

Induction of the specific allergic immune response is independent of proteases from the fungus *Alternaria alternata*

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We have analyzed the importance of proteases for the induction of allergic responses against the mold *Alternaria alternata*. Responses induced in vivo with untreated or heat treated (protease inactivated) extracts were compared in BALB/c, C57BL/6, TLR4 KO, and MyD88 KO mice. In BALB/c mice, both extracts induced similar lung inflammation, upregulation of inflammatory mediators, Th2 cytokines, and *Alternaria*-specific antibodies. However heat inactivation abrogated polyclonal IgE production. Similar results were obtained in C57BL/6 albeit lung expression of some Th2 mediators was decreased in mice stimulated with the heat-treated extract. Treatment of the extract with protease inhibitors did not affect the induction of the allergic response either, except again for the polyclonal IgE response. Th2 responses and lung inflammation were readily induced in TLR4 knockout mice. In contrast, lung inflammation, Th2 responses, cytokine productions, and antibody synthesis were strongly suppressed in MyD88-deficient mice. Early lung IL-33 and IL-1- α expression were also suppressed. In conclusion, albeit some heat labile proteases are required for the stimulation of the polyclonal IgE secretion, fungal proteases, and TLR4 signaling are not required while MyD88 is essential for triggering the systemic immune response and for the development of lung allergic inflammation in response to *Alternaria* extracts.

Keywords: *Alternaria alternata* · Animal models · Asthma · Eosinophils · proteases



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Introduction

Allergy is the clinical manifestation of a dysregulated immune response characterized by the production of IgE directed against foreign molecules. In this context, allergic asthma has become

a major public healthcare problem. Asthma patients suffer from repeated attacks of breathlessness, cough, and wheeze occurring secondary to bronchoconstriction in the setting of airway hyper-responsiveness, mucus hypersecretion, and airway remodeling. Their airways are infiltrated by Th2 cells, which predominantly secrete IL-4, IL-5, and IL-13. These cytokines induce IgE production, cause eosinophilia, stimulate mast cells, promote leukocytosis, provoke airway hyper-reactivity, and may also participate in the characteristic airway remodeling of asthma [1, 2].

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In allergic asthma, these responses are initially triggered by aeroallergens entering into the lungs. Obviously the respiratory mucosa is continuously exposed to a wide variety of environmental, nonreplicating, nonpathogenic antigens. Usually these encounters induce a state of peripheral tolerance or hyporesponsiveness rather than active immunity. This T-cell tolerance induced by respiratory exposure is partially mediated by pulmonary DCs secreting IL-10 [3].

Respiratory allergens, however, have the striking capacity to overcome these tolerance mechanisms and to induce the activation of Th2 cells when inhaled. The precise mechanism that causes this shift from tolerance to immune activation is still unclear but numerous experimental results have pointed to the presence of proteases in allergenic preparations as nonspecific activators [4]. Indeed allergenic preparations from house dust mite (HDM), cockroach, pollen, and molds share the common property of presenting intrinsic protease activity which gives them the ability to permeabilize the epithelial defence barriers of the host through disruption of tight junctions [5–7] and to stimulate the production of cytokines by airway cells [8–10]. Wills-Karp et al. have recently reviewed the mechanisms of innate immunity activation by proteolytic allergens through cleavage of several key immune molecules such as CD40, DC-SIGN, CD25, CD23, PAR2, lung surfactant proteins, and unknown basophil receptors [11]. Moreover, proteases in general are potent initiators of allergic lung inflammation and are therefore potential adjuvant factors accountable for the early triggering of allergic responses [12]. All these studies support the notion that the proteolytic activity of allergens is in some manner linked to their ability to stimulate Th2-cell activation. Proteases are also essential for the infectious and reproductive cycle of helminths [13]. Hence proteases might be an essential link between Th2-type allergic immune responses and anti-helminth immunity. Indeed basophils directly activated by papain are able to induce a Th2 immune response through production of IL-4, thymic stromal lymphopoietin (TSLP), and other Th2-associated cytokines and chemokines [14]. These findings have strengthened the idea that the immune system has evolved a specific detection mechanism based on the sensing of abnormal protease activities as a trigger for Th2 responses.

Although the stimulating/allergenic properties of proteases have been clearly demonstrated in various in vitro models, their requirement for the development of Th2 responses and lung inflammation in vivo using a complex aeroallergen is not clearly established. Here we have investigated the potential implication of proteases in the overcoming of lung tolerance and in the induction of Th2-biased immune responses using an *Alternaria* model. The mold *Alternaria alternata* is a ubiquitous saprophyte commonly found in soil and plants and one of the most common fungi associated with allergic rhinitis and asthma [15]. Spores from *Alternaria* do not colonize the lungs and are rapidly cleared, in contrast to *Aspergillus* which is an opportunistic pathogen causing allergic and invasive diseases.

In previous studies, we demonstrated that chronic instillations of an *Alternaria* extract in naïve mice induced a typical Th2 immune response, a characteristic lung allergic inflammation, the

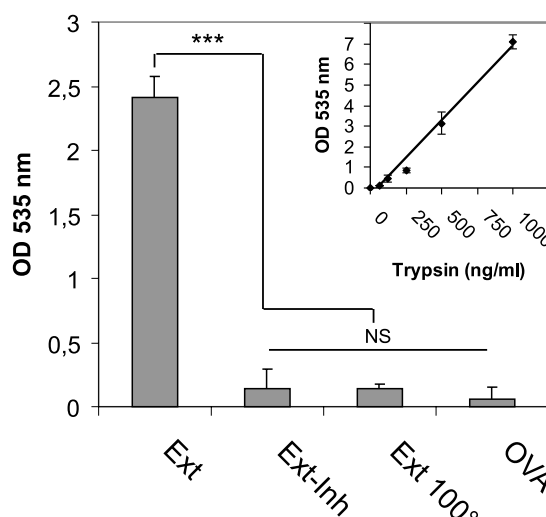


Figure 1. Proteolytic activities of the untreated, protease-inhibited and heat-treated *Alternaria* extract. Proteolytic activities were assessed using a protease fluorescent detection kit. Untreated, protease-inhibited, or heat-inactivated (100°C for 10 min) samples (50 µg/mL) were incubated at 37°C with the fluorescent protease substrate. Analyses were performed 18 h later by recording the OD at 535 nm in the supernatants after acetic acid precipitation. Insert: Dilution curve of a trypsin solution provided by the manufacturer. Data are shown as mean ± SD of three determinations, and are representative of three different experiments. *** $p < 0.001$; two-tailed Mann–Whitney U-test.

development of an airway hyper reactivity (AHR), and an important remodeling of the airway wall [16, 17]. Here we show that, in this *Alternaria* model, proteases are not necessary for the full development of the specific allergic response in vivo, although the loss of proteolytic activities in the extract is concomitant with an inhibition of the polyclonal IgE response. LPS potentially contaminating the extracts and the TLR4 signaling pathway is not involved in the development of this allergic response either, while Myd88 signaling is.

