at day 30 after the conidia challenge. Whole lung samples were processed as described in the Materials and Methods section, and ELISA was used to determine specific KC levels in cell free supernatants. Data are mean $\pm$ SEM of $n = 5-10$ /group. * $P \leq 0.05$ compared with BAL neutrophil percentages or whole lung KC levels detected in the TLR2 $^{+/+}$ group at day 30 after conidia challenge.

The absence of TLR2 at day 3 after conidia challenge in *A. fumigatus*-sensitized TLR2 $^{-/-}$ mice had a major effect on the presence of immunoreactive levels of TNF- α and MIP-2 in whole lung samples. This finding is of interest because it mirrors an observation made by Balloy *et al.* [13] who showed that neutrophil-depleted TLR2 $^{-/-}$ mice were susceptible to invasive aspergillosis, due in part, to reduced whole lung levels of TNF- α , IL-12, and MIP-2. It is conceivable that the lack of these potent anti-fungal cytokines [20, 21] at day 3 after conidia allowed for the persistence of fungal material in the lungs of *A. fumigatus*-sensitized TLR2 $^{-/-}$ mice, which was most dramatic at day 30 after the conidia challenge. While neutrophil-depleted TLR2 $^{-/-}$ mice are susceptible to invasive aspergillosis [14], we observed similar neutrophil recruitment at day 3 in both groups of mice (data not shown) and significantly increased neutrophil recruitment at day 30 after conidia challenge in *A. fumigatus*-sensitized TLR2 $^{-/-}$ mice compared with the wildtype group at this time. The increased neutrophil influx into the alveolar space at day 30 in TLR2 $^{-/-}$ mice was also associated with an increase in KC, a potent neutrophil recruitment factor in the mouse. It is unclear why this CXC chemokine was increased and MIP-2 was not at this time in the model. Thus, the absence of TLR2 has a major effect on the development of anti-fungal cytokine responses under allergic conditions.

The role of TLR2 in Th2-type immune responses has been explored previously in OVA-induced models of allergic airway disease, and TLR2 agonists have been shown to either induce [35] or inhibit [36] the Th2 disease evoked by this soluble antigen. Total IgE levels were significantly increased in TLR2 $^{-/-}$ mice at all times after conidia challenge suggesting

a disconnection between levels of this Ig and the decreased lung-associated Th2 immune response in these mice. We do not have an explanation as to why airway responsiveness improved (at days 7 and 14) although IgE remained elevated. However, we have observed this phenomenon in a number of previous studies with this model of fungal asthma in which we have therapeutically intervened; airway hyperresponsiveness was reduced but IgE levels remained elevated [15, 23, 37]. Circulating levels of IgG2a, an Ig that is sensitive to the presence of Th1-type cytokines, showed a trend towards elevation at all times after conidia challenge in TLR2 $^{-/-}$ mice. Specifically, we observed that whole lung IL-4 levels were significantly decreased in the TLR2 $^{-/-}$ group compared with the TLR2 $^{+/+}$ group at all times after the conidia challenge. This modulation of the Th2 immune response was consistent with the decreased airway-remodeling present in the TLR2 $^{-/-}$ group, particularly at day 30 after conidia. Our previous studies have highlighted the importance of Th2-type cytokines in the airway remodeling response associated with chronic fungal asthma [16, 17, 23]. Clearly, the decreased Th2 immunity in TLR2 $^{-/-}$ mice did not prevent the increased airway hyperresponsiveness in this group at day 30. While we speculate that elements of the innate immune response (namely neutrophils) are driving the airway reactivity at day 30, the disconnect between airway hyperresponsiveness and remodeling has been observed in prior studies of CCR4 $^{-/-}$ mice with chronic fungal asthma [25].

In summary, many Th2-type immune and remodeling features of chronic fungal asthma in mice were markedly altered by the complete absence of TLR2. We confirmed that fungal sensitization dynamically altered the level of TLR2

expression in the lung [14], appearing to inhibit TLR2 expression over the early time points in this model while dramatically increasing levels at the latest time point examined (i.e. day 30 after conidia challenge). In the complete absence of TLR2 due to gene deletion, *A. fumigatus*-sensitized mice fail to successfully remove fungal components following a conidia challenge [16, 19]. Based on this finding, a therapeutic option in the treatment of fungus-induced allergic disease might pertain to increasing either TLR2 expression or activity. Clearly, the caveat with this approach is highlighted in the present study in which the complete absence of TLR2 attenuated the Th2 immune response and the airway remodeling associated with fungal asthma. Given the innate and adaptive immune roles for TLR2 in allergic airway disease clearly more research is required in order to determine whether cell specific targeting of TLR2 (i.e. TLR2 on T cells versus TLR2 on innate immune cells) is an attainable therapeutic option.

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Conflict of Interest. All authors are free of any conflict of interest pertaining to the studies provided herein.

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Abbreviations: bronchoalveolar lavage, BAL.

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