

Original article

A new mechanism regulating the initiation of allergic airway inflammation

Attila Kiss, MD,^{a,*} Martin Montes, MD,^a Sarat Susarla, MD,^b Elin A. Jaensson, BA,^c Scott M. Drouin, PhD,^d Rick A. Wetsel, PhD,^d Zhengbin Yao, PhD,^e Rachel Martin, PhD,^e Nabeel Hamzeh, MD,^a Rebecca Adelagun, BS,^a Sheila Amar, MD,^a Farrah Kheradmand, MD,^{a,c} and David B. Corry, MD^{a,c} Houston, Tex

Background: The earliest immune events induced by allergens are poorly understood, yet are likely essential to understanding how allergic inflammation is established.

Objective: We sought to describe the earliest signaling events activated by allergen and determine their significance to allergic inflammation.

Methods: A fungal-associated allergenic proteinase (FAP) or ovalbumin was administered once intranasally to wild-type mice to determine their ability to induce allergy-associated genes and initiate allergic lung inflammation. Mice deficient in recombina-activating gene 1, C3a, the C3a anaphylatoxin receptor, and MyD88 were challenged similarly to understand the requirement of these molecules and T and B cells for allergic inflammation. Adoptive T-cell transfer experiments were further performed to determine whether signal transducer and activator of transcription 6 (STAT6) was required for cell recruitment and allergic inflammation.

Results: FAP, but not ovalbumin, induced eosinophilic airway inflammation and lung IL-4 production in the absence of adaptive immune cells after the transcriptional induction of allergy-specific airway chemokines. Allergen-mediated chemokine secretion and innate allergic lung inflammation occurred in the absence of STAT6, recombina-activating gene 1, C3a, C3a anaphylatoxin receptor, Toll-like receptor 4, and MyD88 but required intact proteinase activity. Furthermore, FAP induced recruitment of T_H2 cells and eosinophils to lungs independently of STAT6, which was previously thought to be required for T_H2 cell homing.

Conclusion: FAP induces allergic lung inflammation through a previously unrecognized innate immune signaling mechanism.

Clinical implications: These findings reveal a new paradigm for understanding how allergic inflammation begins and suggest novel possibilities for the prevention and treatment of allergic diseases, such as asthma. (J Allergy Clin Immunol 2007;119:1016-1025.)

Key words: T_H1/T_H2 cells, chemokines, allergy, inflammation, lung

Allergic inflammation is observed in human subjects and experimental animals in response to characteristic pathogens that include helminths and fungi and after exposure to certain shed or secreted products that include pollen and a wide variety of insect, animal, and mite antigens. Although T and B cells are clearly required for allergic lung inflammation, innate signaling molecules, especially the C3a anaphylatoxin,^{1,2} are also essential.³

Of additional importance to the control of allergic inflammation is the signal transducer and activator of transcription 6 (STAT6) signaling pathway, which is critical for the development of T_H2 cells, eosinophils, and IgE-secreting B cells and is an important mediator of experimental allergic lung disease that resembles allergic asthma.^{4,5} STAT6 is further required for the homing of T_H2 cells to the lung after allergen challenge by transcriptionally regulating production of allergy-specific chemokines, such as CCL17 and CCL11.⁶ However, although pathogen-associated molecular patterns, such as endotoxin, have been linked to allergic lung inflammation under some experimental conditions,⁷ neither they nor allergenic pathogens induce STAT6 directly; indeed, no signaling pathway activated by allergenic organisms has been specifically linked to allergic inflammation and disease. Consequently, the innate response to allergenic organisms that might be important for the control of allergic lung disease remains unknown.

Experimental respiratory allergens are distinguished by their ability to elicit allergic lung inflammation when inhaled. Ovalbumin (OVA), a commonly used experimental allergen, is incapable of eliciting allergic inflammation if administered strictly by means of inhalation, whereas pollen and fungal-derived allergens readily induce allergic responses when administered through the respiratory tract.⁸ Previously, we showed that pollen and fungal allergens are biochemically linked through their abundance of exogenous proteinases, which are in turn

From the Departments of ^aMedicine, ^bPediatrics, and ^cImmunology, Baylor College of Medicine; ^dthe Institute of Molecular Medicine, University of Texas Health Science Center; and ^eTanox, Inc.