Results

Protease activity of native and heat-denatured *Alternaria* extracts

Proteases are very common in various allergen preparations and in mold extract in particular. The proteolytic activity in the *Alternaria* extracts used in this study was measured by production of fluorescence upon cleavage of an FITC-casein substrate (Fig. 1). As compared with the trypsin standard curve, the untreated *Alternaria* extracts showed a proteolytic activity equivalent to that obtained at a concentration of 500–600 ng/mL of trypsin (corresponding to 50–60 ng of trypsin for each 100 µL extract instillation). After heat inactivation at 100°C for 10 min or after inactivation using a protease inhibitor cocktail, the proteolytic activity in the extract was reduced to background levels (below 7 ng in each instillation). A control OVA preparation showed only background protease activity.

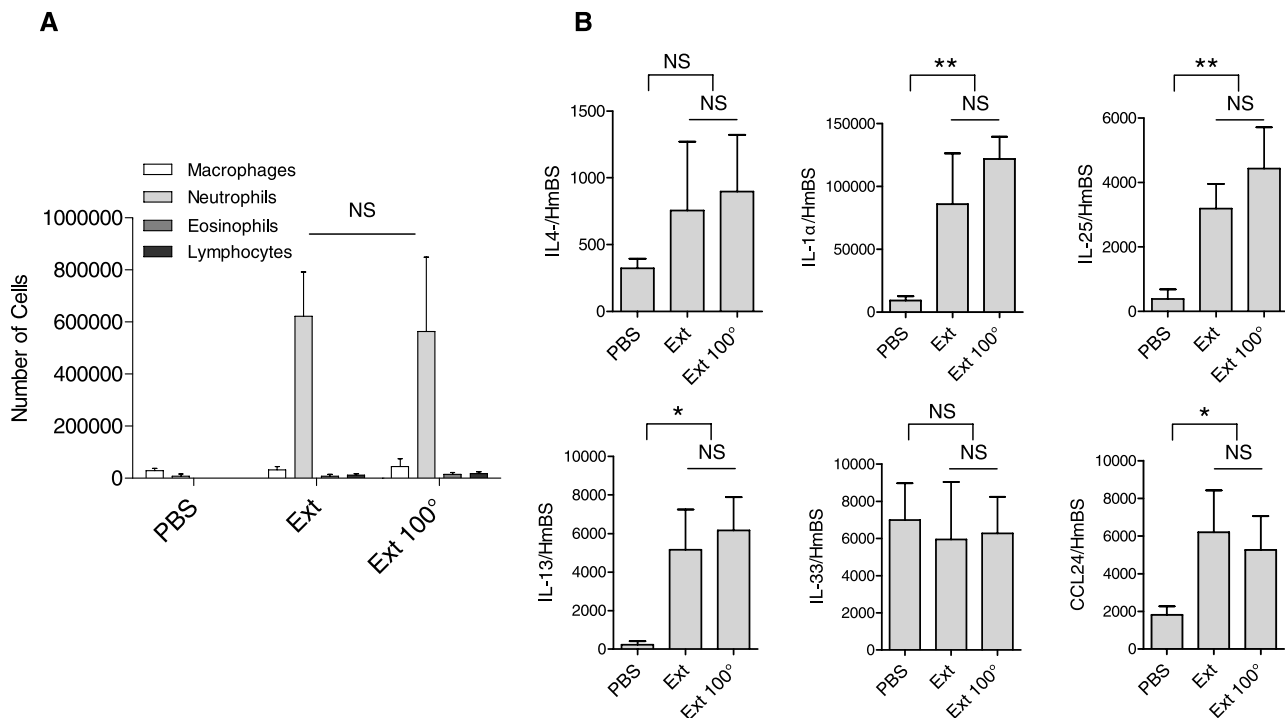


Figure 2. Early lung inflammation and cytokine/chemokine mRNA lung expression in BALB/c mice after stimulation with native or heat-treated *Alternaria* extracts. BALB/c mice were instilled during 3 days with PBS, untreated or heat-treated *Alternaria* extract. The graphs show the inflammatory cell recruitment in the BALF after 3 days (A) and qRT-PCR analysis of lung chemokine/cytokine mRNA expression levels (B). Total RNA was extracted from the whole lungs and transcriptional levels of the indicated mediators were analyzed by qRT-PCR. (A,B) Data are shown as mean + SD of five individual mice and are representative of two independent experiments. NS = nonsignificant, * $p < 0.05$, ** $p < 0.01$; two-tailed Mann–Whitney U-test.

Allergic responses are not altered in BALB/c mice instilled with heat-inactivated *Alternaria* extract

The role of chemokines and cytokines in the inflammatory network responsible for allergy and asthma is clearly established. Cytokines and chemokines can promote the selective recruitment of different effector cell subsets, including Th2 cells, eosinophils, and neutrophils in the inflamed tissue and can also directly cause cellular activation and release of inflammatory mediators. The expression of pro-allergic and pro-inflammatory mediators in the lungs of mice stimulated with untreated or heat-inactivated *Alternaria* extract was compared by quantitative real time polymerase chain reaction (qRT-PCR). To investigate the innate component of the response, analyses were performed 24 h after the first three extract instillations, when neutrophils and a few macrophages accumulated in the bronchoalveolar lavage fluid (BALF) (Fig. 2). When stimulated with the native extract, pro-allergic mediators such as IL-4, IL-33 (Fig. 2B), IL-5, CCL11, and TSLP (Supporting Information Fig. 1A) showed no significant upregulation at day 3. IL-25, IL-1α, and IL-13, however, were strongly increased and this pattern of expression was identical when mice were treated with the heat-treated extract. A similar neutrophil accumulation in the BALF was also obtained with the native and the heat-treated extract.

Similar analyses were also performed after 4 weeks of instillations in order to investigate the specific component of the immune response during this chronic phase. At this late time point, the inflammatory cell subsets recruited in the BALF were mainly eosinophils with some macrophages, neutrophils, and lymphocytes (Fig. 3A). Heat inactivation of the extract did not affect the eosinophil accumulation. Both native and heat-treated extracts also strongly increased the lung expression of IL-4, IL-33, CCL11, CCL24 (Fig. 3B), and IL-5, while IL-25 expression was completely suppressed at this late time point (Supporting Information Fig. 1B). Lung IL-4 and IL-13 mRNA expression correlated with the levels of IL-4 and IL-13 detected in the BALF by ELISA (Fig. 3C and D).

