Selective cleavage of fibrinogen by diverse proteinases initiates innate allergic and antifungal immunity through CD11b

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Running title: Fibrinogen cleavage initiates allergy through CD11b

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ABSTRACT

Proteinases are essential drivers of allergic airway disease and innate antifungal immunity in part through their ability cleave the clotting factor fibrinogen (FBG) into fibrinogen cleavage products (FCPs) that signal through Toll like receptor 4 (TLR4). However, the mechanism by which FCPs engage TLR4 remains unknown. Here we show that the proteinases from Aspergillus melleus (PAM) and other allergenic organisms rapidly hydrolyze FBG to yield relatively few FCPs that drive distinct antifungal mechanisms through TLR4. Functional FCPs, termed cryptokines, were characterized by rapid loss of the FBG alpha
chain with substantial preservation of the beta and gamma chains, including a gamma chain sequence (Fibγ390-396) that binds the integrin Mac-1 (CD11b/CD18). PAM-derived cryptokines could be generated from multiple FBG domains and the ability of cryptokines to induce fungistasis in vitro and innate allergic airway disease in vivo strongly depended on both Mac-1 and the Mac-1 binding domain of FBG (Fibγ390-396). Our findings illustrate the essential concept of proteinase-activated immune responses (PAIRs) and for the first time link Mac-1, cryptokines and TLR4 to innate antifungal immunity and allergic airway disease.

Allergic airway disease, comprised of the strongly associated conditions asthma and chronic rhinosinusitis (CRS), represents a rapidly increasing morbidity and healthcare burden across the world. More than 1 in 12 persons in the United States have asthma, with higher rates seen among children, the impoverished, and women(1). Despite the epidemic nature of allergic airway disease and an intensive, international effort to understand its causes, modern standard management remains entirely palliative, offering no chance of long-term remission or cure(2). Nonetheless, important advances in our understanding of allergic airway disease pathogenesis have been made.

Environmental proteinases are increasingly recognized to be critical drivers of both allergies, the subset of allergic diseases that are driven by IgE-dependent type 1 hypersensitivity reactions, and allergic airway disease that is mediated by both lymphocytes and IgE-dependent immune reactions(3). Canonical environmental allergens such as pollens, dust mites, cockroaches and other arthropods, and pet danders are all important sources of active proteinases that alone or combined as part of whole-organism extracts readily induce experimental allergic airway disease in rodents(4). A particularly important source of exogenous, allergic proteinases, however, is the fungi, which are linked to asthma through remarkably diverse environmental contexts that include thunderstorms, mold-contaminated domestic and work environments, and moldy automobiles (2,5-10).

The pathophysiology of fungal-related allergic airway disease is likely related to several factors, including fungal hypersensitivity, which does not involve actual infection, and true airway infection by fungi that involves the non-invasive growth of fungi in either the upper or lower airways, a unique type of superficial fungal infection termed airway mycosis (11). We and others have previously established that airway mycosis can be readily established in mice(12-15) and that airway mycosis-dependent allergic airway disease requires the secretion of active proteinases by the fungi(13). Moreover, an identical disease phenotype can be induced simply by challenging mice with single proteinases derived from diverse species (16-19).

Several proposed, non-mutually exclusive mechanisms potentially explain how the airway immune system recognizes allergenic proteinases from fungi and potentially other sources to initiate allergic airway disease. These mechanisms include disruption of tight junction proteins of airway epithelial cells to facilitate antigen presentation by dendritic cells(20,21), induction of signaling from protease activated receptor 2 (PAR2)(22-24), and activation of innate pattern recognition receptors such as TLR4(25-29). We showed previously that proteinase-dependent induction of allergic airway disease requires in part the activation of the coagulation factor fibrinogen, cleaved products from which (FCPs) initiate both allergic airway disease and activate innate immune cells such as macrophages to limit the growth of fungi in vitro, termed fungistasis.

Both innate fungistatic responses and allergic airway disease are driven by FCPs obligatorily through TLR4(25), but it remains unclear what structural features of FCPs determine their ability to engage this immune receptor. Moreover, multiple ligands including LPS interact with TLR4 indirectly through binding partners, raising the possibility that FCPs engage TLR4 through a similar indirect mechanism. The leukocyte integrin Mac-1
CD18/CD11b has previously been shown to bind to fibrinogen and regulate macrophage-dependent innate immunity (30). Moreover, Mac-1 interacts with TLR4 through an unknown mechanism to promote LPS-dependent inflammation and T cell activation (31). Considering these prior observations with our current findings, we reasoned that Mac-1 could facilitate the FCP-TLR4 interaction that promotes antifungal immunity and allergic airway disease.

In this study, we have combined proteinase cleavage assays with mass spectrometry to demonstrate the fundamental structure of FCPs that determines their ability to interact with TLR4. We further demonstrate that FCPs most likely engage TLR4 indirectly through a mechanism involving the integrin Mac-1 (CD18/CD11b).

Results

Fungal Proteinase-derived FCPs promote fungistatic immunity in vitro through diverse mechanisms

Our prior studies demonstrated that FBG can be cleaved by the endogenous proteinase thrombin to produce FCPs that stimulate fungistatic immunity (25). We reasoned that a more clinically relevant source of fibrinogen cleavage could be fungi that, through their secreted proteinases, are likely causes of human asthma and sinusitis (6). To determine if FCPs can be generated from exogenous fungal proteinases, we first determined how FBG is degraded in the presence of the allergenic proteinase from Aspergillus melleus (PAM) using SDS-PAGE. This analysis confirmed that PAM yields numerous FCPs ranging in size from a few kDa to nearly 250 kDa (Fig. 1A).

