



Cleavage of Fibrinogen by Proteinases Elicits Allergic Responses Through Toll-Like Receptor 4
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transcription PCR (qRT-PCR) on RNA isolated from nuclear or cytosolic fractions (fig. S14). Using RNA fluorescence in situ hybridization (FISH), substantial amounts of lincRNA-Cox2 were visible in both the nuclear and the cytosolic compartments of macrophages (Fig. 4A and fig. S15, A and B). Analysis of published RNA-seq data from nuclear and cytosolic fractions of macrophages (20) confirmed that lincRNA-Cox2 was present in both compartments, unlike *Neat1*, which was exclusively nuclear (fig. S16). RNA FISH analysis demonstrated efficient targeting of lincRNA-Cox2 in the nuclear fractions of our shRNA-silenced cells (fig. S15, C to F).

Many lncRNAs regulate transcription through their interactions with chromatin-modifying complexes or with heterogeneous nuclear ribonucleoproteins (hnRNPs) (5, 21–23). To identify binding partners for lincRNA-Cox2, we incubated in vitro-transcribed biotinylated lincRNA-Cox2 as well as an antisense lincRNA-Cox2 control RNA with nuclear or cytosolic extracts of macrophages and subjected RNA binding proteins to mass spectrometry for identification. hnRNP-A/B and hnRNP-A2/B1 were identified as specific binding partners for lincRNA-Cox2 in both the nuclear and cytosolic fractions (fig. S17). The ability of hnRNP-A/B and hnRNP-A2/B1 to bind lincRNA-Cox2 was confirmed by Western blot analysis (Fig. 4, B and C).

hnRNPs are multifunctional nuclear RNA binding proteins involved in various aspects of RNA biology (24, 25). hnRNPs also act as mediators of lncRNA-induced transcriptional repression (22, 23). hnRNP-A/B has been linked to transcriptional repression of some genes (26, 27), and hnRNP-A2/B1 and hnRNP-A/B have been shown to associate (28). Therefore, we hypothesized that lincRNA-Cox2 regulates the transcription of immune genes by associating with these hnRNPs. To test this directly, we generated macrophages lacking expression of hnRNP-A/B or hnRNP-A2/B1 by shRNA (fig. S18, A and B). Knockdown of hnRNP-A/B and hnRNP-A2/B1 did not modulate the levels of lincRNA-Cox2 itself (fig. S19) but did result in an enhancement of *Ccl5* protein levels in both unstimulated and Pam₃CSK₄-stimulated cells (Fig. 4D). Multiplex RNA analysis also revealed elevated levels of *Ccl5*, *Stat1*, *Tlr7*, *Icam1*, and *Ikb* in hnRNP-silenced macrophages (Fig. 4E). There was considerable overlap between genes that were regulated by lincRNA-Cox2 and these two hnRNP proteins (fig. S20 and table S5). We also examined the occupancy of RNA polymerase II at the promoters of the *Ccl5* and *Irf7* genes using chromatin immunoprecipitation (ChIP) and found increased levels of RNA polymerase II (Pol II) at the *Ccl5* and *Irf7* promoters when lincRNA-Cox2 or either of the hnRNPs were silenced in unstimulated cells (Fig. 4, F and G, and fig. S21). The occupancy of RNA Pol II on the promoter of *Irf7* was reduced when lincRNA-Cox2 but not hnRNP-A/B or A2/B1 was knocked down in Pam₃CSK₄-stimulated cells (fig. S22); this effect

was consistent with hnRNPs having no effect on Pam₃CSK₄-induced *Irf7* mRNA levels (Fig. 4E). Silencing of hnRNP-A2/B1 or hnRNP-A/B in cells that overexpressed lincRNA-Cox2 also reversed the inhibitory effect of lincRNA-Cox2 on *Ccl5* expression (Fig. 4H and fig. S23). Taken together, these experiments confirm that hnRNP-A/B and hnRNP-A2/B1 form a complex with lincRNA-Cox2 to repress the transcription of immune genes. The identification of hnRNP-A/B and hnRNP-A2/B1 further underscores the importance of hnRNPs in lncRNA function (21–23).

Innate immune responses have the capacity to both combat infectious microbes and drive pathological inflammation, which contributes to diseases such as atherosclerosis, autoimmunity, and cancer. A multitude of regulatory checkpoints control TLR signaling and inflammatory responses. We propose a model whereby TLR signaling induces lncRNAs, such as lincRNA-Cox2, that serve as repressors and activators of genes through their physical interactions with various regulatory complexes. As such, lncRNAs represent a component of the innate immune response that can both restrain and promote aspects of inflammatory signaling. Further characterization of these regulatory networks is likely to reveal novel drug targets and opportunities for therapeutic intervention in infectious and inflammatory diseases.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1240925/DC1
Materials and Methods
Figs. S1 to S23
Tables S1 to S5
References (29–35)

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Cleavage of Fibrinogen by Proteinases Elicits Allergic Responses Through Toll-Like Receptor 4

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Proteinases and the innate immune receptor Toll-like receptor 4 (TLR4) are essential for expression of allergic inflammation and diseases such as asthma. A mechanism that links these inflammatory mediators is essential for explaining the fundamental basis of allergic disease but has been elusive. Here, we demonstrate that TLR4 is activated by airway proteinase activity to initiate both allergic airway disease and antifungal immunity. These outcomes were induced by proteinase cleavage of the clotting protein fibrinogen, yielding fibrinogen cleavage products that acted as TLR4 ligands on airway epithelial cells and macrophages. Thus, allergic airway inflammation represents an antifungal defensive strategy that is driven by fibrinogen cleavage and TLR4 activation. These findings clarify the molecular basis of allergic disease and suggest new therapeutic strategies.

Allergic asthma is a chronic inflammatory airway disease that is characterized by both airway obstruction and enhanced

systemic and airway allergic inflammation marked by interleukin-4 (IL-4)-secreting T helper 2 (T_H2) cells, eosinophils, and serum immunoglobulin

E (IgE). Proteinases able to elicit T_H2 cell-driven allergic responses are secreted by fungi (1) and can be found in natural sources linked to allergic disease such as pollens (2) and dust mite antigens like Der p 1 (3, 4). Nonetheless, nonproteinase allergens such as ovalbumin also possess allergenic activity. Prior studies have linked TLR4, a microbial pattern-recognition receptor, to both proteinase-dependent and -independent allergic responses in mice (5–7), but a mechanism that explains the importance of proteinases and TLR4 in diverse allergic contexts involving both proteinase-active and -inactive allergens remains unknown.

To study the role of TLR4 in the context of proteinase-dependent allergic inflammation, we assessed wild-type (WT) and *Tlr4*^{-/-} mice after intranasal exposure to a fungal proteinase derived from *Aspergillus oryzae* (PAO) [endotoxin content: 1.7×10^{-3} endotoxin units (EU)/ μ g]. Consistent with our prior observations (2), WT mice challenged with PAO developed canonical features of asthma, including airway hyperresponsiveness, airway infiltration by eosinophils, enhanced production of the mucin gene transcript *Muc5ac*, goblet cell metaplasia of the airway, and enhanced production of transcripts for the proinflammatory cytokines IL-4, IL-5, and IL-13 (fig. S1, A to G). In contrast, all of these allergic parameters, with the exception of lung IL-4 transcripts, were either attenuated or abrogated in *Tlr4*^{-/-} mice. Thus, TLR4 is essential for the expression of proteinase-dependent asthma-like disease in mice.

Quantification of IL-4-secreting cells from whole lungs of proteinase-challenged mice (fig. S1H) confirmed the equivalent presence of IL-4-producing cells in the lung regardless of mouse genotype, suggesting that T_H2 cell development and recruitment occurred independently of TLR4. We then immunized mice against ovalbumin and confirmed that ovalbumin-specific T_H2 cell development was equivalent or enhanced in *Tlr4*^{-/-} mice compared with controls (fig. S1I). Induction of total IgE, an IL-4- and T_H2 cell-dependent process (8–11), was also identical between WT and *Tlr4*^{-/-} mice (fig. S1J).

Both the fungus *A. niger* and proteinase-free ovalbumin (1.8×10^{-3} EU/ μ g) also induced TLR4-dependent allergic lung disease in controls compared with proteinase-challenged WT mice (fig. S2). The consistent defect in disease expression seen in *Tlr4*^{-/-} mice was durable because identical

reductions in allergic disease parameters were seen after 2 and 4 weeks of ovalbumin immunization (fig. S2). Thus, TLR4 was required for the development of allergic airway disease, regardless of

allergen proteinase content, but was dispensable for T_H2 responses.