Lung expression of the pro-inflammatory mediators IL-1α, IL-17A and IL-17F, CXCL-1, CXCL-2, CCL3, TNF-α, and IFN-γ was strongly upregulated during the early phase and decreased during the chronic phase. Again, this pattern of expression was very similar in mice instilled with the native and with the heat-treated extract, except for IL-1α and IL-17 which were slightly more expressed during the chronic phase in mice stimulated with the heat-treated extract (Fig. 3B and Supporting Information Fig. 1B).

Chronic instillations of the *Alternaria* extract stimulated a polyclonal production of IgE leading to an increase of total IgE serum

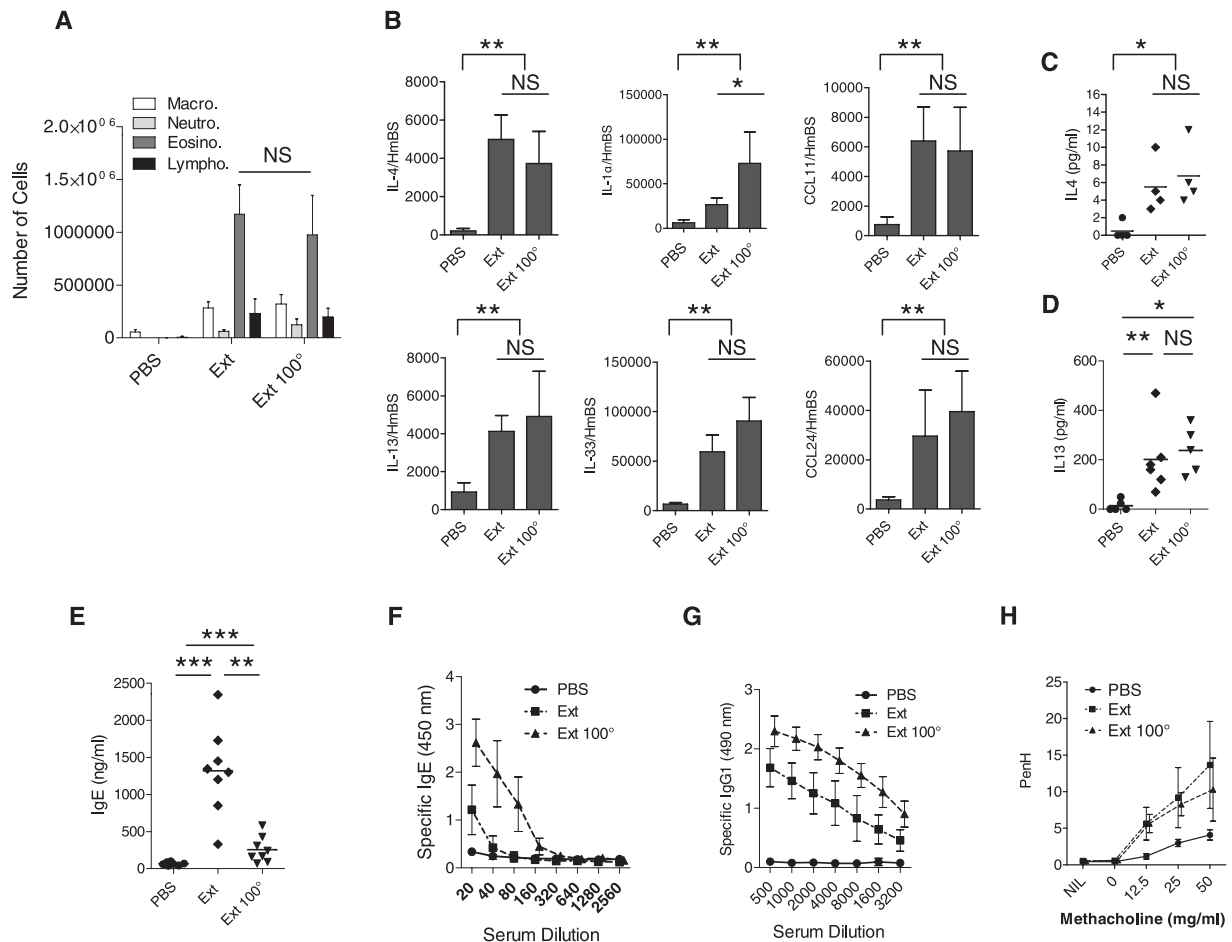


Figure 3. Late lung inflammation, cytokine/chemokine mRNA lung expression, and systemic response in BALB/c mice after stimulation with untreated or heat-treated *Alternaria* extracts. BALB/c mice were chronically instilled with PBS, untreated or heat-treated *Alternaria* extract during 4 (for qRT-PCR analysis) to 5 weeks and then systemic and lung responses were investigated. The graphs show the inflammatory cell recruitment in the BALF (A), qRT-PCR analysis of lung chemokine/cytokine mRNA expression levels (B) and IL-4 (C) and IL-13 (D) levels in BALF. Results are shown as mean $\pm$ SD of four to eight individual mice. Total IgE concentrations in the serum (E), *Alternaria*-specific IgE (F) and IgG1 (G) antibody response were determined by ELISA; results are the mean $\pm$ SEM of eight individual mice. AHR was analyzed by whole body plethysmography after nebulization of increasing doses of methacholine. Data are shown as mean $\pm$ SD of six individual mice and are representative of two independent experiments. NS = nonsignificant, *** p < 0.001; two-tailed Mann-Whitney U-test.

levels after 5 weeks. Heat treatment of the extract almost completely abolished this production of polyclonal IgE (Fig. 3E). However stimulation with either untreated or heat-inactivated extract led to a synthesis of specific IgE and a strong production of specific IgG1, these productions being even higher in mice instilled with the heat-inactivated than with the untreated extract (Fig. 3F and G). No IgG2a production was induced (data not shown). Thus, heat treatment of the *Alternaria* extract inhibits the proteolytic activities of these preparations and their capacity to induce a systemic polyclonal IgE response while antigen-specific IgE and IgG1 responses are not affected. AHR was increased similarly in mice stimulated with the native or the heat denatured *Alternaria* extract after 5 weeks (Fig. 3H). Histological examination of lungs from mice chronically instilled with untreated or denatured *Alternaria* extracts did not show significant differences between the two groups either (Supporting Information Fig. 2). Intense inflammation foci containing eosinophils in both

the perivascular and the peribronchial areas, numerous goblet cells, and a pronounced thickening of the airway epithelium were observed in mice instilled with the extracts as compared with control PBS-treated mice.