When added at a constant concentration of 6µg/mL to FBG diluted to 5mg/mL, PAM gradually cleaved FBG from 340 kDa to ~200 kDa, yielding FCPs of many sizes. However, even after two hours of incubation in these strongly hydrolyzing conditions, PAM yielded only three major FCPs of ~75, 150, and 200 kDa in size that persisted out to at least 6 hours (data not shown). After 24 hours, PAM had reduced fibrinogen to fragments no larger than ~75 kDa (Fig. 1B). We found that similar trends in FBG fragmentation could be observed by modulating the concentration of PAM while keeping the incubation time constant (30 mins; Fig. 1C). Thus, an allergenic fungal proteinase rapidly cleaves fibrinogen into FCPs of discrete size that remain relatively stable once formed.

We next determined the functional importance of PAM-derived FCPs using a modification of an in vitro assay that quantifies the fungistatic ability of immune cells, i.e., their ability to inhibit fungal growth in vitro (25). First, we verified that addition of bovine serum enhances the ability of mouse splenocytes to inhibit the growth of A. niger in vitro as indicated by a reduction in the number of fungal germination events (Fig. 1D) (32). We further confirmed that supplementing serum-free cultures of murine splenocytes with 100nM whole FBG or 100nM PAM-FCPs resulted in similar fungal inhibition (Fig. 1E). We conducted additional functional experiments to confirm that the ability of PAM-FCPs to promote innate antifungal immunity cannot be accounted for by the residual proteinase remaining in the FCP mixture following their preparation (Fig. 1F). PAM-FCPs were further capable of promoting fungistasis in both murine bone-marrow derived macrophages (BMDMs) and splenocytes in a manner that absolutely depended on expression of TLR4 (Fig. 1G,H and (25)). Thus, PAM-derived FCPs are functionally equivalent to thrombin-derived FCPs with respect to induction of in vitro fungistatic activity from diverse immune cells.

We further evaluated the difference between whole FBG and FCPs in this assay and whether FCP-dependent fungistasis was cell contact-mediated or the result of secreted antimicrobial soluble products. Naïve mouse splenocytes were primed with fibrinogen, FCPs, or sham, and after 18 hours the conditioned supernatants were transferred to separate wells and fresh media was added to the remaining splenocytes. The ability of primed splenocytes, conditioned media, or sham to inhibit A. niger growth was then determined (Fig. 2A). We conducted additional experiments in which splenocytes were incubated with A. niger spores,
but kept separated by a semi-permeable transwell membrane (Fig. 2B). These studies demonstrated that only FCP-not FBG-primed splenocytes mediate fungistasis and that splenocytes inhibit fungal growth through a mechanism that requires contact with the fungus and not secretion of a soluble antifungal substance (Fig 2A,B). We further conclude from these experiments that the ability of intact FBG to mediate fungistasis when added to cultures that simultaneously contain splenocytes and fungi (Fig. 1E) is most likely due to fungus-dependent cleavage of FBG to produce FCPs.

In contrast to splenocytes, although PAM-FCPs enhanced BMDM fungistatic immunity, these cells secreted a soluble factor that accounted for most of this fungistatic activity (Fig. 2C). Thus, fungal proteinase-derived FCPs, but not fibrinogen, induce fungistatic immunity from diverse cell types through a variety of mechanisms involving either cell contact or the secretion of soluble antifungal factors.

**Proteinase from Aspergillus Melleus (PAM) is an alkaline serine proteinase**

We have previously demonstrated that *Aspergillus* spp. proteinases are potent allergens that strongly induce allergic airway disease in mice, including profound airway hyperresponsiveness and eosinophilia when delivered intranasally (13,16,25). Although long available from commercial sources, the identity of PAM as a proteinase has not previously been characterized. By SDS-PAGE, PAM consists of at least 3 dominant protein bands of approximately 11, 22, and 33 kDa (Fig 3A,B). Mass spectrometry (MS) performed on all of these protein bands revealed nearly identical sequence information, indicating that the different bands represented multimers of a single gene product. We observed similar multimerization occurring with the functionally similar allergenic proteinase derived from *A. oryzae* (5). MS-based sequencing indicated that the *A. melleus* proteinase is an extracellular alkaline serine proteinase that is substantially similar to the proteinases produced by other *Aspergillus* species (Fig. 3C-D). We further conducted proteinase inhibition experiments involving PAM and multiple class-specific proteinase inhibitors that confirmed that PAM is only susceptible to inhibition by the serine class-specific inhibitor AEBSF (Fig. S1). Therefore, PAM is an alkaline serine proteinase, similar to other fungal proteinases (e.g., Asp f13) that have previously been linked to allergic airway disease in mice (20,33).

**Proteinases from diverse species produce FCPs by preferentially cleaving the fibrinogen alpha chain**

In addition to fungi, allergic airway diseases such as asthma and allergic rhinitis are linked to many other organisms including plants (especially pollens), arthropods (e.g. environmental mites) and bacteria, all of which produce proteinases. Therefore, we next examined if allergenic proteinases derived from non-fungal sources could induce similar fibrinogen-dependent fungistatic immunity in vitro. We found that FCPs produced from PAM, pronase from the bacterium *Streptomyces griseus*, an extract derived from the dust mite *Dermatophagoides farinae*, and ragweed pollen from *Ambrosia artemisiifolia* (ragweed) induced similar fungistatic activity from splenocytes (Fig. 4A).