*Dr Kiss is currently affiliated with the Department of Respiratory Medicine, Semmelweis University, Budapest, Hungary.

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Reprint requests: David B. Corry, MD, Baylor College of Medicine, One Baylor Plaza, BCM285, Houston, TX 77030. E-mail: dcorry@bcm.tmc.edu. 0091-6749/\$32.00

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Abbreviations used

- CFSE: Carboxyfluorescein succinimidyl ester
 C_t: Threshold cycle
 FAP: Fungal associate allergenic proteinase
 OVA: Ovalbumin
 RAG-1: Recombinase activating gene 1
 STAT6: Signal transducer and activator of transcription 6
 TLR: Toll-like receptor

required for establishing allergic lung inflammation when administered through the physiologically relevant airway exposure route.⁸ Additional studies have confirmed that allergic responses are induced by helminth larvae, which are obligate producers of exogenous proteinases.^{9,10} Together, these findings indicate that pathogen-specific molecules distinct from pathogen-associated molecular patterns, especially exogenous proteinases, might be critical for initiating relevant allergic responses, but mechanistic insight linking pathogen-derived proteinases to specific allergy-associated innate immune events has been lacking.

We hypothesized that a fungal-associated allergenic proteinase (FAP) from *Aspergillus oryzae*, a potent respiratory allergen,⁸ induces allergic inflammation through a specific innate immune signaling pathway. Our findings confirm that FAP induces eosinophilic inflammation innately, in part by coordinating the production of chemokines necessary for recruitment of eosinophils and other allergic effector cells, through a novel signaling pathway. These findings clarify the pathogenesis of allergic lung inflammation and suggest new approaches to the treatment of allergic diseases, such as asthma.

METHODS

Mice

Inbred C57BL/6, BALB/c, C3H.OuJ, and C3H.HeJ mice and STAT6 (BALB/c background)-null and recombinase activating gene 1 (RAG-1)-null (C57BL/6 background) mice were purchased from the Harlan Company or Jackson Laboratories. Myd88^{-/-} mice (C57BL/6 background) were produced as previously described¹¹ and generously provided by D Golenbock. Mice homozygous null for the C3 and C3a receptor genes (C57BL/6 background) were generated as previously described.^{12,13} All mice were bred and housed at either the American Association for Accreditation of Laboratory Animal Care-accredited vivarium at Baylor College of Medicine or the University of Texas Health Sciences Center Institute of Molecular Medicine under pathogen-free conditions. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and the UT Health Sciences Centre and followed federal guidelines.

Antigens, antibodies, and other reagents

Chicken egg OVA (grade V; Sigma, St Louis, Mo) was precipitated in alum (OVA/alum), as previously described.¹⁴ Lyophilized allergenic fungal proteinase (FAP) derived from *Aspergillus oryzae* (Sigma) was reconstituted to 1 mg/mL with sterile PBS and stored at -20°C. Proteinase activity of this FAP was inhibited by more

than 95% by repeated addition of phosphoramidon (500 μmol/L; Roche Molecular Diagnostics, Pleasanton, Calif) and 1,10-phenanthroline (10 mmol/L, Sigma) for 2 hours at room temperature, followed by overnight dialysis against PBS. Chicken egg OVA was reconstituted in sterile PBS at 500 μg/mL and stored at -20°C. For intranasal challenge, 5 μL (5 μg) of FAP was added to 45 μL (22.5 μg) of OVA immediately before intranasal administration. *Escherichia coli* endotoxin (Sigma) and synthetic CpG deoxyoligonucleotides (TCCATGACGTTCTGACGT) were reconstituted in PBS to a concentration of 100 μg/mL and stored at -20°C. Fluorescent polystyrene beads for calibration (Flow-Check; Beckman Coulter, Fullerton, Calif; 10-μm average diameter) were washed 3 times by means of centrifugation and resuspended in sodium azide/PBS (with 0.1% sodium azide), and the concentration was adjusted to 2 × 10⁶ beads/mL.

Intranasal challenge

Anesthetized mice were instilled intranasally once with 50 μL of endotoxin and CpG oligonucleotides (each 10 μg/mL), FAP prepared with OVA, or OVA alone, as previously described.¹⁵ Mice were killed 6 hours later for microarray analysis; 2, 8, and 12 hours later for quantitative mRNA analysis; and 18 hours later for BAL fluid protein and cellular analyses. These time points were chosen based on preliminary studies to show the maximal response for each variable after a single challenge with FAP.