Importance of proteases for allergic responses induced in chronically stimulated C57BL/6 mice

Genetic background of mice is important for the characteristics of inflammatory diseases such as asthma. Phenotypic differences between the two commonly used strains, C57BL/6 and BALB/c, have been observed in asthma models with different distributions of pro-inflammatory cytokines, inflammatory cells, and AHR [18]. We therefore analyzed the effect of protease inactivation on the chronic allergic responses induced in C57BL/6 mice. As in BALB/c mice, heat treatment of the extract had no effect on the

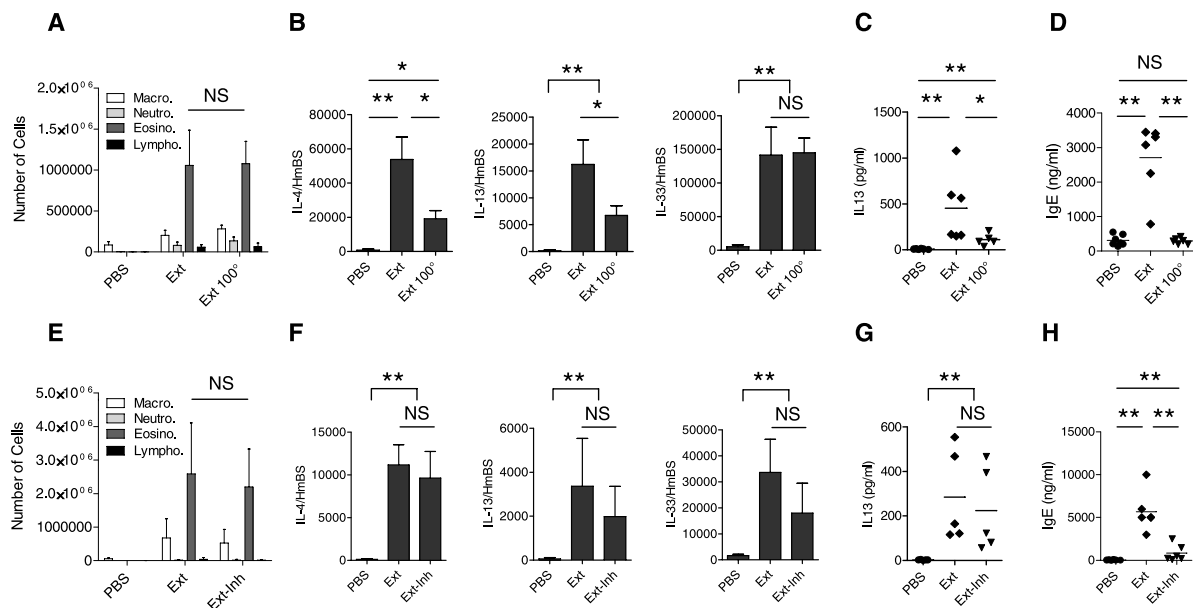


Figure 4. Late lung inflammation, cytokine/chemokine mRNA lung expression and systemic response in C57BL/6 mice after stimulation with untreated, heat-treated, or protease-inhibited *Alternaria* extracts. In the upper panel C57BL/6 mice were instilled during 4 weeks with PBS, untreated or heat-treated *Alternaria* extract. The graphs show the inflammatory cell recruitment in the BALF (A), qRT-PCR analysis of lung chemokine/cytokine mRNA expression levels (B), IL-13 levels in BALF (C), and total IgE concentrations in the serum (D). In the lower panel C57BL/6 mice were instilled during 4 weeks with PBS, untreated or protease-inhibited extract. The graphs show the inflammatory cell recruitment in the BALF (E), qRT-PCR analysis of lung chemokine/cytokine mRNA expression levels (F), IL-13 levels in BALF (G) and total IgE concentrations in the serum (H). Results are shown as the mean $\pm$ SD values of five to seven individual mice and are representative of two independent experiments. NS = nonsignificant, * $p < 0.05$, ** $p < 0.01$; two-tailed Mann–Whitney U-test.

accumulation of eosinophils in the BALF of C57BL/6 mice instilled during 4 weeks (Fig. 4A). However, in contrast to BALB/c mice, lung IL-4 and IL-13 expression and IL-13 levels in BALF were decreased (Fig. 4B and C). Heat treatment of the extract completely inhibited the polyclonal IgE production, as observed in BALB/c mice. Lung CCL11 expression was also suppressed but not IL-1 α or CCL24 while specific IgE and IgG1 production was not affected by denaturation of the extract (Supporting Information Fig. 3). Since many compounds from the extract can be affected by the heat treatment, proteases from the *Alternaria* extract were specifically inhibited using a protease inhibitor cocktail. Mice chronically stimulated with untreated or protease-inhibited extract mounted lung allergic responses with similar eosinophil accumulation in the BALF (Fig. 4E), lung cytokine expression (Fig. 4F and Supporting Information Fig. 3), and IL-13 levels in the BALF (Fig. 4G). However, and as observed with the heat-treated extract, specific protease inhibition induced a strong inhibition of the polyclonal IgE synthesis (Fig. 4H).

Importance of TLR4 and MyD88 signaling for the development of *Alternaria*-specific allergic responses

In the mouse, LPS has variable effects on allergic pulmonary inflammation depending on the model used and in the OVA model of asthma, exposure to LPS can either protect against asthma or exacerbate it. Inhalation of antigen with high levels of LPS results in Th1 lung responses while LPS at low concentrations has the ability to promote the induction of DC-driven Th2-cell

responses to harmless inhaled antigens [19,20]. Moreover, recognition of LPS contaminating house dust mite preparations by TLR4 on lung epithelial cells is necessary and sufficient for priming of specific Th2 immune responses [21,22]. To rule out the assumption that the allergic responses induced by the extracts were due to their LPS contamination, LPS content in the *Alternaria* extract was analyzed. The LPS concentration in extract was very low (0.804 EU/mL, 0.0804 EU LPS for each instillation resulting in a total amount of 1.04 EU instilled during the 5 week period). In contrast to mice chronically instilled with the *Alternaria* extract, stimulation with an OVA preparation containing a similar LPS concentration or spiked with 2 μ g/mL of LPS did not trigger any allergic lung inflammation, polyclonal IgE synthesis, or IL-13 production (Supporting Information Fig. 4).