After normalizing for proteinase activity, FCPs produced from these sources were analyzed by SDS-PAGE under reducing conditions to reveal the individual FBG polypeptide chains (alpha, beta and gamma chains; Fig. 4B). We further included in this analysis additional FCPs derived from the dust mite *Dermatophagoides pteronyssinus* and the storage mites *Glyciphagus domesticus*, and *Tyrophagus putrescentiae*. This analysis revealed that each of the tested proteinases preferentially degraded the fibrinogen alpha chain (Fig. 4C). Additional analyses of PAM-derived FCPs revealed that whereas the alpha chain was almost completely degraded immediately upon addition of proteinase, the beta and gamma chains remained substantially resistant to hydrolysis even after two hours (Fig. 4D). Thus, although we cannot exclude the possibility that beta or gamma chain cleavage is
also essential, our findings indicate that proteinases derived from diverse organisms cleave FBG in a highly stereotypical manner, with predominantly the alpha chain being rapidly degraded to reveal the relatively proteinase-resistant beta and gamma chains.

**Cleavage products produced from the fibrinogen D and E fragments also promote fungistasis.**

We next sought to determine the FBG region from which functional FCPs derive. Our initial attempts to identify functionally active FCPs from PAM-digested whole FBG were unsuccessful because standard isolation techniques (molecular sieving; column chromatography) inevitably resulted in denatured and inactive FCPs (data not shown). We therefore determined the ability of distinct FBG regions (e.g., D and E domains, Fig. 4B) to yield active FCPs. At a constant 100 nM, PAM-derived FCPs derived from whole fibrinogen and D and E domains all induced significant fungistatic responses from murine splenocytes as compared to sham-activated cells (Fig. 5A). As noted previously, fibrinogen readily induced fungistasis independent of the addition of PAM, likely due to the cleavage induced by fungal proteinases secreted into the culture media.

In contrast, whole D and E domains behaved distinctly under the same conditions. Whereas D domains had no intrinsic ability to induce fungistasis, E domains showed a non-significant trend to promote fungistasis prior to the addition of PAM, suggesting that E domains might be either partially competent or more readily induced by ambient fungal proteinases to become competent to induce fungistatic responses (Fig. 5A). Thus, multiple domains of FBG, including D and E domains, are capable of inducing fungistatic responses from immune cells following proteolytic activation. Whole FBG is more potent in inducing fungistasis under these conditions that control for equivalent molar addition of FBG product as whole FBG contains stoichiometrically more fungistasis-inducing regions than the individual domains.

In contrast to whole FBG, SDS-PAGE analysis of D and E domains indicated that PAM-directed proteolysis resulted in relatively minor structural changes (Fig. 5B). Mass spectrometry was subsequently performed on the ~85kDa fragment of the cleaved D domain that again indicated predominant loss of the alpha chain and substantial preservation of the beta and gamma chains as was observed with whole FBG (Fig. 4D). Moreover, the 85 kDa fragment retained a region in the fibrinogen gamma chain (amino acids 390-396) that is known to be critical for binding to Mac-1 (data not shown) (30,34).

**CD11b critically regulates FCP-dependent fungistatic immunity and allergic airway disease**

To determine if FCPs could interact with Mac-1, we first compared the ability of wild type and CD11b-deficient mouse (itgam–/–) splenocytes to control fungal growth in response to FCP challenge in vitro. We found that although FCPs induced significant fungistatic responses from itgam–/– splenocytes, these responses were markedly attenuated as compared to wild-type cells (Fig. 6A).

We next determined the ability of CD11b-deficient mice to develop allergic airway disease, a model of asthma in which key disease features such as airway hyperresponsiveness, airway eosinophil recruitment, mucus hyper-production, and type 2 cytokine secretion are induced by airway fungi in part through TLR4 ((16,25)). Wild-type (WT) and itgam–/– mice were challenged with 400,000 conidia of *A. niger* or sham every other day for two weeks and compared to identically treated Fibγ390-396A mice in which the amino acids of the FBG binding site have been converted to alanines (35). All mice were assessed for key allergic airway disease features 24 hours following the final challenge. Compared to wild-type mice, both Fibγ390-396A and itgam–/– mice challenged with fungus demonstrated significantly less airway hyperresponsiveness as assessed by increases in respiratory system resistance (R_{RS}) following provocative challenge with escalating doses of acetylcholine (Fig. 6C).
**itgam**<sup>−/−</sup> mice challenged with fungi also displayed a significant reduction in total cell and eosinophil infiltration into the airways as compared to wild-type mice, but Fibγ390-396A mice showed no defect in cell trafficking (Fig. 6D). Quantitative real-time PCR analysis of RNA isolated from mouse lung homogenates further revealed no significant differences between WT, Fibγ390-396A, and **itgam**<sup>−/−</sup> mice with regards to Muc5AC or IL-13 expression (Fig. 6E,F). Finally, IL-4 secretion from whole lung homogenates was not impaired by the absence of Mac-1 or in the presence of mutant fibrinogen (Fig. 6G).

Together, these observations demonstrate that a cognate interaction between the FBG gamma chain and Mac-1 is essential for the expression of antifungal responses from splenocytes, and airway hyperresponsiveness. Our findings further confirm the independent role played by Mac-1 in eosinophil recruitment in diverse contexts (36-38).