We confirmed the reduced IL-5 transcript production in *Tlr4*^{-/-} mice by assessing secreted

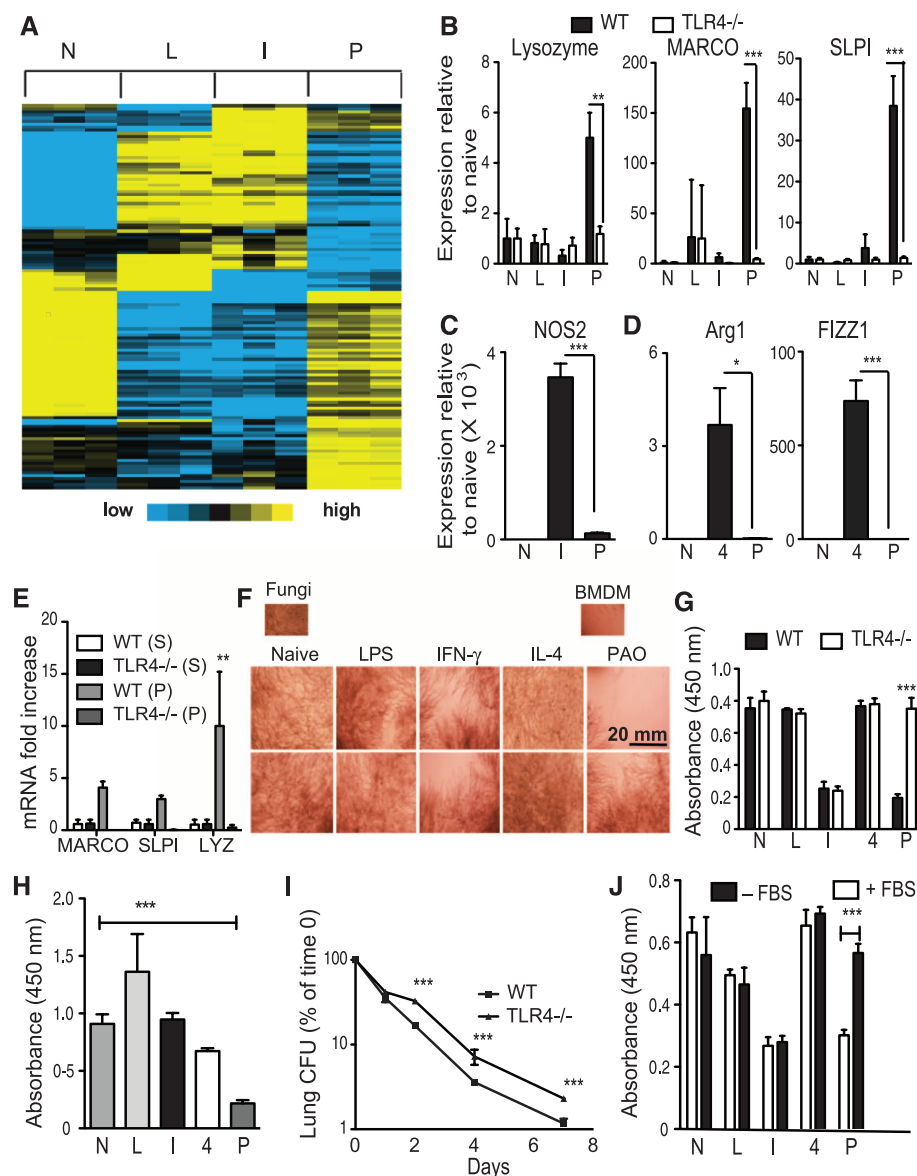


Fig. 1. PAO induces a distinct fungistatic macrophage phenotype through TLR4. (A to D) BMDMs from WT or *Tlr4*^{-/-} mice were left unstimulated (N) or cultured in the presence of lipopolysaccharide (L), IFN- γ (I), PAO (P), or IL-4 (4) as indicated. (A) Heat map depicting the relative expression of 252 gene probes, as assessed by microarray ($P < 0.01$ and fold change > 1.5 , comparing PAO to each of the other groups) of WT BMDMs. (B) Polymerase chain reaction (PCR)-based analysis of lysozyme, MARCO, and SLPI. (C) NOS2 expression in IFN- γ - and PAO-activated WT macrophages. (D) Arg1 and Fizz1 expression in IL-4- and PAO-activated WT macrophages. (E) MARCO, SLPI, and lysozyme (LYZ) mRNA expression in alveolar macrophages derived from mice challenged with PBS (S) or PAO (P). (F) BMDMs from WT (top row) and *Tlr4*^{-/-} (bottom row) mice were treated with IFN- γ , LPS, IL-4, or PAO for 24 hours and then cultured with *A. niger* conidia. Photomicrographs depict filamentous fungal growth. (G) Quantification of fungal growth in the same experiment, as assessed by XTT assay ($n = 3$ replicates per group). (H) PAO-treated human peripheral blood monocyte-derived macrophages were cultured under the same conditions as in (F) and assessed by XTT assay for their ability to restrain fungal growth ($n = 3$ patients). (I) WT and *Tlr4*^{-/-} mice were intranasally challenged with 400,000 *A. niger* conidia, and lungs were harvested and fungal colony forming units (CFU) were determined over 7 days. (J) Fungistatic potential of BMDMs was determined as in (G), but in the presence (+) and absence (-) of fetal bovine serum (FBS). Data are presented as means $\pm$ SEM (error bars) from one of three comparable experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Mann-Whitney (two group comparisons) and Kruskal-Wallis (three or more group comparisons) tests.

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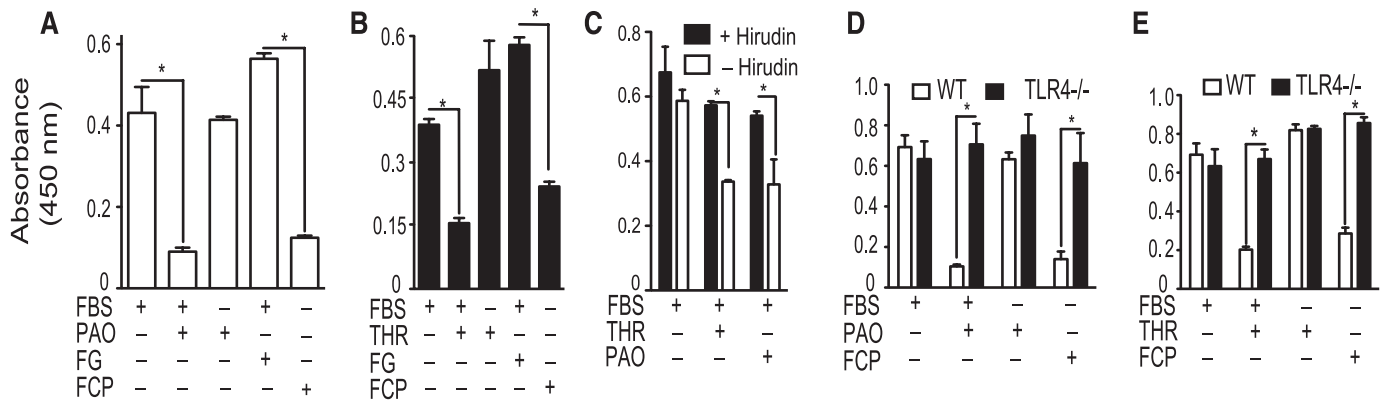


Fig. 2. Fibrinogen mediates PAO-dependent fungistasis through TLR4. BMDMs were cultured for 24 hours in the presence of FBS, PAO, fibrinogen (FG), FCP, or thrombin (THR), as indicated, and then inoculated with the conidia of *A. niger* for 24 hours, testing the requirement of (A) PAO and (B) thrombin for induction of fungistasis and (C) the effect of the thrombin inhibitor hirudin on

PAO- and thrombin-dependent fungistasis, as assessed by XTT assay. (D) PAO and (E) thrombin were further compared with FCPs alone for their ability to induce fungistasis in the presence or absence of the *Tlr4* gene ($n = 3$ replicates per group). Data are presented as means $\pm$ SEM (error bars) from one of four comparable experiments. * $P < 0.05$ by Mann-Whitney test.

IL-5 levels in bronchoalveolar lavage fluid (fig. S3A). Type 2 innate lymphoid cells (ILCs) secrete IL-5 and IL-13, but not IL-4, in the setting of airway proteinase challenge (12), suggesting that these cells might be influenced by TLR4. Indeed, relative to vehicle-challenged animals, ILCs failed to be recruited as robustly into bronchoalveolar lavage fluid in *Tlr4*^{-/-} mice relative to WT animals after proteinase challenge, potentially accounting in part for the reduced T_H2 cytokine production in *Tlr4*^{-/-} mice (fig. S3, B and C).

We next considered whether other TLRs played a role in proteinase-dependent allergic lung disease. Most TLRs signal through one of two major adapter proteins, MyD88 and TRIF, whereas TLR4 signals through both adapters (13). Mice deficient in either MyD88 or TRIF showed an enhanced or identical disease phenotype as genotype matched control mice when challenged with *A. niger* spores (fig. S4). In contrast, mice deficient in both MyD88 and TRIF showed complete disease abrogation. Thus, proteinase-dependent allergic lung disease is mediated through TLR4 and not other TLRs.