Next, TLR4 deficient, Myd88 deficient and WT C57BL/6 mice were stimulated with *Alternaria* extract to further rule out the role of LPS in the induction of allergic lung inflammation in this model. As shown in Figure 5A, TLR4 deficiency did not affect the development of lung inflammation and eosinophils were similarly recruited in the BALF of WT and TLR4-deficient mice upon chronic intranasal (i.n.) stimulations with *Alternaria* extracts. However, eosinophil recruitment was completely inhibited in Myd88-deficient mice. Lung cytokine gene expression analysis using qRT-PCR demonstrated an important upregulation of IL-4, IL-13, IL-33, CCL11, CCL24, and IL-1 α mRNA expression in chronically instilled WT mice (Fig. 5B). TLR4 deficiency did not modify the IL-33, CCL11, CCL24, and IL-1 α mRNA upregulation. Although IL-4 and IL-13 mRNA expression was significantly lower

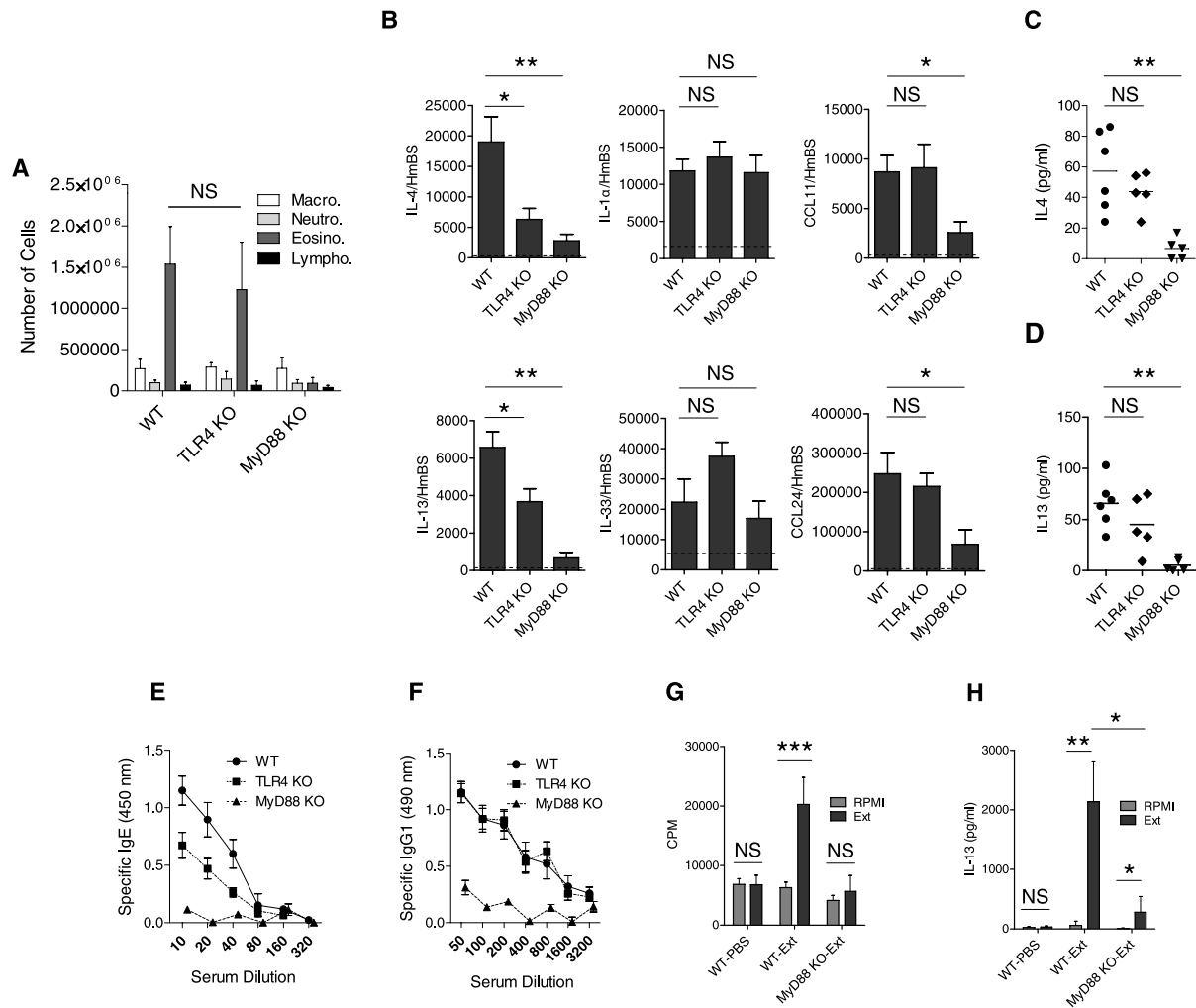


Figure 5. Analysis of lung inflammation and cytokine/chemokine mRNA lung expression induced by the *Alternaria* extract in TLR4 KO, MyD88 KO and WT C57BL/6 mice. C57BL/6, TLR4 KO, or MyD88 KO mice were chronically instilled with *Alternaria* extract during 5 weeks. The graphs show the inflammatory cell recruitment in the BALF (A), qRT-PCR analysis of lung chemokine/cytokine mRNA expression levels (B; dotted lines denote mRNA levels obtained from C57BL/6 naive mice), IL-4 (C), and IL-13 levels in BALF (D), *Alternaria*-specific IgE (E) and IgG1 (F). Graph (G) and (H) show the lymphoproliferation and IL-13 production of in vitro restimulated spleen cells from WT and MyD88-deficient mice treated during 4 weeks with the *Alternaria* extract. Results are shown as mean $\pm$ SD of four to five individual mice and are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed Mann-Whitney U-test.

in TLR4 knockout mice than in WT mice, levels of IL-4 and IL-13 detected by ELISA in BALF were not significantly different (Fig. 5C and D). In MyD88-deficient mice IL-4 and IL-13 mRNA expression were more strongly suppressed and this was reflected by strongly decreased IL-4 and IL-13 concentrations in the BALF. CCL11 and CCL24 expression were also lower in MyD88-deficient mice while IL-33 and IL-1 α expression were not affected by the MyD88 mutation. The TLR4 deficiency slightly decreased the specific IgE antibody response as compared with the WT mice (Fig. 5E) whereas the IgG1 antibody production was not affected (Fig. 5F). In contrast, specific IgE production and IgG1 antibody serum levels were strongly suppressed in MyD88-deficient mice (Fig. 5E and F). Analysis of spleen cell proliferative responses and IL-13 production after in vitro restimulation with the *Alternaria* extract showed that both T-cell specific proliferation and IL-13

synthesis were strongly suppressed in MyD88-deficient mice as compared with WT (Fig. 5G and H). To investigate a potential impairment of the innate component of the response, WT, TLR4, and MyD88-deficient mice were stimulated during 3 days i.n. with *Alternaria* extract. Twenty-four hours later, neutrophils were present in the BALF from WT and TLR4-deficient mice but were almost completely absent in the BALF from MyD88-deficient mice (Fig. 6A). This lack of neutrophil recruitment was correlated with a strong inhibition of lung pro-inflammatory mediator (IL-17A, IL-17F, TNF- α , and CXCL2) expression in MyD88-deficient mice (Fig. 6B). In contrast to BALB/c mice, WT C57BL/6 mice did not show any increase in lung IL-25 and IL-13 expression but had a clear upregulation of IL-33 lung expression in this early phase of the response. This early lung IL-33 upregulation was completely inhibited in MyD88 but not in TLR4-deficient mice.