Quantitation of allergic inflammation

Collection of bronchoalveolar lavage fluid and mouse lungs, analysis of bronchoalveolar lavage fluid cells, ELISPOT, and ELISA were all performed as previously described.¹⁵

Adoptive CD4⁺ T-cell transfer and quantitation

CD4⁺ T cells from immunized wild-type BALB/c mice were collected and adoptively transferred to recipient mice, as previously described,¹⁶ with the following modifications. Isolated CD4⁺ T cells were adjusted to 20 × 10⁶/mL in PBS and labeled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, Ore), according to the manufacturer's directions. CFSE-labeled cells (12 × 10⁶) were injected intraperitoneally into recipient mice, which were challenged intranasally with either FAP or vehicle immediately after cell transfer and then daily for 3 consecutive days and analyzed 18 hours after the final allergen challenge. CFSE-labeled T cells were quantitated from whole lung cells by means of single bead-enhanced quantitative flow cytometry, as previously described.¹⁷

Statistics

Data are presented as means ± SEMs and are representative of at least 2 independent experiments that used at least 4 mice in each group, unless otherwise indicated. Significant differences are expressed relative to OVA-challenged mice ($P \leq .05$) by using the Kruskal-Wallis test for multiple-group comparisons.

RESULTS

Innate recruitment to the lung of allergic effector cells by FAP

To determine whether the FAP derived from *A. oryzae* induces an innate allergic inflammatory response, we quantitated total bronchoalveolar lavage fluid cells and total IL-4- and IFN-γ-producing cells from whole lungs of RAG-1^{-/-} mice that lack adaptive immune cells