Because TLR4 does not determine T_H2 responses, we turned to macrophages to further explore how TLR4 controls allergic disease. Relative to naïve cells, bone marrow–derived macrophages (BMDMs) expressed distinct transcriptional programs when activated by lipopolysaccharide (LPS), interferon- γ (IFN- γ), and PAO (Fig. 1A). Specific genes induced by PAO included lysozyme (*Ly2*), macrophage receptor with a collagenous structure (*Marco*), and secretory leukoproteinase inhibitor (*Slpi*), all of which have been linked to antifungal immunity (14–16), and their induction was dependent on TLR4 (Fig. 1B). PAO did not induce genes linked to previously characterized macrophage phenotypes, including IFN- γ -activated type 1 macrophages (M1) (nitric oxide synthase 2 (NOS2)) and IL-4-activated M2 (arginase 1 and FIZZ1) (17) (Fig. 1, C and D). Alveolar macrophages from mice treated with PAO

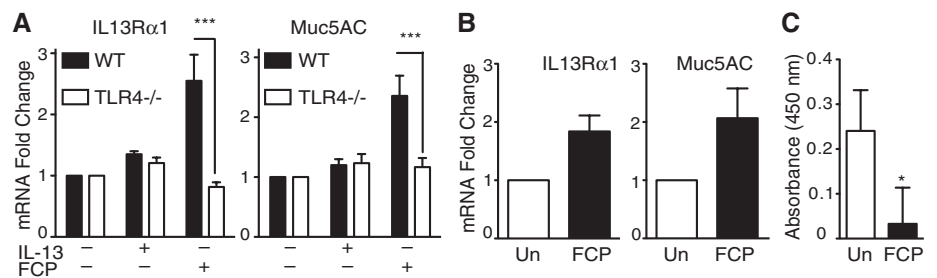


Fig. 3. FCPs up-regulate IL-13 α 1 and Muc5AC on airway epithelium. (A) WT and *Tlr4*^{-/-} mouse primary airway epithelial cells were cultured in the presence of IL-13 or FCPs for 24 hours, and *Il13ra1* and *Muc5ac* gene transcripts were analyzed by quantitative PCR ($n = 3$). (B) *Il13ra1* and *Muc5ac* transcripts were similarly analyzed from human primary airway epithelial cells 24 hours after treatment with FCPs or left unstimulated (Un) ($n = 4$). (C) Unstimulated and FCP-pretreated human airway epithelial cells were assessed for their ability to inhibit the growth of *A. niger* by XTT assay ($n = 3$ replicates per group). Data are presented as means $\pm$ SEM (error bars) from one of three (murine) or two (human) comparable experiments. * $P < 0.05$; *** $P < 0.001$ by Mann-Whitney test.

similarly showed up-regulation of lysozyme, MARCO, and SLPI (Fig. 1E). Thus, PAO induced a macrophage phenotype marked by expression of a distinct transcriptional profile that included genes with antifungal properties.

We next determined if PAO-activated macrophages were capable of restraining fungal growth in vitro. Relative to naïve BMDMs, as assessed by both microscopy and colorimetric quantification, only IFN- γ - and PAO-activated macrophages efficiently controlled fungal growth when the conidia of *A. niger* were added to cultures (Fig. 1, F and G, and fig. S5). Human monocyte-derived macrophages were similarly responsive to PAO treatment, although less so to IFN- γ compared with mouse BMDMs (Fig. 1H). However, control of fungal growth through PAO, but not IFN- γ , required the presence of TLR4 (Fig. 1, F to H). Again, MyD88 and TRIF were individually dispensable for control of fungal growth in macrophages activated by PAO, but deletion of both adapters abrogated the ability of mouse macrophages to control fungal growth (fig. S6). These in vitro findings correlated with a reduced ability

of *Tlr4*^{-/-} mice to clear *A. niger* conidia from the airway after a single inhalational challenge (Fig. 1I).

Unlike other macrophage activators, PAO-dependent inhibition of fungal growth (fungistasis) required the presence of serum, suggesting that fungal proteinases acted through both a serum factor and TLR4 to induce macrophage antifungal immunity (Fig. 1J). Fibrinogen, a proposed TLR4 ligand (18), is the functional mammalian analog of the arthropod factors pro-Spätzle and coagulogen, which regulate antifungal immunity through Toll (19).

To determine if fibrinogen mediates proteinase-dependent fungistasis, we added it (1.8×10^{-5} EU/ μ g) to BMDM cultures, with and without fungal and endogenous proteinases, and the conidia of *A. niger*. Only when stimulated by PAO in the presence of serum did BMDMs exhibit robust fungistatic activity (Fig. 2A). Identical results were obtained in experiments in which PAO was substituted with the endogenous proteinase thrombin, which converts fibrinogen to fibrin as the terminal step in the clotting cascade while also creating additional cleavage products that do not

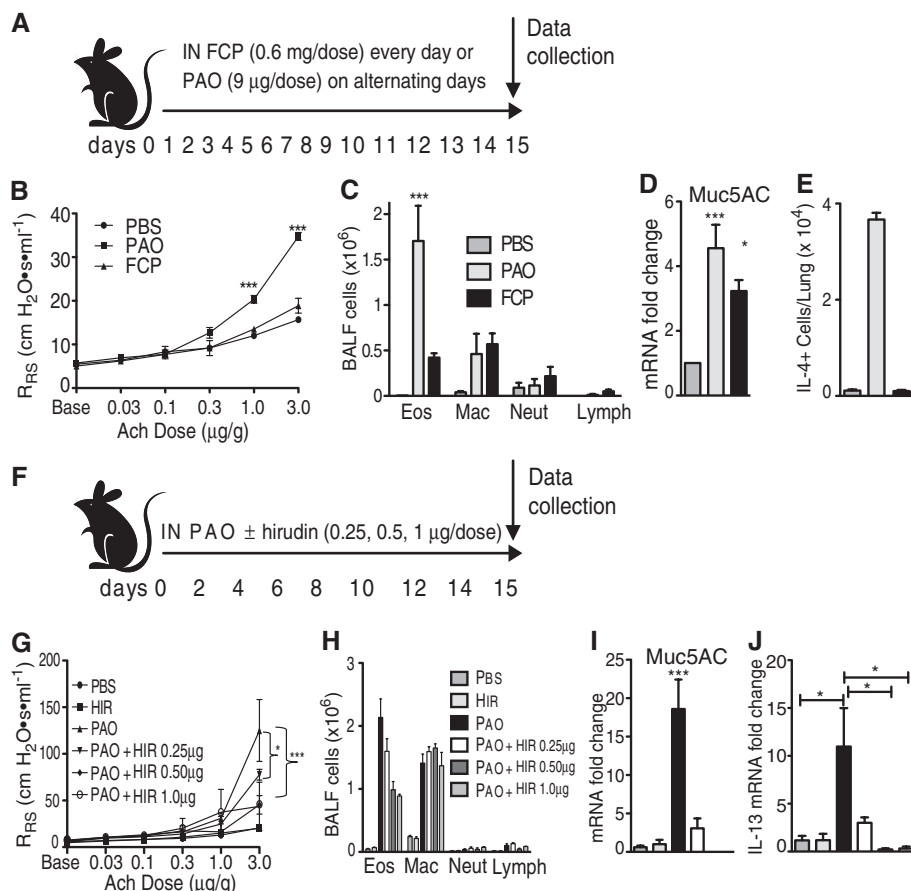


Fig. 4. Fibrinogenolysis is necessary but insufficient for expression of robust allergic airway disease. (A) C57BL/6 mice were challenged intranasally with PAO or FCPs as indicated, after which (B) airway hyperresponsiveness, (C) total bronchoalveolar lavage fluid (BALF) inflammatory cells, (D) lung Muc5AC transcripts, and (E) total lung IL-4-secreting cells were quantitated. (F to J) C57BL/6 mice were intranasally challenged with PAO without and with hirudin or hirudin alone on alternating days for 2 weeks, and the indicated parameters were assessed ($n \geq 3$ mice per group). Data are presented as means $\pm$ SEM (error bars) from one of three comparable experiments. Data are averages $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$ by Kruskal-Wallis test. R_{RS} , respiratory system resistance.

participate in clot formation (Fig. 2B). These results suggested that rather than fibrinogen in per se, fibrinogen cleavage products (FCPs) were required to induce fungistasis. FCPs created by incubating fibrinogen with PAO or thrombin induced fungistasis to a comparable degree as whole serum and proteinase when added to BMDMs (Fig. 2, A and B). Moreover, the thrombin inhibitor hirudin (3.4×10^{-3} EU/ μ g) neutralized both PAO- and thrombin-dependent fungistasis that was induced in the presence of serum (Fig. 2C). Another abundant serum protein and putative TLR4 ligand, fibronectin (20), had no effect on macrophage fungistasis in either native or cleaved forms (fig. S7).