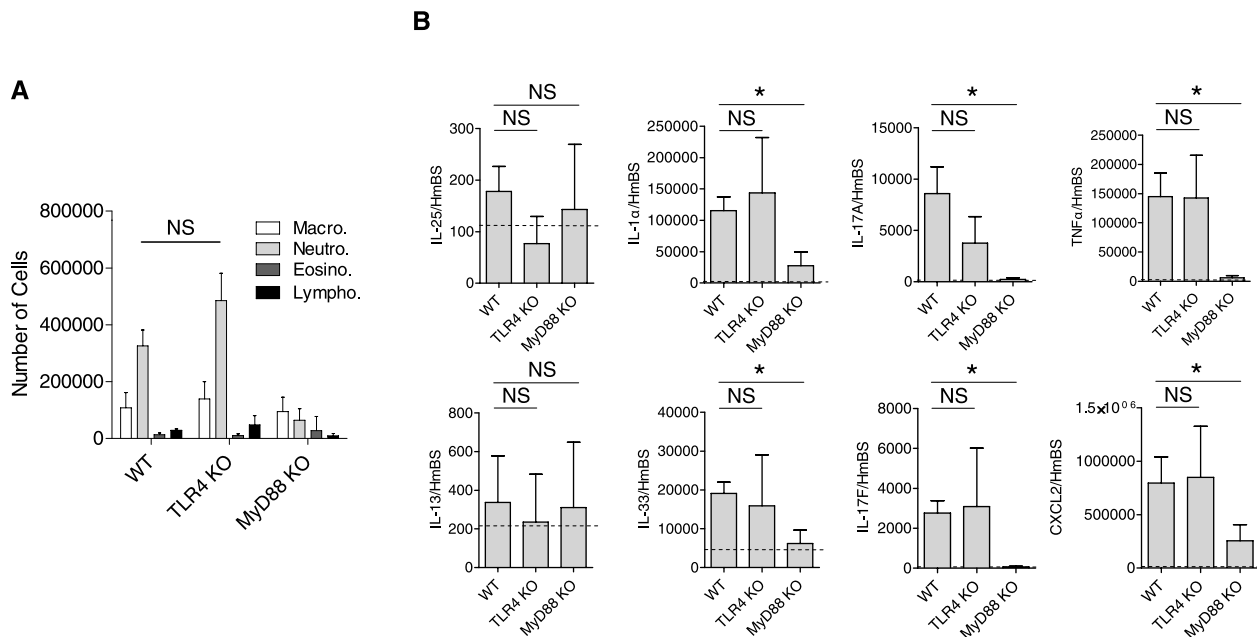


Figure 6. Early lung inflammation and cytokine/chemokine mRNA lung expression after stimulation of WT C56BL/6, TLR4, or MyD88-deficient mice with *Alternaria* extracts. Mice were instilled during 3 days with *Alternaria* extract. The graphs show the inflammatory cell recruitment in the BALF (A) and qRT-PCR analysis of lung chemokine/cytokine mRNA expression levels (B). Dotted lines denote mRNA levels obtained from naïve C57BL/6 mice. Results are shown as the mean + SD of five individual mice and are representative of two independent experiments. NS = non-significant, * $p < 0.05$; two-tailed Mann–Whitney U-test.

Finally lung IL-1- α expression was also strongly suppressed in MyD88-deficient mice (Fig. 6B).

Discussion

Even if numerous studies have highlighted the activating properties of proteases *in vitro*, their importance in the development of allergic responses *in vivo* remains largely unknown. In this study, we have used an *Alternaria* model to evaluate the importance of proteolytic activities present in an *Alternaria* extract for the triggering of Th2 responses *in vivo* and for the development of lung allergy. Previous studies have shown that proteases present in *Alternaria* extracts induce the production of TSLP by airway epithelial cells [23] and activate human eosinophils [24]. It is also known that *Alternaria* extracts are potent stimulators of DCs leading to a type-2 activation of T cells but the fungal components involved in this skewing are still unknown [25]. In this study, we have compared *in vivo* the Th2-activating properties of native (protease containing) and heat-inactivated (protease inactivated) *Alternaria* extracts. Our results clearly demonstrated a relationship between heat inactivation of the extract and a reduced stimulation of polyclonal IgE production. In the same way, Gough et al. found that specific inhibition of the proteolytic activity of Der p1 decreased the polyclonal IgE synthesis [26] but the mechanism(s) involved are unclear.

Despite the reduced polyclonal IgE response induced with the heat denatured extract, both protease containing and heated *Alternaria* extracts induced in BALB/c mice the same allergic

response in the chronic phase (mold-specific IgG1 and IgE antibodies, lung inflammation with eosinophilia, mucus production, thickening of the epithelium and AHR). Also, during the early phase (3 days of stimulations), native or heat-denatured extract induced similar neutrophil accumulation in the BALF. In this early phase, the strong lung mRNA expression of the pro-allergic mediators IL-13 and IL-25 and of pro-inflammatory mediators induced by the *Alternaria* extract was not affected by heat treatment either. mRNA expression of pro-inflammatory mediators decreased in the chronic phase, while expression of the pro-allergic mediators IL-4, IL-33, CCL11, and CCL24 increased. Again, lung expression of these mediators was not modified by the heat treatment of the extract. Similar results were also obtained in C57BL/6 mice when allergic responses were compared after a chronic stimulation with native, heat-treated, or proteases-inhibited *Alternaria* extracts. Therefore, proteolytic activities seem dispensable for the development of the systemic and the lung allergic immune response to the *Alternaria* extract. Our results seem to be in conflict with those reported by Boitano et al. showing that *Alternaria* proteases were able to activate PAR-2 *in vitro* and arguing that this activation will result in protease-dependent inflammatory cell recruitment to the airways [27]. In Boitano's study, inflammatory recruitment was indeed completely inhibited by heat inactivation or use of protease inhibitors but cells recruited in the BALF were mainly macrophages and neutrophils with very few eosinophils. Also, instead of an extract, they used a culture filtrate of *Alternaria* and with only a short course protocol, two differences with our study that might explain the contradiction. Moreover, in a very acute model of activation, *Alternaria* extract