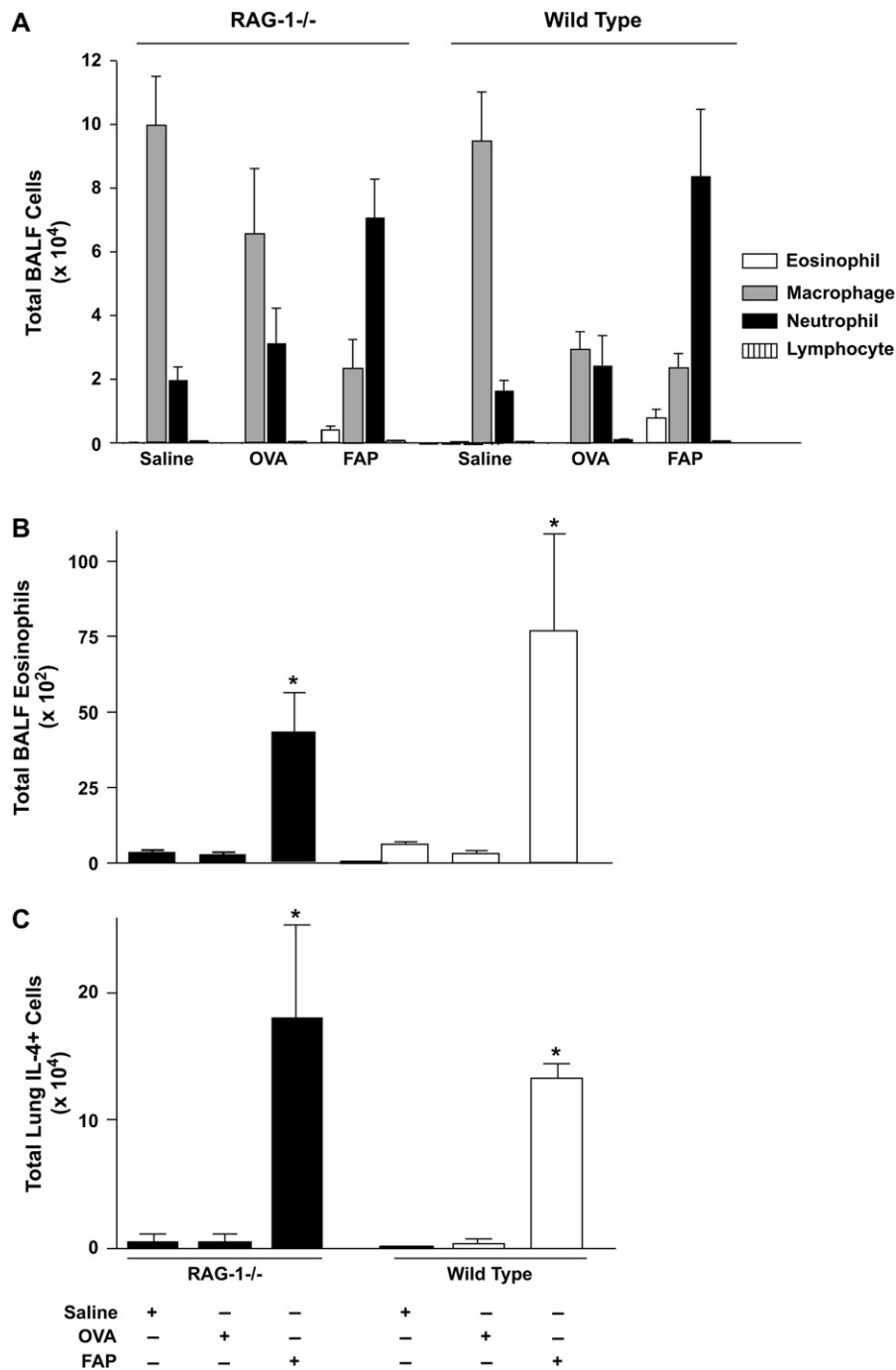


FIG 1. Innate recruitment of allergic inflammatory cells by FAP. RAG-1^{-/-} and wild-type mice were challenged once intranasally with either vehicle, OVA, or FAP. Eighteen hours later, the total numbers of bronchoalveolar lavage fluid (BALF) cells, including eosinophils, macrophages, neutrophils, and lymphocytes (**A**); BALF eosinophils (**B**); and whole lung homogenate IL-4-secreting cells (**C**), were assessed. **P* < .05 compared with OVA-challenged mice. Representative of 4 independent experiments (n = 4 mice per group).

(T and B cells) and wild-type mice challenged once with FAP or OVA. Bronchoalveolar lavage fluid contained predominantly macrophages and neutrophils, irrespective of the challenge (Fig 1, A). However, significant numbers of eosinophils were elicited by FAP but not by OVA or saline (Fig 1, A and B). Furthermore, FAP induced

recruitment of many IL-4-producing cells but few if any IFN- γ -secreting cells, regardless of mouse genotype (Fig 1, C, and data not shown). Thus although FAP and OVA can induce strong T_H2-driven allergic responses in mice, only FAP induced innate allergic lung inflammation.

FAP transcriptionally activates allergy-specific genes

Because recruitment of T_H2 cells is regulated primarily through chemokine receptor signaling,¹⁸ we reasoned that the innate allergic response to FAP was most likely transcriptionally driven. To demonstrate this, in preliminary experiments we first intranasally challenged RAG-1^{-/-} mice with FAP and determined the global induction of lung genes induced by Affymetrix Microarray (Affymetrix, Santa Clara, Calif). Eight hours after a single challenge, 1475 distinct genes, most with unknown function, were induced at least 2-fold above the levels of vehicle-challenged animals (see Fig E1, A, in the Online Repository at www.jacionline.org). The induced genes with known or putative functions are summarized in Fig E1, B, and Table E1 (available in the Online Repository at www.jacionline.org). Several of these FAP-induced genes were related to IL-4, T_H2 cells, or other allergic effector cells and included suppressor of cytokine signaling 3, vascular cell adhesion molecular 1, IL-1 β , and an IL-4-inducible gene of unknown function. However, the single largest family of induced genes was chemokines, including chemoattractants for T_H1 and T_H2 cells, eosinophils, and neutrophils (see Table E1 in the Online Repository at www.jacionline.org). Thus diverse lung genes were induced by FAP, but the prominent induction of chemokines and adhesion molecules suggested that homing of eosinophils and T_H2 cells was a major innate function regulated by this allergen.