In addition to fungistasis (Fig. 2, D and E), FCPs also induced in BMDM the expression of mRNA for IL-13 α 1, a component of the IL-13 receptor that is required for expression of allergic airway disease (21), and the airway mucin gene *Muc5ac* through TLR4 (Fig. 3A). FCPs yielded similar findings and also induced fungistatic activity in human primary airway epithelial cells (Fig. 3, B and C).

Together, these findings support a model in which both endogenous and exogenous airway proteinase activities with allergenic potential produce alternate TLR4 ligands from fibrinogen that license innate immune cells to respond to T_H2 cells, as required for full expression of allergic airway disease. To test this model, we first administered intranasally to mice the maximum tolerated dose of FCPs, 0.6 mg per dose (Fig. 4A), which induced modest airway eosinophil recruitment and *Muc5ac* gene expression but failed to induce airway hyperresponsiveness and IL-4-secreting cells (Fig. 4, B to F). Thus, FCPs appeared to influence only innate immune cells and specifically did not induce T_H2 responses that are required for robust allergic lung disease.

We conducted additional studies to confirm that airway proteinase activity was required for allergic lung disease using the proteinase inhibitor hirudin. Our in vitro studies indicated that hirudin, a known thrombin antagonist, could also inhibit PAO-mediated fungistasis (Fig. 2C), suggesting that hirudin may possess broad-spectrum

antiproteinase activity. In a dose-dependent manner, hirudin progressively and significantly attenuated PAO-dependent allergic lung disease while leaving unaffected robust lung IL-4 responses (Fig. 4, G to K), a phenotype that resembles that of *Tlr4*^{-/-} mice challenged with diverse allergens (figs. S1 and S2). Hirudin further inhibited ovalbumin-dependent allergic airway disease, suggesting that ovalbumin challenge activates an endogenous proteinase, possibly thrombin, to achieve the fibrinogenolysis that is necessary for disease expression (fig. S8). Together, these studies confirm the importance of airway fibrinogenolysis for the expression of allergic lung disease, regardless of the proteinase content of the inhaled allergen.

Although previous studies have shown that TLR4 contributes to T_H2 responses (5, 22, 23), we have shown here that TLR4 is not required for T_H2 cell development but rather is required for responsiveness of innate airway cells to T_H2 cells. Our findings do not exclude the possibility that bacterial endotoxin, a canonical TLR4 ligand, could mediate T_H2 responses under some conditions, as shown previously (5), but additional studies are needed to determine the contribution of fibrinogenolysis to this observation. Although independent of TLR4, T_H2 cells nonetheless develop through a proteinase-dependent pathway (24), suggesting that proteinases coordinate both innate and adaptive allergic pathways that together lead to allergic inflammation and disease (fig. S9).

Ultimately, mammalian TLR4 preserves the crucial role of arthropod Toll by linking proteinase-dependent fibrinogenolysis to antifungal immunity. However, in addition to fungi, mammals must also defend against other proteinase-associated pathogens such as helminth parasites (25) and, potentially, viruses (26). Although highly effective, the ancient proteinase-Toll-based defensive strategy is also susceptible to aberrant activation in response to innocuous proteinase sources such as pollens and many allergens, both with and without intrinsic proteinase activity. Clarification of the contribution of true infections to common allergic airway disorders such as allergic rhinitis, asthma, and chronic rhinosinusitis will determine the usefulness of interrupting FCP-TLR4 signaling as a therapeutic strategy.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/341/6147/792/DC1
Materials and Methods
Figs. S1 to S9
References (27–31)

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Recurrent Insect Outbreaks Caused by Temperature-Driven Changes in System Stability

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Insects often undergo regular outbreaks in population density but identifying the causal mechanism for such outbreaks in any particular species has proven difficult. Here, we show that outbreak cycles in the tea tortrix *Adoxophyes honmai* can be explained by temperature-driven changes in system stability. Wavelet analysis of a 51-year time series spanning more than 200 outbreaks reveals a threshold in outbreak amplitude each spring when temperature exceeds 15°C and a secession of outbreaks each fall as temperature decreases. This is in close agreement with our independently parameterized mathematical model that predicts the system crosses a Hopf bifurcation from stability to sustained cycles as temperature increases. These results suggest that temperature can alter system stability and provide an explanation for generation cycles in multivoltine insects.

One of the fundamental tenants in insect ecology is that temperature is a pace-maker of all vital rates (1, 2). Temperature has a direct impact on ontogenetic development, survival, and reproduction and, through these, an indirect impact on generation time and population growth rate (3, 4). The reliability by which temperature can predict development in insects is used to predict the number of generations per year (voltinism), and the timing at which different life stages appear (5, 6). The latter is a critical component for scheduling pest control. While the influence of temperature on individual-level life-history traits is well understood, the impact on population-level dynamics, such as population cycles or outbreak frequency, is less clear. For multivoltine insects, temperature is thought to contribute to insect outbreaks early in the season when climate has helped synchronize the population stage

structure through either induction of diapause (7, 8) or differential winter mortality (9). Mathematical theory suggests that temperature could also destabilize dynamics of ectotherms (10–13); however, the role that temperature plays in sustained cycles or overall dynamics in real systems is poorly understood.

The smaller tea tortrix, *Adoxophyes honmai*, is a multivoltine lepidopteran that undergoes multiple outbreak cycles each year (14). The insect is a pest on tea plantations and of significant economic concern throughout Japan. Outbreak densities are higher at the peaks than the troughs by a rate between 100- and 4000-fold (Fig. 1). Our previous work (15) showed that summer outbreaks are most consistent with intraspecific mechanisms that involve the strongly stage-structured nature of the insect life cycle. The outbreak dynamics themselves, however, are highly variable throughout a season (Fig. 1), with outbreaks occurring during the warmer months of the season (typically May to September) but not during the cooler months. The classic explanation for such a pattern is that cool winter temperatures synchronize population stage-structure, which leads to transient generation cycles (5). This mechanism

generally results in cohort synchrony and outbreak amplitudes that are greatest at the start of the season and decay through time. The decay in cohort synchrony through time is observed in other structured systems (16) and occurs because developmental plasticity and environmental variability cause development to become increasingly uncorrelated. However, in the smaller tea tortrix, developmental synchrony is enhanced rather than eroded through the season. We, therefore, studied an alternative explanation, motivated by mathematical models, whereby sustained cycles are the result of temperature-dependent changes in system stability—seasonal temperature increases cause the system to destabilize each spring, and cooling temperatures cause the system to restabilize each fall.

To test this idea, we studied the relation between temperature and stability in a stage-structured model of the tortrix life cycle (supplementary text). The model, which is a set of coupled integral delay-differential equations parameterized with individual-scale laboratory data, provides theoretical predictions that are fully independent of the time-series data and so can be used for a rigorous test of the hypotheses. Our model represents a dynamic description of life history that incorporates a more realistic nonlinear function for ontogenetic development than is found in the classic degree-day model from insect ecology (17). We studied local stability of the models using characteristic equations (supplementary text). The model predicts that the population dynamics should be strongly temperature-dependent with decay to extinction at low temperatures, transient fluctuations around a stable equilibrium at intermediate temperatures, and sustained generation cycles at higher temperatures (Fig. 2 and supplementary text). The predicted transition from stability to cycles is via a supercritical Hopf bifurcation at 12.6°C. This class of bifurcation generates a smooth increase in amplitude with increasing temperature once past the critical temperature threshold. The model provides two testable predictions that can be used to evaluate if the dynamics are driven by temperature-dependent destabilization: (i) cycle amplitude should show a threshold response to temperature, and (ii) above the thresh-

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Supplementary Materials for

**Cleavage of Fibrinogen by Proteinases Elicits Allergic Responses
Through Toll-Like Receptor 4**

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This PDF file includes:

Materials and Methods
Figs. S1 to S9
Full Reference List

Materials and Methods

Mice.

TLR4^{-/-} (B6.B10ScN-Tlr4^{lps-del}/JthJ), C57BL/6, and BALB6 mice were purchased from Jackson Laboratory (Bar Harbor, MA). All mice were bred and maintained in the specific pathogen free Transgenic Mouse Facility at Baylor College of Medicine. Age and sex matched 4-8 wks old females were used for all experiments. All animal experiments were performed in accordance with all Federal and Institutional Animal Care and Use Committee regulations.

Allergens and intranasal Challenge.