**FCPs can induce “innate” asthma in Fibγ390-396A mice, but not itgam**<sup>−/−</sup> mice.**

Full expression of proteinase or fungal-induced allergic airway disease in mice requires generation of both FCPs and TH2 cells, the latter of which drive the strong expression of airway hyperresponsiveness, marked eosinophilia, mucus hypersecretion, and IgE antibodies that are markers of advanced disease(25). However, although they only trigger innate immune responses, FCPs can alone drive an attenuated or “innate” form of allergic airway disease that includes modest induction of airway hyperresponsiveness and non-allergic airway inflammation through a mechanism that involves the activation of signal transducer and activator of transcription 6 (STAT6) (32).

We therefore hypothesized that providing exogenous FCPs would overcome the inability of Fibγ390-396A mice, but not **itgam**<sup>−/−</sup> mice, to express innate allergic airway disease. To test this, we challenged syngeneic Fibγ390-396A mice, **itgam**<sup>−/−</sup>, and wild type mice for 5 consecutive days with FCPs or a vehicle control intranasally and quantified key features of the allergic airway disease phenotype one day after the final challenge (Fig. 7A). We found that both wild type and Fibγ390-396A mice developed modest, but significant airway hyperresponsiveness, whereas **itgam**<sup>−/−</sup> mice did not (Fig. 7B-D). Moreover, we found that **itgam**<sup>−/−</sup> mice challenged with FCPs yielded fewer total leukocytes from the airways as compared to Fibγ390-396A mice, indicating that homing defects were restricted to the **itgam**<sup>−/−</sup> mice (Fig. 7E). Mucus gene induction as assessed by quantifying muc5ac transcripts were similarly enhanced in all three genotypes of mice after FCP challenge (Fig. 7F). Together, these findings demonstrate that “innate” allergic airway disease, similar to leukocyte fungistatic responses, is in part mediated by FCPs through a cognate interaction with CD11b.

**Discussion**

Increasing evidence implicates coagulation factors in the development and perpetuation of allergic airway disease(25,39-42). These studies underscore specifically the importance of the antimicrobial role of clotting factors that can now be seen as a primitive immune response that is orthologous to the coagulogen-Spaetzle-Toll system of arthropods(43,44). In distinct contexts, fibrinogen has been linked to immunity against bacteria and fungi through its interactions with CD11b/Mac-1(45-47) and TLR4(25,48,49). Proteinases are also known to initiate inflammation in the settings of allergic asthma(2,6,20,50) and innate antifungal immunity(25,51), but it is also now clear that the distinct and complex inflammatory pathways that comprise allergic inflammation also serve complex antifungal roles (12). Thus, fibrinogen activation by proteinases leading to allergic and antifungal responses through TLR4 and CD11b as shown here unifies major elements of this ancient immune paradigm and provides new insight into the pathogenesis of allergic airway diseases.

Our findings recapitulate and extend our previous findings(25) that the formation of fibrinogen cleavage products from proteinases promotes antifungal immunity in vitro in a manner that requires TLR4. Using protein gel electrophoresis, we have characterized the
ordered degradation of fibrinogen by the allergenic fungal proteinase PAM, which was determined to be an alkaline serine proteinase. FCPs were found to induce antifungal immunity by both contact-dependent and contact-independent means. Splenocytes, which represent a diverse cell population consisting predominantly of B cells and T cells and to a lesser extent monocytes, granulocytes, and dendritic cells, were found to require direct contact with fungi to inhibit fungal growth. In contrast, fully mature BMDM secreted soluble antimicrobial factors in response to FCPs that accounted for most of their antifungal activity, although we cannot rule out the possibility that these cells can inhibit fungal growth through additional mechanisms that require contact. Whereas FCPs could prime splenocytes to become fungistatically active, intact fibrinogen required contact with the fungus to initiate fungistatic activity, likely representing the production of FCPs in situ from whole fibrinogen. These observations suggest that surface-bound FBG may bind leukocytes with low affinity, perhaps through Mac-1 or TLR4, but with much higher affinity to Mac-1 in the presence of proteinases produced by germinating fungi that convert FBG to FCPs. These newly formed FCPs would already be present at the cell surface for rapid transfer to other critical receptors such as TLR4 to complete a highly efficient signaling complex (Fig. 8).

Once formed, the putative Mac-1/TLR4/FCP complex then initiates a signaling cascade that coordinates two effector functions, innate fungistatic immunity as illustrated by splenocytes and BMDM, and innate allergic airway disease that includes modest airway hyperresponsiveness and predominant neutrophilia (Fig. 7), but also limited eosinophilia (32). The latter events likely also serve an antifungal role (12), but despite their similar functions, we have shown in parallel studies that leukocyte-based fungistatic immunity and innate allergic airway disease are coordinated through distinct transcription factors, nuclear factor kappa B (NF-kB) and signal transducer and activator of transcription 6 (STAT6), respectively (32) (Fig. 8).