has been shown to induce the expression on the airway epithelium of FIZZ1, a resistin-like molecule involved in lung remodeling and early eosinophil recruitment in PAR-2-deficient mice [28]. Porter et al. investigated in vivo the relationship between proteases of *Aspergillus niger* (a pathogenic fungus) and allergic asthma [29]. In their study, allergic lung inflammation was defined as a protective mechanism against potentially lethal invasive airway disease. Importantly, allergic lung inflammation required both lung infection and in situ protease secretion. Indeed active fungal infection and not conidia hypersensitivity was required for allergic lung disease. Moreover, an aqueous extract containing significant protease activity induced exclusively a neutrophil-dominated inflammatory response devoid of allergic features [29]. This mechanism may be relevant in the case of invasive molds as for instance *Aspergillus spp.*, but it is not pertinent for soluble *Alternaria* extract or even *Alternaria* spores that are noninfectious and do not colonize the lungs [30] but can indeed induce an allergic lung inflammation.

In addition to proteases, LPS has been shown to be able to bypass the lung tolerogenic mechanisms and to stimulate allergic inflammations when given together with proteins [20]. However, it is very unlikely that contaminating LPS activated Th2 responses in the *Alternaria* model described here. Indeed neither instillation of OVA in the presence of low nor of high levels of LPS induced the same full allergic response as the *Alternaria* extract. Experiments in TLR4 and MyD88 mutated mice confirmed that LPS was not essential in our model. Even if lung IL-4 and IL-13 mRNA expression levels were reduced in TLR4-deficient mice, the concentrations of IL-4 and IL-13 in the BALF were not significantly suppressed during the chronic phase. In addition, lung IL-33, CCL11, CCL24, IL-1- α mRNA expression and, more importantly, lung eosinophil recruitment were not affected. These results clearly indicate that in the *Alternaria* model, TLR4 signaling is not as critical as it is in other models [21,22] but may contribute to the optimal expression of some mediators. However in MyD88-deficient mice, eosinophil accumulation in BALF was completely abrogated, IL-4, IL-13, CCL11, and CCL24 lung mRNA expression was strongly decreased, as were specific IgE and IgG1 levels in the serum in the chronic phase. Moreover, these mice showed a defective adaptive immune response since spleen cells from MyD88-deficient mice showed a reduced capacity to proliferate and to produce IL-13 when restimulated in vitro with the *Alternaria* extract.

In contrast to BALB/c mice, an upregulation IL-33 lung expression instead of IL-25 was observed in the early phase of the response in C57BL/6 mice. However, both strains strongly upregulated lung IL-1- α expression, a cytokine that has recently been shown to control allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33 [31]. IL-25 and IL-33 are powerful activators of innate lymphoid cells that produce very rapidly large amounts of IL-5 and IL-13 that in turn trigger the activation of Th2 cells [32–34]. Thus, in this *Alternaria* model, different activating pathways could be operative in BALB/c and C57BL/6 mice leading finally to similar allergic responses. The early upregulation of pulmonary IL-33 was not impaired by TLR4 deficiency. However, early lung IL-33 and IL-1- α expression were fully dependent on MyD88 signaling and were completely

inhibited in MyD88-deficient mice. Of note, late IL-33 and IL-1- α lung expression were not affected. Also in human corneal epithelium, IL-33 has been shown to be largely induced by microbial components through TLR and NF- κ B signaling pathways [35]. In contrast, Kouzaki et al. reported no modification in the early IL-33 production (but found a decreased IL-5 and IL-13 secretion) in MyD88-deficient mice exposed to *Alternaria* [36]. The fact that these results were obtained at a very early time point (1 and 12 h) after a single exposure to a high allergen concentration (100 μ g of extract) might explain the discrepancy. Therefore, signaling through other TLRs or through the IL-1 receptor seems clearly decisive for the late lung recruitment of eosinophils. In addition, basophils could also be important players in these responses since IL-18 and IL-33 are capable to trigger the release of IL-4 and IL-13 by these cells using a MyD88-dependent mechanism [37].

Recently, the ability of chitin to serve as a potent multifaceted adjuvant for the induction of Th1, Th2, and Th17 immune responses has been clearly demonstrated [38]. Effects of chitin have been shown to be mediated by a TLR2-dependent pathway involving the production of IL-17A or by a rapid (2 days) MyD88-independent pathway [39,40]. Obviously, this compound is of particular importance in the case of mold allergy since chitin is a major constituent of fungal cell wall. However, we found that the allergic lung inflammation induced by *Alternaria* extract was TLR2 and IL-17RA independent (data not shown) and (as already mentioned), induction of the lung eosinophilia by *Alternaria* extract required more than 2 weeks and was indeed MyD88 dependent. Therefore, it is very unlikely that chitin is involved in the triggering of allergic lung inflammation by *Alternaria*.

If proteases, TLR4- and TLR2-activating compounds (such as LPS and chitin, respectively) are not implicated, the adjuvant substance(s) in the *Alternaria* extract mediating the initiation of the allergic response remain to be formally identified. Recently, HDM allergens have been shown to induce in vitro the production of CCL20 from airway epithelial cells which is neither protease nor TLR2/4 dependent but relies on β -glucan moieties in the HDM allergens [41]. Moreover, in vitro derived human DCs have been shown to internalize HDM allergens through DC-SIGN. After maturation, this internalization also promoted a Th2 polarization of naive CD4⁺ T cells [42]. Glycosylated moieties from the peanut allergen Ara h 1 are also ligands for DC-SIGN, act as Th2 adjuvant in vitro and can also directly activate DCs [43]. Data in the literature also suggest that complex carbohydrate from helminths such as Lacto-N-fucopentaose III are key molecules responsible for activating anti-inflammatory antigen presenting cells and driving CD4⁺ T-cell responses toward the Th2-type [44,45]. Therefore, one can hypothesize that the heat resistant activity present in the *Alternaria* extract responsible for the activation of the Th2 immune response might be fungal carbohydrates able to activate directly or indirectly a MyD88-dependent signaling pathway. Validation of this hypothesis and precise identification of the Th2 activating components will, however, require further studies.

In conclusion, our data support the finding that, although heat labile protease components of the extract are required for the

stimulation of the polyclonal IgE secretion in our *Alternaria* model, fungal proteases are clearly not required for the triggering of the systemic and lung allergic immune response. TLR4 signaling is also dispensable for these responses to develop. However, early lung IL-33 expression and late eosinophil lung recruitment, Th2 cytokines expression, antibody production, and T-cell priming are clearly MyD88 dependent.