Fungal proteinase from *Aspergillus melleus* (PAO, 1.7×10^{-3} endotoxin units (EU)/ μ g; Sigma Chemical, St Louis, MO) was suspended in sterile, pyrogen free phosphate buffered saline (PBS) and stored at a concentration of 1mg/ml and administered intranasally at 9 μ l/dose as previously described (1). Mature conidia of a household isolate of *A. niger* were administered intranasally at 400,000 conidia/dose as previously described (1). Ovalbumin (1.8×10^{-3} EU/ μ g; Sigma) was prepared sterilely at a concentration of 1mg/ml and administered intranasally at 50 μ l /dose after first immunizing mice intraperitoneally with ovalbumin precipitated in alum (OVA/alum) weekly for two weeks. Human fibrinogen (Sigma) in PBS was prepared with human thrombin ($< 2.0 \times 10^{-5}$ EU/ μ g Sigma) at a 1:100 dilution to generate a fibrin clot, after which supernatants, henceforth referred to as fibrinogen hydrolysis products (FCPs; 1.9×10^{-5} EU/ μ g), were collected. FCP were then intranasally administered to anesthetized mice over 15 consecutive days. For indicated hirudin experiments, mice received intranasal administrations of varying concentrations of purified hirudin (3.4×10^{-3} EU/ μ g; Sigma) simultaneous with intranasal ovalbumin. Twenty-four hours after the final IN challenge, the allergic disease phenotype was assessed. C57BL/6 mice were intranasally challenged with PAO with and without hirudin on alternating days for a total of 2 weeks. Allergic lung disease phenotype was assessed 24 hours after the final challenge.

Assessment of allergic lung disease in mice.

Bronchial constriction in response to increasing acetylcholine doses (airway hyperresponsiveness (AHR)), bronchoalveolar lavage cell differential counting, lung specific IL-4 and IFN, Muc5AC (Mm01276718_m1; Applied Biosystems; Carlsbad, CA) gene transcripts, and lung histopathology were assessed in response to control and provocative challenges as previously described (1). Briefly, mice were anesthetized with etomidate and intravenous acetylcholine was administered via tail vein to assess changes in airway constriction as measured by respiratory system resistance (R_{RS}). Cytokine and chemokine analyses was performed on bronchoalveolar lavage fluid (BALF) from these animals with MILLIPLEX MAP Kit Mouse Cytokine/Chemokine Immunoassay (Millipore, Billerica, MA) in a Bioplex analyzer (BioRad, Hercules, CA) as per the manufacturers' specifications. Lungs were fixed in paraffin for histology, prepared for RNA extraction or homogenized and assessed for IL-4 producing cells via ELISPOT (BioRad, Hercules, CA).

Flow cytometry of innate lymphoid cells.

TLR4^{-/-} and WT mice were intranasally challenged with PBS or PAO for 1 week. 24 hours after the last challenge, BAL cells were collected, non-specific antibody binding was blocked by addition of 2ug/sample mouse Fc Block, labeled with fluorescent Percp-Cy5.5 conjugated anti-mouse lineage (CD4, CD8, CD19, CD56), phycoerythrin conjugated anti-CD25 and Alexa Fluor-488 conjugated anti-CD127 (AF488) antibodies (BD Biosciences; San Jose, California), and signal was detected using an Accuri C6 analytical flow cytometer (BD Biosciences; San Jose, California).

Splenocyte cultures, cytokine and IgE quantitation.

Splenocytes were collected and restimulated with whole ovalbumin as previously described (1). Secreted cytokines were quantified using mouse cytokine/chemokine panel I or II kits (Millipore) using a Luminex-based multianalyte platform (Bio-Plex; Bio-Rad, Hercules, CA) according to the manufacturer's protocols. Total IgE was determined from mouse serum as previously described using a sandwich ELISA (2).

Macrophage cultures.

C57BL/6 or TLR4^{-/-} bone marrow derived macrophages were generated as previously described (27). Briefly, marrow from femurs and tibias of 6-8 week old female mice were flushed and cultured at 37° in Dulbecco modified Eagle's minimal essential medium (DMEM) supplemented with 20% fetal calf serum (Invitrogen) and L929 fibroblast cell line secreting macrophage colony stimulating factor. After 5 days in culture, mature macrophages were harvested for further study. Human peripheral blood mononuclear cells (PBMC) were collected via ficoll gradient centrifugation as previously reported and cultured in DMEM containing GM-CSF (10ng/mL) for seven days. Cells were then harvested using cell dissociation buffer (Millipore) for subsequent studies. Macrophages were stimulated at 37° for 24 hours with recombinant mouse/human IL-4 (10ng/mL; Applied Biosystems, Carlsbad, CA), recombinant mouse/human IFN (20ng/mL; Applied Biosystems, Carlsbad, CA), lipopolysaccharide (LPS; 10ug/mL; SIGMA), PAO (10ug/mL) in CDMEM (DMEM, 10% FCS, 1% penicillin/streptomycin), PAO + fibrinogen (10ug/ml and 5ug/mL) or FCP in serum or serum deficient media prior to phenotypic and functional characterization. Murine experiments each involved three mice and human experiments were each performed using cells from at least three patients to generate biological replicates and co-cultures were performed in triplicate.

Microarray and confirmatory PCR.

Bone marrow derived macrophages from three animals were harvested and treated as described above. Total RNA was extracted with combination of RNA TRIzol reagent (Invitrogen) and the RNeasy Mini kit (QIAGEN). Gene expression profiling was performed using the Illumina MouseWG-6 v2 Expression BeadChip Array technology (Expression Analysis); differential expression was assessed using t-test and fold change; probes with average signal <20 units were pre-filtered. Confirmational real-time PCR analysis was performed using Taqman probes and the ABI7900HT Detection System (Applied Biosystems, Carlsbad, CA) using probes for MARCO (m00440265_m1) SLPI (Mm00441530_g1), Lysozyme (Lyz; Mm00657323_m1), retlna (Mm00445109_m1), Arg1 (Mm00475988_m1), and Nos2 (Mm00440502_m1). The threshold, CT, of each gene in comparison to the Ct of the 18s ribosomal RNA was used to calculate the relative

abundance. Gene expression was normalized with ribosomal 18s as an internal standard. Fold induction was expressed relative to values for naïve cells.

Fungistasis Assay.

Macrophages were co-cultured in 96-well flat tissue culture plates with *Aspergillus niger* conidia at a ratio of 100:1 in a 37° C/5% CO₂ incubator. Qualitative analysis of fungal growth was performed 24 hours later using light microscopy to document hyphal growth. Quantitation of fungal growth was performed by adding the colorimetric substrate XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfoph-2H-tetrazolium-5-carboxyanilide inner salt; Sigma) to cultures at 24 hours per the manufacturer's specifications. Fungal growth is proportional to the XTT-dependent optical density at 450nm (OD450). Normalized data are reported according to the formula: (OD450 of fungus incubated with macrophages – OD450 of macrophages alone).

Murine Epithelial Cell cultures:

Isolation and culture of mouse tracheal cells from C57BL6 and TLR4^{-/-} female mice aged 6 weeks old was performed as previously described (28). Briefly, tracheas were resected, cut longitudinally, and cultured in Ham's F-12 media with 100U/mL penicillin, 100ug/mL streptomycin, and 1.5mg/mL pronase overnight. 10% FBS was added after overnight incubation and fibroblasts were excluded by adherence. Non-adherent cells were cultured on 0.4uM porous corning polycarbonate and polyester membranes (transwell clear; Corning) coated with sterile 50ug/mL rat tail collagen in DMEM-Ham's F-12, 15mM HEPES, 3.6mM sodium bicarbonate, 4mM L-glutamine, 100 U/mL penicillin, 100ug/ml streptomycin, and 0.25 ug/mL fungizone) for three weeks. Cells were then serum starved for 24 hours prior to experiments.

Human Epithelial Cell cultures:

Primary human sinus tissue was collected from patients and dissociated with collagenase / DNase. After fibroblast depletion, epithelial cells were cultured to confluency in complete bronchial epithelial cell growth medium (BEGM; ATCC, Manassas, VA) containing hydrocortisone in collagen I coated T-25 flasks. Cells were then trypsinised and cultured at 25K – 30K per well in collagen-coated 96 well plates in complete BEGM. After reaching confluency, cells were re-cultured in BEGM without hydrocortisone or amphotericin B 24 hours prior to treatment. All epithelial cells were stimulated with different concentrations of recombinant IL-13 (Applied Biosystems, Ca), PAO, and FCP for different time points then protein lysates and RNA was prepared using RIPA buffer and RNeasy Mini kits (Qiagen).

Endotoxin assays.

The endotoxin content of all reagents was determined by the limulus amoebocyte lysate assay according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL).

Statistics.

Data are presented as means ± SEM with between 3 and 5 mice/group for in vivo experiments and a minimum of 3 replicates/condition for in vitro experiments.

Significant differences for paired groups were determined using the Mann-Whitney test and for multiple comparisons using the Kruskal-Wallis test. A P value of less than 0.05 was considered significant.