Our combined findings thus reveal unexpected complexity with regard to the antimicrobial programs that are deployed in response to airway mycosis and which trace back to a unique cleaved form of fibrinogen that results in the presence of fungal and many other exogenous proteinases derived from 4 kingdoms of life. Given the pleiotropic, cytokine-like properties of FCPs that extend well beyond the clotting function of FBG, we propose the new term cryptokine (“hidden cytokine”) to more functionally describe this unique form of immunologically active fibrinogen fragment (Fig. 8). Our findings further illustrate the broader concept of proteinase-activated immune responses (PAIRs) in which endogenous proteinases cleave a variety of pro-cytokines to yield their fully active forms (e.g., IL-1 family (52) and IL-25 (53)), i.e., enPAIRs, which presumably are formed downstream of exogenous proteinase activated immune responses, i.e., exPAIRs, exemplified through fibrinogen cryptokines. Of special interest is the IL-1 family member IL-33, which may be proteolytically activated by both endogenous and exogenous proteinases (54). A more complete understanding of exPAIRs and enPAIRS and their molecular relationships is likely to clarify our understanding of how innate and adaptive immune responses are coordinately regulated.

A limitation of this study is that we were unable to directly demonstrate the binding of FCPs to Mac-1. This is due to the marked affinity that fibrinogen, and FCPs, have for immunoglobulin, especially IgG (55,56). This issue of non-specificity precludes use of immunological assays that are routinely used to demonstrate specific binding between two molecules, including western blotting and flow cytometry. Thus, although the model presented in Figure 8 is strongly supported through the indirect evidence presented herein, it remains speculative pending additional studies that overcome this issue.

Lack of Mac-1 did not completely abrogate leukocyte fungistatic responses or airway hyperresponsiveness, in contrast to the more severe such defects seen in TLR4−/− mice.
(Fig. 1H and (25)). It is thus possible that closely related integrins that also bind FBG such as CD11a/CD18 (leukocyte function associated antigen 1; LFA-1) may be in part compensating for the lack of CD11b in these experiments. Moreover, ICAM-1 (CD54), an endogenous ligand for LFA-1 and Mac-1 that is also expressed on leukocytes, also recognizes FBG, albeit through a site distinct from that recognized by CD11b(57). Cognate interactions between cryptokines, LFA-1 and ICAM-1 may therefore suffice to support cellular infiltration seen in itgam<sup>−/−</sup> mice, yet be insufficient for the induction of airway hyperresponsiveness. We previously showed that allergic airway disease develops poorly in mice deficient in all CD18 integrins and specifically LFA-1 and ICAM-1 due to a defect in T<sub>H</sub>2 cell homing to lung, but we cannot exclude the possibility that perturbations in cryptokine signaling related to lack of these adhesion molecules also contributed to this phenotype(58,59).

Additional studies have examined the role of Mac-1 in allergic airway disease. Kanwar, et al., found that compared to wild type, Mac-1 deficient mice developed increased airway hyperresponsiveness and eosinophilic inflammation in an ovalbumin-based model after 28 days(60). However, because this protocol involves no exPAIR-based mechanism for the creation of cryptokines (ovalbumin is not proteolytically active), it is possible that the function of Mac-1 in this context is entirely distinct, resulting in a substantially different phenotype.

Our findings here and elsewhere(32) suggest that cryptokines coordinate a physical relationship between TLR4 and CD11b to yield a highly efficient signaling complex that coordinates the development of allergic airway disease and innate antifungal immunity (Fig. 8). Additional studies that document the association of fibrinogen with the CD11b I domain and Mac-1 with TLR4 in remarkably distinct contexts that include endotoxemia and malaria strongly support this model(31,61-63). Thus, although our work has emphasized the importance of this pathway to fungal-related allergic inflammation and disease, it should be noted that cryptokine-TLR4 signaling broadly shapes the immune response to very different pathogens. Additional studies are required to understand how a fundamentally similar ligand-receptor interaction translates into diverse immune responses that are tailored to distinct pathogens.

As the healthcare costs associated with allergic airway disease continue to rise(64), the need for understanding the mechanisms that initiate and propagate these ailments increase commensurately. Future research will further clarify the mechanisms by which fungi and proteinases coordinate the expression of allergic inflammation and diseases at innate and adaptive levels and ultimately translate into more effective and durable therapies.
Experimental Procedures

**Mice.** C57Bl6J (JAX #000664), TLR4⁻/⁻ (B6.10ScN-Tlr4<sup>tm1<sup>del</sup>/JthJ ; JAX #007227)(65,66), and CD11b⁻/⁻ (B6.129S4-I<sub>Itgam</sub><sup>tm1Myd</sup>/J ; JAX #003991)(67) mice were purchased from Jackson Laboratory (Bar Harbor, MA). Fibγ<sub>3</sub>90-396A mice developed as previously described(45). Mice were housed and bred under specific pathogen free conditions at the Baylor College of Medicine Transgenic Mouse Facility. Female mice 4-10 weeks old were used for all experiments and matched for age and sex. Animal studies were conducted under Institutional Animal Care and Use Committee approved protocols and conformed to all Federal and institutional guidelines.

**Genotyping of Mice.** TLR4⁻/⁻ (B6.10ScN-Tlr4<sup>tm1<sup>del</sup>/JthJ), CD11b⁻/⁻ (B6.129S4-I<sub>Itgam</sub><sup>tm1Myd</sup>/J), and Fibγ390-396A mice were routinely genotyped using the following primers (All 5'<sup>3</sup>): For TLR4⁻/⁻, Mutant Forward (GCAAGTTTCTATATGCATTCTC), Mutant Reverse (CCTCCATTTCCAATAGGTAG), Wild type Forward (ATATGCATGATCAACACCACAG), Wild type Reverse (TTTCCATTGCTGCCCTATAG). For CD11b⁻/⁻, Mutant Reverse (TGATTCCCACTTTGTGGTTC), Common (TGTTTTTACCCCTCCCTCCT), Wild type Reverse (CCTTTGATCTCTCCCCACCT). For Fibγ390-396A, Gamma F(+) (ATTGACATGATCACCAAAATTGCTTATTG), Gamma E (-) (CCATTTAAGGCTAGTATGTTAAGAAG), followed by the addition of 1 µL of PvuII for 2 hours at 37° C to target the novel mutant restriction site, as previously described.(45) PCR products were electrophoresed on 1% agarose gels for genotype determination.

**SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).** Protein electrophoresis was performed using NuPAGE 4-12% Bis-Tris Protein Gels, MES SDS Running Buffer, and SimplyBlue SafeStain (all Invitrogen, Carlsbad, CA). Samples for protein electrophoresis were diluted at least 1:2 in Tricine sample buffer and Precision Plus Protein Kaleidoscope Prestained Protein Standards were used as a protein electrophoresis molecular weight marker (both Bio-Rad, Hercules, CA).

**Isolation of murine splenocytes and generation of murine bone marrow-derived macrophages (BMDMs).** Murine splenocytes were isolated using ACK lysing buffer-treatment of homogenized mouse spleens in RPMI-1640 cell culture media (without serum) passed through a 40 µm nylon cell strainer (Corning, Corning, NY). BMDMs were isolated by suspending syringe-flushed marrow from murine femurs and tibias in RPMI-1640 cell culture media supplemented with 10% fetal bovine serum and passing the suspension through a 40 µm cell strainer. The marrow was then cultured in RPMI-1640 cell culture media supplemented with 10% fetal bovine serum and 20 ng/mL recombinant murine GM-CSF (R&D Systems, Minneapolis, MN) for one week with one media wash and replenishment on day 4. All cell culture media contained Penicillin-Streptomycin (Invitrogen) 100 units/mL.

**Proteinases.** Proteinase from Aspergillus Melleus (PAM; P4032; Sigma-Aldrich, St. Louis, MO) and Pronase from Streptomyces griseus (PRON-RO; Sigma-Aldrich, St. Louis, MO) were purchased, aliquoted in PBS, and stored at -80°C. Ragweed extract (Short Ragweed, *Ambrosia artemisiifolia*) was obtained from Greer (Cambridge, MA), aliquoted and suspended in PBS and stored at -80°C. Lyophilized mite samples of *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Glyciphagus domesticus*, and *Tyrophagus putrescentiae* were generously provided by Fabrizio Ottoboni (Life Science Knowledge Consulting, Milan, Italy). Mite samples were suspended in cold PBS, and protein was extracted using zirconium beads in a Retsch PM 100 Planetary Ball Mill for 3 5-minute cycles at 550 rpm, each followed by 5 minutes on ice. Sample slurries were then centrifuged to remove undissolved solids at 2200xG for 30 minutes at 4°C, followed by a second centrifugation of the supernatant at 10000xG for 30 minutes at 4°C. The remaining supernatants were aliquoted and stored at -80°C.

**Mass Spectrometry Protein Sequencing.** Samples were excised from SDS-PAGE
preparations and mass spectrometry protein sequencing was performed by the Baylor College of Medicine Mass Spectrometry Proteomics Core Facility. Protein identity was confirmed by aligning detected protein in the samples to known sequences.

**Generation of Fibrinogen Cleavage Products (FCPs).** FCPs were made by suspending Human Fibrinogen (HCI-0150R; Haematologic Technologies, Essex Junction, Vermont) at 5 mg/mL in PBS. PAM was added to the fibrinogen solution at a concentration of 6 µg/mL for 30 minutes at 37°C. Fibrinogen fragments D (HCI-0150D) and E (HCI-0150E) were also acquired from Haematologic Technologies and processed by the same methods. For in vivo studies, fibrinogen was incubated with PAM for 6 hours. Other proteinases, concentrations, and incubation durations were performed using similar methods as indicated in the figure legends.

**Fungistasis Assay.** Splenocytes or BMDMs were cultured in 24 well flat bottom tissue culture plates (353047, Corning, New York) or 24-well transwell plates (351152, Corning, New York) as indicated and incubated for 24 hours with FCPs or other described preparations and controls in a 37°C/5% CO2 incubator using RPMI-1640 cell culture media without serum. 200 conidia of *Aspergillus niger* were then added to each well. After 18-22 hours, allowing for sufficient growth of the spores, fungal germination events (FGEs) were enumerated. The percentage of fungal growth inhibition was calculated by the difference in the number of FGEs in control no murine cell wells versus the experimental wells, divided by the number of FGEs in no cell control wells, multiplied by 100%. 100%*(FGE_{NC} - FGE_{Exp}) / FGE_{NC}

**Murine model of FCP-induced acute allergic airway inflammation.** Anesthetized mice were intranasally challenged with 0.25 mg PAM-FCPs (6 hour degradation) diluted in 50µL PBS or vehicle control (0.3 µg PAM) in 50µL PBS every day for 5 days. 24 hours following the final challenge, allergic airway disease was assessed.

**Assessment of allergic airway disease.** 24 hours following the final challenge of the allergic airway disease models described above, mice were anesthetized with etomidate, and increasing concentrations of acetylcholine were administered intravenously via the tail vein to determine airway hyperresponsiveness using airway resistance as previously described(6,25). After these data were collected, bronchoalveolar lavage fluid (BALF) and whole lung for RNA and cell culture were obtained. Total cells were counted in the BALF and cell differential analysis was performed using modified Giemsa-stained cytospin preparations. Homogenized whole lung was prepared for RNA extraction in TRIzol (ThermoFisher Scientific, Waltham, MA) or for cell culture for detection of cytokines. Cell culture supernatants were analyzed for IL-4 concentration by ELISA using anti-IL-4 (Clone 11B11, 1:100) and anti-IL-4-Biotin (BD Biosciences, 554390, 1:200) antibodies.