Materials and methods

Mold extract preparation, determination of proteolytic activity, and LPS detection

Alternaria alternata (strain 18586) was obtained from the BCCMTM/IHEM (WIV-ISP, Brussels, Belgium). Mold extracts were prepared as previously described [46]. Proteolytic activities were assessed using the protease fluorescent detection kit according to the specifications of the manufacturer (Sigma Aldrich). Samples were incubated for 18 h at 37°C. Proteases in the extract were inactivated by heating the extract at 100°C for 10 min. Proteases were also inactivated by incubating the extract with a protease inhibitor cocktail (Sigma Aldrich, 10% vol/vol) containing 100 mM AEBSF, 1.4 mM E-64, 2.2 mM Pepstatin A and 500 mM of 1,10-phenanthroline. After 1 h of treatment, extract was dialyzed against PBS. OVA (Imject OVA) was obtained from Pierce. LPS (*E. coli*, serotype EH100 Ra TLR grade) was obtained from Alexis Biochemicals. For LPS determination in the antigenic preparations, aliquots (50 µg/mL) of *Alternaria* extract, OVA prepared in PBS or OVA in PBS spiked with 2 µg/mL of LPS were sent to LONZA (Verviers, Belgium). The kinetic colorimetric LAL assay (Kinetic-QCL[®], sensitivity = 0.005 EU/mL, D method of the European Pharmacopeia was used) was used. The reactivity of LAL to β -1,3-glucans present in the samples was inhibited with β -G-Blocker.

Animals and immunizations

Female BALB/c and C57BL/6 mice were obtained from the Elvage Janvier (Le Genest Saint Isle, France) and maintained under standard laboratory conditions. TLR4- and MyD88-deficient mice (on a C57BL/6 background) were kindly given to us by Dr. S. Goriely (Institute for Medical Immunology, ULB, Gosselies, Belgium) and Dr. C. Desmet (Cellular and Molecular Physiology, GIGA-Research, ULg, Belgium). To induce the allergic lung inflammation, mice were instilled intranasally (i.n.) with 5 µg of extract (in 100 µL of PBS) from *Alternaria* on three consecutive days for the first week and then twice a week, every Monday and Friday during three to four more weeks. Control mice were instilled with PBS. Samplings were performed 24 h after the last instillation. In some experiments, groups of BALB/c mice were also instilled with OVA (5 µg) with or without LPS (0.2 µg). All experiments were performed in accordance to the local ethics committee.

ELISA

Mice were bled in the retro-orbital plexus 24 h after the last i.n. instillation. Total serum IgE levels were determined using a sandwich ELISA made of LO-ME-2 and peroxidase-labeled LO-ME-3 mAbs (IMEX, UCL, Brussels, Belgium). A purified monoclonal mouse IgE (LB-4, IMEX) was used as a standard [16]. Specific antibodies were analyzed using a modified indirect ELISA as already described [17]. Briefly, plates were coated overnight with native *Alternaria* extract (20 µg/mL for IgE detection and 2 µg/mL for IgG1) in borate buffer, washed in PBS Tween 0.1%, and saturated for 2 h with skimmed milk. Then, serial twofold dilutions of serum were applied for 2 h. After washing peroxidase-labeled rat anti-mouse IgE (LO-ME-3) or anti-mouse IgG1 (LO-MG1-13) were then added for 2 h. Finally, plates were washed and developed by the addition of 100 µL of TMB (for IgE) or OPD (for IgG1). The reaction was stopped with 50 µL of 2N H₂SO₄ and od was read at 450 nm (IgE) or 490 nm (IgG1).

IL-4 and IL-13 levels were measured by ELISA in BALF obtained 24 h after the last i.n. inoculation or in three days culture supernatants. Quantikine[®] kits (sensitivity 2 and 1.5 pg/mL, respectively) were used according to the instructions provided by the manufacturers (R&D systems, MN, USA).

Measurement of airway reactivity

AHR was measured within 24 h after the last instillation, in response to methacholine inhalation using a whole-body plethysmography system (EMKA, Paris, France) as already described [17].

Bronchoalveolar lavages

Mice were euthanized by cervical dislocation 48 h after the last i.n. instillation. The trachea was exposed and incised. A needle (1.2 × 40 mm) was inserted into the trachea and BALF were harvested by rinsing the lungs twice with 1 mL of PBS. Total cell counts were determined with a hemocytometer. Differential cell counts were obtained by examining at least 300 cells on cytopsin slides stained with Diff-Quick (Dade Behring, Deerfield, IL, USA).

Histological analysis

Histological samples were prepared as already described [17].

mRNA cytokine quantification

Total RNA was extracted from homogenized lung cells with Tri-reagent (Sigma-Aldrich), according to the manufacturer's instructions 24 h after the last instillation. cDNA was synthesized using the SuperScript II Rnase H⁻ Reverse Transcriptase from Invitrogen. qRT-PCR was performed on a Biorad iCycler using a qPCR Core

Kit for Sybr Green I (Eurogentec). Each Real Time quantitative Polymerase Chain Reaction (RT qPCR) amplification was performed in duplicate under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The forward and reverse primers used are described in the Supporting Information Table 1. Hydroxymethyl-bilane synthase (HmBS) mRNA was used as reference housekeeping gene for normalization. The level of target mRNA, relative to the mean of the reference housekeeping gene, was calculated as previously described [47].

In vitro spleen cell stimulation

Spleens were removed aseptically 48 h after the last instillation. Spleen leukocytes (5.10^5 in 200 μ L) were cultured in triplicate in 96-well round-bottom microwell plates in DMEM medium supplemented with gentamycin, 2 mM L-glutamine, 5.10^{-5} M 2-mercaptoethanol, 10% fetal calf serum with or without 5 μ g of the stimulating antigens (native *Alternaria* extracts or OVA). Cultures were maintained in 5% CO₂ at 37°C for 3 days and then supernatants were collected and analyzed by ELISA for the presence of IL-13. Lymphoproliferative responses were measured using uptake of tritiated thymidin.

Statistical analysis

Results are presented as the mean $\pm$ SD (or SEM for ELISA results) of the indicated determinations. Data were analyzed by a two-tailed Mann–Whitney U-test (GraphPad Prism software). Significance was assumed at a *p* value of less than 0.05.

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Abbreviations: AHR: airway hyper reactivity · BALF: Broncho Alveolar Lavage Fluid · HDM: house dust mite · qRT-PCR: quantitative real time polymerase chain reaction · TSLP: thymic stromal lymphopoietin

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