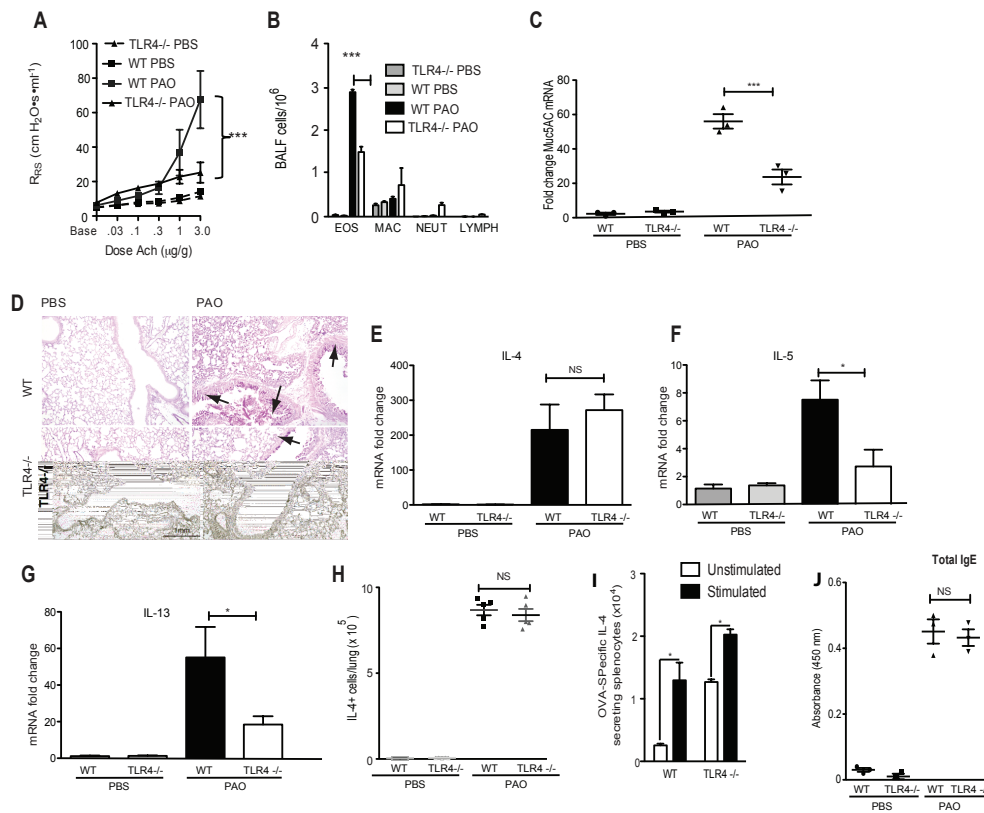


Figure S1. TLR4 is required for allergic airway disease but not Th2 responses. Wildtype C57BL/6 and TLR4 knockout (Tlr4^{-/-}) mice were intranasally challenged with PAO derived from *A.oryzae* or PBS daily over two weeks after which (A) airway responsiveness to acetylcholine provocation, (B) total airway inflammatory cells (eosinophils:EOS; macrophages: MACS; neutrophils: NEUTS; lymphocytes: LYMPH), and (C) lung Muc5AC gene expression were quantified. (D) Representative lung histology of the same mice. (E-G) Quantitative real-time PCR quantitation of transcripts for IL-4, IL-5, and IL-13 from whole lung, respectively. (H) Total IL-4-secreting cells from whole lung. WT and Tlr4^{-/-} mice were immunized with ovalbumin (OVA) weekly for 3 weeks after which (I) OVA-specific IL-4-secreting splenocytes were quantitated by ELISpot and (J) total serum IgE responses were determined. Data are means $\pm$ S.E.M. from one of three comparable experiments*, P < 0.05; **, P < 0.01; ***, P < 0.001 by Kruskal-Wallis test (n=5/group). Arrows point to mucus-producing goblet cells.

Fig. S1.

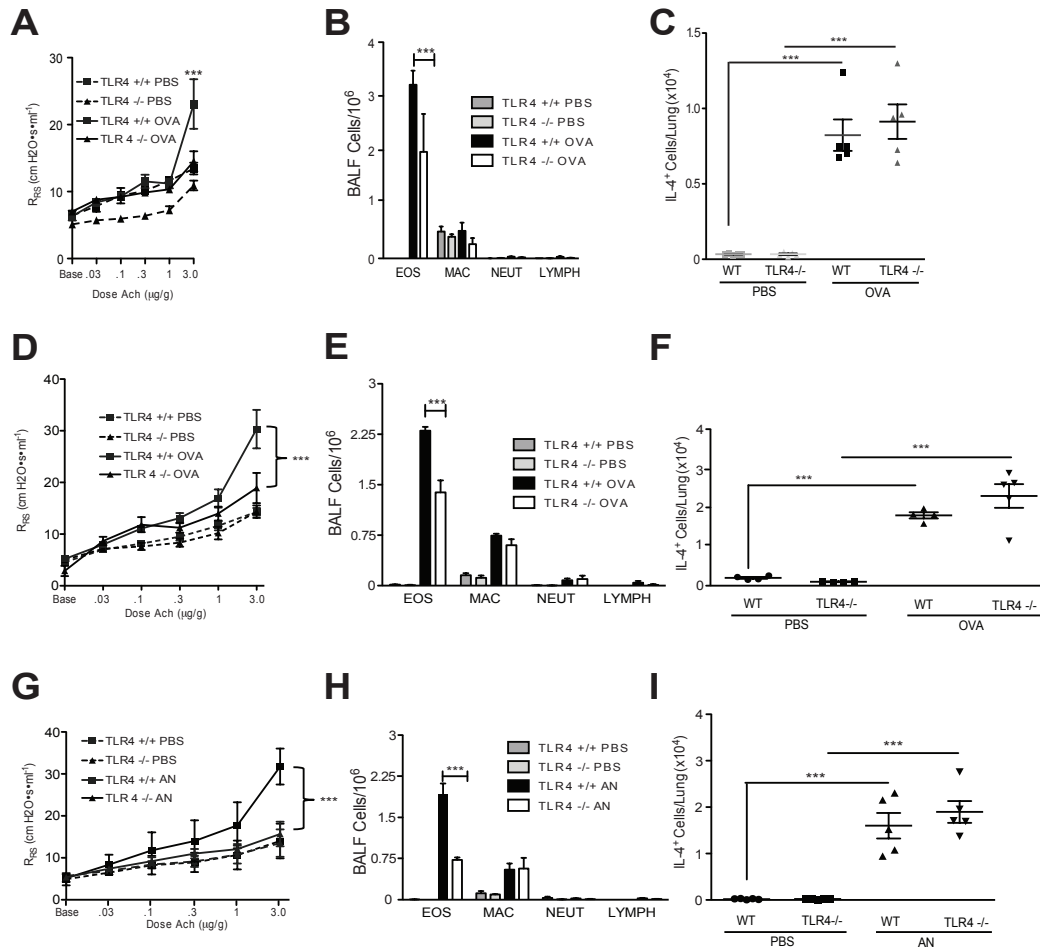


Figure S2. TLR4 is required for allergic airway disease due to diverse allergens. (A-F) Airway hyperresponsiveness (A,D,G), bronchoalveolar lavage fluid (BALF) inflammatory cells (B,E,H; eosinophils:EOS; macrophages: MAC; neutrophils: NEUT; lymphocytes: LYMPH) and lung IL-4-secreting cells (C,F, I) after immunization with ovalbumin (OVA) for two weeks (A-C) or four weeks (D-F) and intranasal challenge with OVA or PBS for four consecutive days, comparing responses of wild type ($+/+$) and TLR4 $-/-$ mice. (G-I) Wild type and TLR4 $-/-$ mice were challenged intranasally with the conidia of *Aspergillus niger* (AN) daily for 16 days after which the same parameters as in A-C were determined. Data are presented as means $\pm$ S.E.M. and are representative of at least 3 independent experiments for each allergen. ***: $P < 0.001$ by Kruskal-Wallis test, ($n=5$ /group).