**Proteinase inhibition.** PAM (50 ng/sample) was incubated with FITC-labeled casein overnight, whole protein precipitated by tricholoroacetic acid, and sample fluorescence was quantified using the SpectraMax i3x (Molecular Devices) in accordance to the Protease fluorescent Detection Kit protocol (PF0100, Sigma-Aldrich). Inhibition of proteolytic activity by serial dilutions of the broad spectrum, class-specific inhibitors E-64 (Sigma-Aldrich, E3132), Pepstatin A (Sigma-Aldrich, P5318), Ilomastat (Selleckchem, S7157), and AEBSF hydrochloride (Santa Cruz Biotechnology, sc-202041) at a starting concentration above the manufacturers recommended working concentration. Repeated experiments were performed at select concentrations to verify the initial analysis.
mRNA isolation, conversion to cDNA, and quantitative PCR. Homogenized murine lung tissue dissolved in TRIzol was extracted with chloroform (Sigma-Aldrich), precipitated in isopropanol (Sigma-Aldrich), washed in ethanol, and then resuspended in nuclease free water. cDNA was generated from RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was then performed using the ABI 7500 Real-Time PCR System (Applied Biosystems) with standard techniques with 18s ribosomal RNA (Applied Biosystems 4318839) as the normalization standard. Muc5ac (Mm01276718_m1) and IL-13 (Mm00434204_m1) Taqman probes (ThermoFisher Scientific) were used in qPCR.

Statistical Analysis. Data were analyzed using GraphPad Prism 5 and are presented as means +/- standard error of the mean (SEM). Significant differences relative to PBS-challenged mice or appropriate controls are expressed by P values of <0.05, as measured by the two tailed Student’s t-test or one-way or two-way ANOVA followed by Bonferroni’s test for multiple comparison using datasets involving similar distribution of variance. Sample sizes, both in vitro and in vivo, were determined empirically. No in vitro data were excluded unless the entire experiment or assay failed due to technical reasons. No mouse data were excluded unless animals died prior to data collection. A process for mouse randomization was not used as all animals within a given genotype were genetically identical and all mice appear identical regardless of genotype.
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Author Contributions: CTL, FK, MJF, and DBC conceived the project; CTL performed most experiments. HYT, JMK, MCM, ZZ, YW, and PP provided critical technical support; MJF provided critical reagents; all authors wrote and edited the manuscript.

Data Availability Statement

Data will be made available by the authors upon reasonable request.
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Figure 1. Proteolytic cleavage of fibrinogen enhances the fungistatic activity of diverse cells in vitro.