Fig. S2

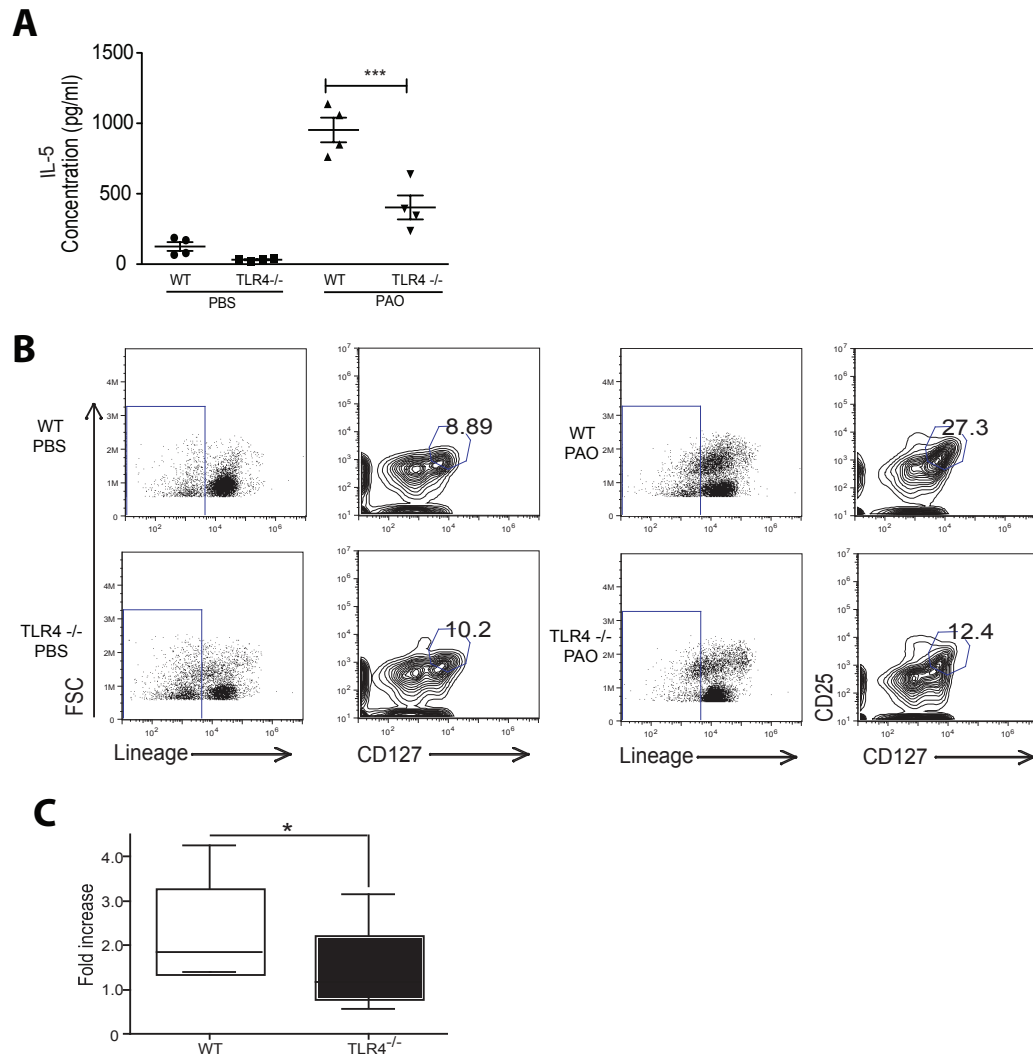


Figure S3. Reduced ILC recruitment into bronchoalveolar lavage fluid after proteinase challenge. Wild type (WT) and TLR4-deficient mice were challenged with PAO intranasally daily for 14 days after which bronchoalveolar lavage fluid was collected for quantitating IL-5 (A) and innate lymphoid cells (ILC; B,C). B. Gating strategy for ILC. C. Aggregate data comparing the fold change in BALF ILC in wild type (WT) and TLR4^{-/-} mice. Data are presented as means $\pm$ SEM and are representative of two independent experiments. *: $P < 0.05$; ***: $P < 0.001$ by Mann-Whitney test, $n=4$ mice/group.

Fig. S3

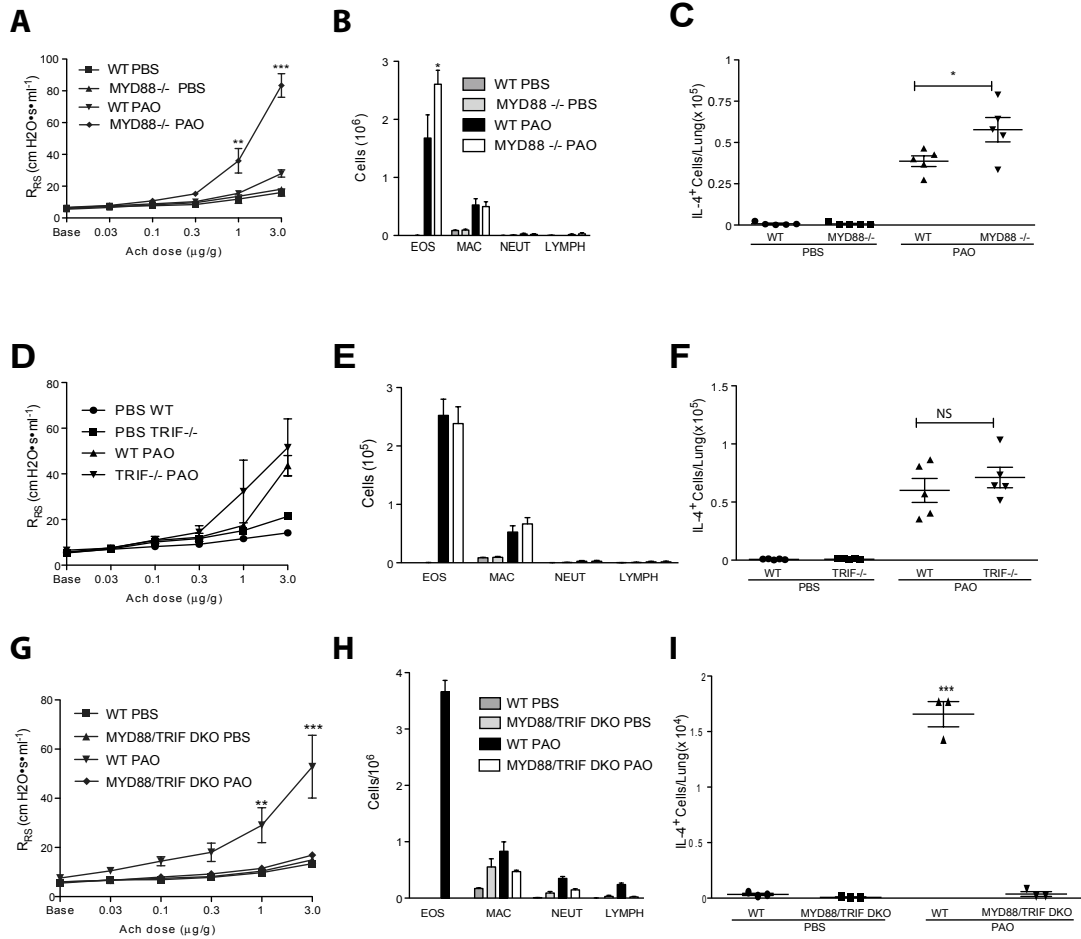


Figure S4. Requirement of MyD88 and TRIF for proteinase-mediated allergic lung disease. Mice deficient in MyD88 (A-D), TRIF (D-F) and both MyD88 and TRIF (DKO; G-I) were challenged daily with PAO for 14 days and then assessed respectively for airway hyperresponsiveness, bronchoalveolar lavage fluid (BALF) inflammatory cells (eosinophils:EOS; macrophages: MAC; neutrophils: NEUT; lymphocytes: LYMPH) and lung IL-4-secreting cells. Data are presented as averages $\pm$ S.E.M. and are representative of three independent experiments for each mouse genotype. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ by Kruskal-Wallis test ($n = 5/\text{group}$).

Fig. S4

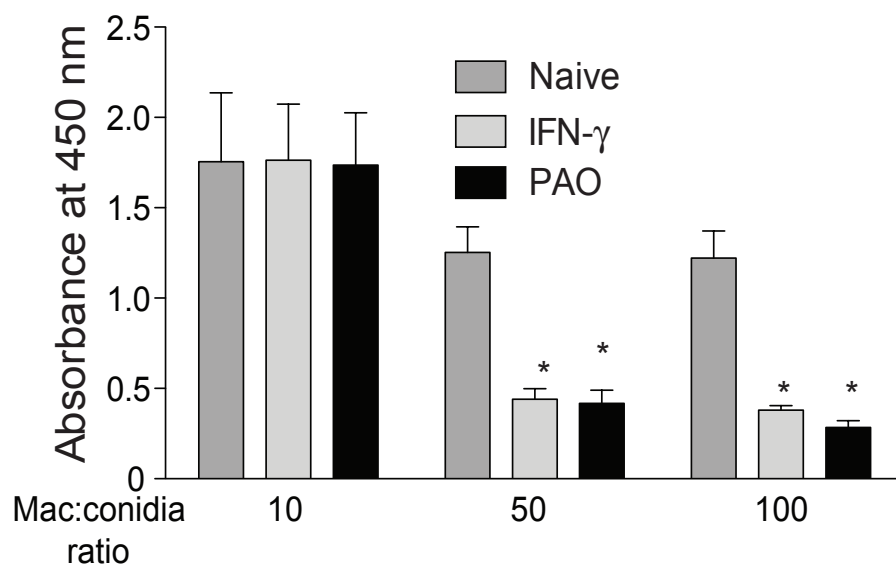


Figure S5. IFN- γ and PAO induce fungistasis in macrophages. Mouse BMDM were cultured for 24 hours as naive cells or in the presence of IFN- γ or PAO after which the the conidia of *A. niger* were added and the cells incubated for 24 more hours at distinct macrophage (Mac):conidia ratios. Fungistasis was then determined by XTT assay. Data are means $\pm$ S.E.M. and are representative of two independent experiments, *, $P < 0.01$. (n=3/group), Kruskal-Wallis test.

Fig. S5

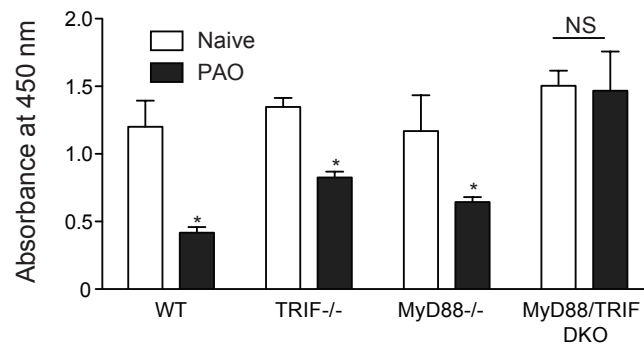


Figure S6. MyD88 and TRIF are together required for proteinase-dependent fungistasis. BMDM were cultured for 24 hours under naive and proteinase activating conditions with PAO and then inoculated with the conidia of *Aspergillus niger* for 24 hours and fungal growth was assessed by XTT assay. Data are means $\pm$ S.E.M. and are representative of three independent experiments. *, $P < 0.01$. (n=3/group), Mann-Whitney test. NS: not significant.

Fig. S6

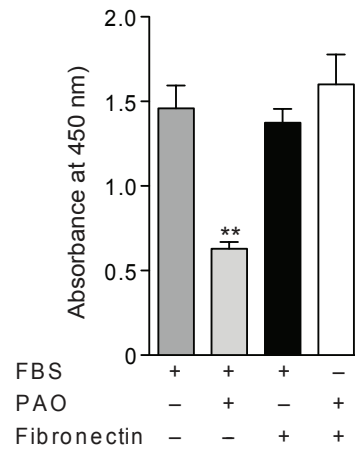


Figure S7. Fibronectin lacks fungistatic activity acting on macrophages. BMDM were cultured with and without fetal bovine serum (FBS), proteinase of *Aspergillus oryzae* (PAO) and fibronectin as indicated and the effect on fungal growth was determined by XTT assay. Data are means $\pm$ S.E.M. and are representative of two independent experiments. **: $P < 0.01$, ($n = 3/\text{group}$), Kruskal-Wallis test.

Fig. S7

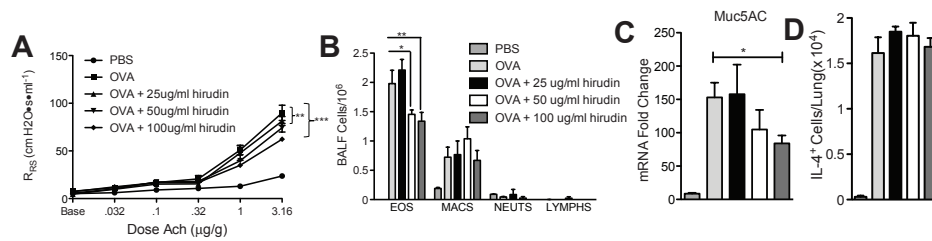


Figure S8. Hirudin blocks ovalbumin-dependent allergic lung disease. C57BL/6 mice were immunized with ovalbumin weekly for two weeks and then challenged intranasally with ovalbumin in the presence or absence of increasing amounts of hirudin on alternating days for 2 weeks. Mice were assessed for (A) airway hyperresponsiveness, (B) bronchoalveolar lavage fluid (BALF) inflammatory cells, (C) Muc5AC mRNA induction and (D) total lung IL-4-secreting cells. Data are means $\pm$ SEM and are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by Kruskal-Wallis test ($n = 3$ or 4 mice/group).

Fig. S8

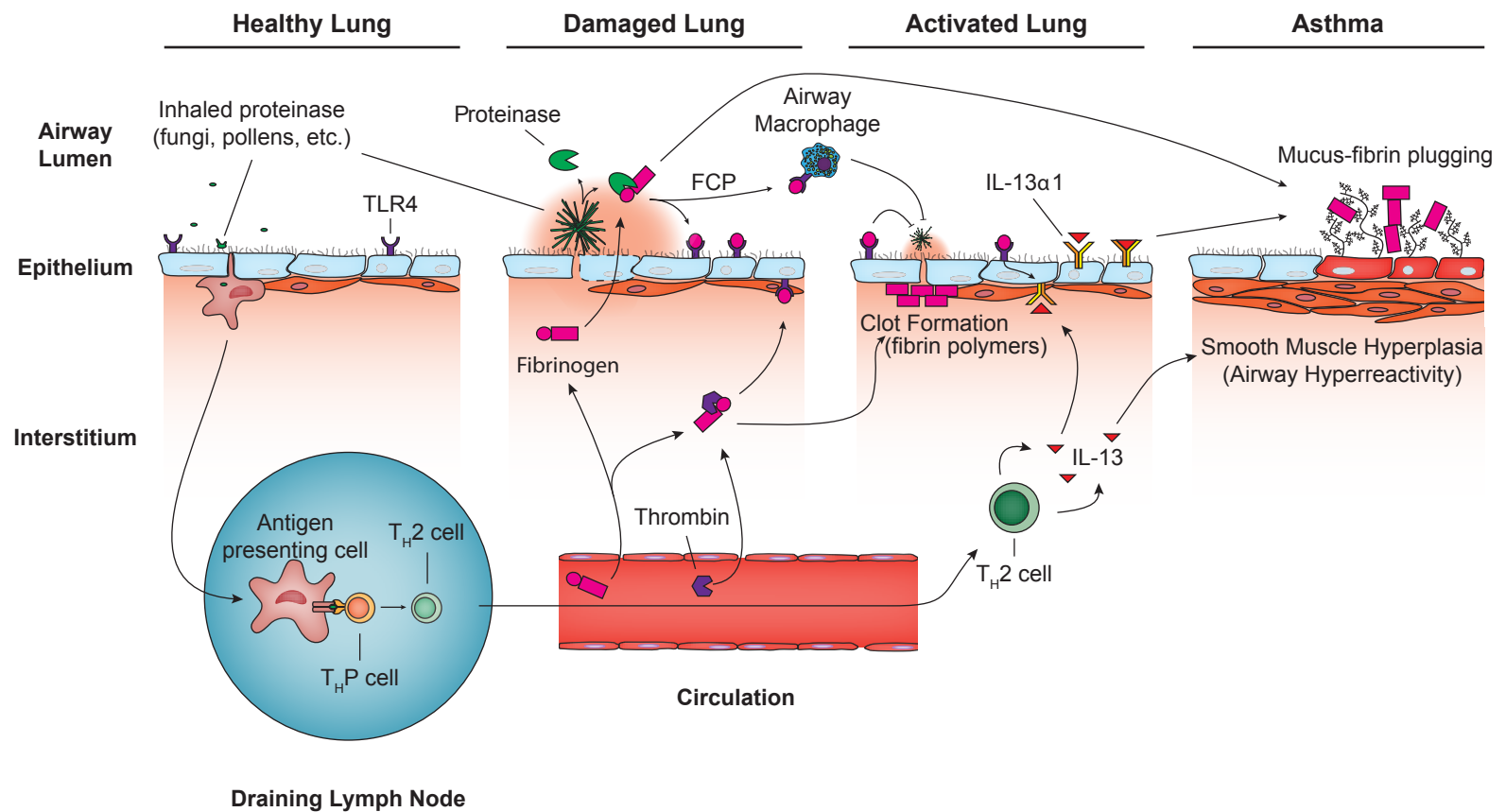


Figure S9. Proteinase model of allergic airway disease pathogenesis. The healthy lung routinely encounters low-grade proteinase activity through inhalation of fungi, pollens and other proteinase sources. Enhanced proteinase exposure, i.e., through airway surface mycotic infections (ASMI), causes lung damage that may include enhanced airway epithelial and vascular endothelial cell permeability that promote release of fibrinogen into the airway interstitium and lumen. Airway epithelial cells may also secrete fibrinogen directly into the airway (29). Exogenous proteinases, but also endogenous thrombin, promote fibrinogen cleavage and the generation of both fibrin clots and fibrinogen cleavage products (FCP) that function as alternate ligands for TLR4, triggering the activation of distinct innate immune cells, including airway epithelium and macrophages. Enhanced anti-fungal immunity induced in FCP-activated innate immune cells in the activated lung promotes resolution of ASMI, and simultaneously increased responsiveness to the cytokines of Th2 cells, e.g., IL-13, through increased expression of the IL-13 receptor subunit IL-13R α 1. Simultaneously, through a non-TLR4-dependent mechanism, airway proteinases initiate the differentiation of Th2 cells (24), which also promote anti-fungal immunity (30). Unchecked airway proteinase activity, i.e., through unresolved ASMI or overwhelming proteinase exposure, leads to allergic airway diseases such as asthma in which FCP-dependent innate immune and FCP-independent Th2 responses (31) contribute equally.

Fig. S9

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