(A) SDS-PAGE gel of fibrinogen, PAM (concentrated), and PAM-FCPs. (B,C) SDS-PAGE showing PAM-mediated degradation of fibrinogen (B) over time and (C) with increasing concentrations of PAM, but limited to 30-minute digests. (D) To mouse splenocytes cultured in serum-containing and serum-free conditions were added (200) *A. niger* conidia after which fungal germination events (FGE) were enumerated after 18 hours. (E) The same fungistasis assay was performed on splenocytes treated with sham (PBS), whole fibrinogen (FBG; 100 nM; 34 ug/mL), or FCPs produced using the proteinase of *Aspergillus melleus* (PAM-FCPs; 100 nM cleaved fibrinogen). Percent (%) of FGE inhibition was calculated as the (# FGE in wells containing no cells – #FGE in wells containing cells/ # FGE in wells containing no cells) x 100%. (F-H) Fungistasis assays measuring the ability of FCPs (100 nM) to prime fungal growth inhibition compared to PAM (FCP-equivalent; 40.8 ng/mL) added to splenocytes (F), bone marrow derived macrophages (BMDMs) (G), and splenocytes (H) from WT and TLR4−/− mice. N=3 biological replicates per group. *p<0.05, **p<0.01, ***p<0.001 by Student’s T-test or One-way ANOVA with Bonferroni’s Multiple Comparisons test. Data are representative of at least two independent experiments.
Figure 2. Splenocyte-mediated fungistasis is contact dependent, while BMDMs secrete soluble antifungal factors. (A) Fungistasis assay comparing the fungistatic activity of PBS, FBG, and FCPs either alone, in treatment-primed cell-conditioned media, or in cultures containing splenocytes primed with each indicated treatment but in fresh media. (B) Fungistasis assay in which splenocytes were primed with PBS or FCPs in a transwell plate that precluded direct contact between fungi and mouse cells. (C) Fungistasis assay using conditioned media from bone marrow derived macrophages primed with sham or FCPs. N=3 biological replicates per group. *p<0.05, **p<0.01, ***p<0.001 by Student’s T-test or One-way ANOVA with Bonferroni’s Multiple Comparisons test. Data are representative of at least two independent experiments.
Figure 3. Proteinase from Aspergillus Melleus (PAM) is an alkaline serine proteinase. (A,B) Protein electrophoreses of PAM indicating the prevalence of 33 kDa, 22 kDa, and 11kDa bands performed (A) using a Novex NuPage 4-12% Gel and (B) by the Baylor College of Medicine Mass Spectrometry Proteomics core facility. (C-D) Protein sequence alignments of PAM in tabular format (C) and in graphical format for the (D) 33 kDa electrophoresis band.
Figure 4. Diverse proteinases preferentially cleave the fibrinogen alpha chain to induce antifungal responses in vitro. (A) Mouse splenocyte fungistasis assays using FCPs (100 nM) derived from the proteolytic cleavage of human fibrinogen using PAM (6 mg/mL), Pronase from *Streptomyces griseus* (PRO; 6 mg/mL), *Dermatophagoides farinae* dust mite extract (DF; 600 mg/mL), and *Artemisia artemisiifolia* (Ragweed) extract (RW; 180 mg/mL). (B) Schematic diagram of fibrinogen demonstrating the alpha, beta, and gamma polypeptide chains as well as the D and E domain, based on crystallography data.(68-70) (C) SDS-PAGE analysis of β-mercaptoethanol-reduced human fibrinogen before addition of proteinases (second lane from left) and following cleavage by PAM (0.6 mg/mL), Pronase (0.6 mg/mL), *D. farinae* extract (DF; 600 mg/mL), *D. pteronyssinus* extract (DP; 600 mg/mL), *G. domesticus* extract (GD; 180 mg/mL), *T. putrescentiae* extract (TP; 180 mg/mL), and *A. artemisiifolia* extract (180 mg/mL). (D) Reducing SDS-PAGE analysis of 6 mg/mL PAM-mediated degradation of fibrinogen over the indicated times. N=3 biologic replicates per group. *p<0.05, **p<0.01, ***p<0.001 by Student’s T-test or One-way ANOVA with Bonferroni’s Multiple Comparisons test. Data are representative of at least two similar, independent experiments.
Figure 5. Fibrinogen D and E Fragments are sufficient substrates through which PAM can generate functional FCPs. (A) Fungistasis assays were performed that compared PAM-induced FCPs derived from whole human fibrinogen, the fibrinogen D Fragment, and the fibrinogen E Fragment as indicated using murine splenocytes. All fibrinogen moieties were added at the same concentration (100 nM). (B) Non-reducing SDS-PAGE analysis of native proteins and PAM-FCPs derived from whole fibrinogen (FBG), and the D and E fragments (Frag) of fibrinogen. N=3 biologic replicates per group. *p<0.05, **p<0.01, ***p<0.001 by One-way ANOVA with Bonferroni’s multiple comparisons test of fragments and FCPs compared to blank and PAM controls. Statistical data are representative of at least two similar, independent experiments.
Figure 6. CD11b is required for the full expression of fungal-induced allergic airway disease. (A) Fungistasis assay comparing splenocytes from wild type and CD11b<sup>−/−</sup> mice. (B) Anesthetized C57BL6/J, Fibγ<sub>390-396A</sub>, and CD11b<sup>−/−</sup> mice were intranasally challenged with 4 × 10<sup>5</sup> live conidia of A. niger (AN) or PBS on alternating days for 15 days for a total of 8 treatments with data collected 24 hours after the final challenge. (C) Respiratory system resistance (R<sub>RS</sub>) as assessed by increasing intravenous acetylcholine (Ach) challenge. (D) Quantification of cells from bronchoalveolar lavage fluid samples (M: macrophages; E: eosinophils; N: neutrophils; L: lymphocytes). (E, F) Quantitative real-time PCR measurements of cDNA derived from mouse whole lung mRNA for Muc5AC and IL-13. (G) IL-4 as measured by ELISA from supernatants derived from whole lung homogenates. N=3 (Figure 6A) or N=4-5 (Figures 6B-G) biologic replicates per group. *p<0.05, **p<0.01, ***p<0.001 by Student’s T-test or One or Two-way ANOVA with Bonferroni’s Multiple Comparisons test. Data are representative of at least two similar, independent experiments.
Figure 7. FCP-induced inflammation promotes allergic airway disease in WT and Fibγ390-396A mice, but not CD11b (Mac-1) knockouts. (A) Anesthetized C57BL6/J, Fibγ390-396A, and itgam−/− mice were challenged intranasally with 0.25 mg of PAM-FCPs or sham (PBS) containing 0.3 mg PAM (FCP dose equivalent) for 5 consecutive days. 24 hours after the final challenge, mice were assessed for development of airway hyperresponsiveness over 5 half-log increments of acetylcholine (B). The same data are shown for the 4th and 5th doses only (C, D). (E) Bronchoalveolar lavage fluid inflammatory cells. (F) muc5ac mRNA expression for the same mouse groups. N=4-7 biologic replicate mice per group. *p<0.05, **p<0.01, ***p<0.001 by One or Two-way ANOVA with Bonferroni’s Multiple Comparisons test. Statistical tests for (7C) and (7D) are carried over from those in (7B). Data represents two combined independent experiments.
Figure 8. FCPs act through Mac-1 and TLR4 to promote allergic and antifungal inflammation. Schematic depiction of the proposed interactions between FBG, FCPs, Mac-1, and TLR4 to trigger allergic and antifungal responses in the airway. Intact FBG may interact weakly with CD11b and TLR4, but high affinity binding to CD11b occurs after proteolytic cleavage of especially the a chain to yield FCPs (cryptokines). Subsequent signaling initiated through the Mac-1/TLR4/FCP complex initiates both innate antifungal and allergic inflammatory programs through distinct transcription factors, NF-kB and STAT6)(32).
Selective fibrinogen cleavage by diverse proteinases initiates innate allergic and antifungal immunity through CD11b
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