Quantitation of mRNA by means of real-time PCR further indicated that genes induced as assessed by DNA microarray were transcriptionally activated by the FAP (see Fig E2, C-E, in the Online Repository at www.jacionline.org and data not shown). The real-time PCR analysis was further notable for the strong induction of chemokines. In addition to mRNA levels for CCL17 and CCL7, both ligands for chemoattractant receptors expressed by eosinophils and T_H2 cells (CCR4 and CCR3, respectively¹⁹), mRNA levels for CXCL10, a T_H1 chemokine,²⁰ was also markedly increased relative to that seen in vehicle-challenged animals (see Fig E2, C-E, in the Online Repository). mRNA levels of these chemokines peaked at 2 hours; remained relatively stable to 8 hours, with the notable exception of CXCL10; and had returned to baseline by 12 hours after challenge (see Fig E2, C-E, in the Online Repository and data not shown).

Based on these preliminary studies, we then assessed bronchoalveolar lavage fluid by means of ELISA for the presence of T-cell and eosinophil chemokines 18 hours after a single allergen challenge. This analysis confirmed the induction of CCL17 and CCL7 by FAP but also revealed that neither vehicle nor OVA alone induced these chemokines (see Fig E2, F and G, in the Online Repository). Consistent with our PCR analysis that showed only transient induction of CXCL10 mRNA, we could not detect CXCL10 protein in bronchoalveolar lavage fluid after challenge with vehicle, OVA, or FAP (data not shown). Thus these findings confirm that FAP preferentially induced T_H2/eosinophil chemokines through

an innate signaling mechanism, whereas OVA failed to induce any of the chemokines. Thus a specific signaling mechanism is activated by an allergenic proteinase that transcriptionally induces chemokines necessary for recruitment of allergic effector cells to the lung.

Innate allergic chemokine secretion requires proteinase activity independent of Toll-like receptor 4 signaling

We performed additional studies to confirm that the previous findings were due to the activity of the fungal proteinase and not other moieties, such as contaminating endotoxin. CCL17, CCL7, and CXCL10 were measured in bronchoalveolar lavage fluid of RAG-1^{-/-} mice challenged with proteinase-intact FAP and an identical preparation in which more than 95% of the proteinase activity was abolished by using specific inhibitors. As in the prior experiments, FAP readily induced secretion of CCL17 and CCL7, although not CXCL10 (Fig 2, A-C). In contrast, the proteinase-inhibited preparation failed to significantly induce secretion of either CCL17 or CCL7; moreover, proteinase inhibition failed to unmask a latent ability to induce CXCL10 (Fig 2, A-C). Thus the enzymatic activity of FAP that is required for allergic lung disease⁸ is also required for the innate immune control of airway chemokine secretion.

To determine whether Toll-like receptor (TLR) ligands could be contributing to the innate immune responses initiated by FAP, we intranasally administered to mice *E coli* endotoxin and unmethylated CpG deoxyoligonucleotides and determined their effect on airway chemokine secretion and cellular recruitment. These molecules (TLR4 and TLR9 agonists, respectively) failed to induce CCL17 levels but strongly induced secretion of CCL7 and, uniquely, CXCL10 (Fig 2, A-C, and data not shown). TLR ligands induced recruitment to the lung of many IFN- γ -secreting cells but no detectable lung IL-4-secreting cells (Fig 2, D, and data not shown). Furthermore, neither TLR ligands nor proteinase-inactive FAP induced recruitment of bronchoalveolar lavage fluid eosinophils (data not shown).⁸ Thus only FAP, but not OVA or TLR ligands, innately induced the secretion of proallergic chemokines and the recruitment of allergy-associated cells to the lung.

Because endotoxin contaminates both the *A oryzae* proteinase and OVA²¹ (0.2-0.8 EU/ μ g for each) and all other allergens tested in our laboratory (data not shown), we performed additional studies in mice deficient in MyD88, an adapter protein required for signaling through most TLR ligands, including endotoxin (a TLR4 ligand), to determine the effect of endotoxin on airway inflammation and allergic chemokine secretion. Compared with wild-type mice, MyD88^{-/-} mice challenged once intranasally with FAP showed markedly enhanced relative and absolute eosinophil responses in BAL fluid (see Fig E2 in the Online Repository at www.jacionline.org). Moreover, BAL fluid neutrophils in response to FAP challenge was markedly suppressed in the absence of MyD88-dependent signaling (see Fig E2 in the Online Repository). Similar

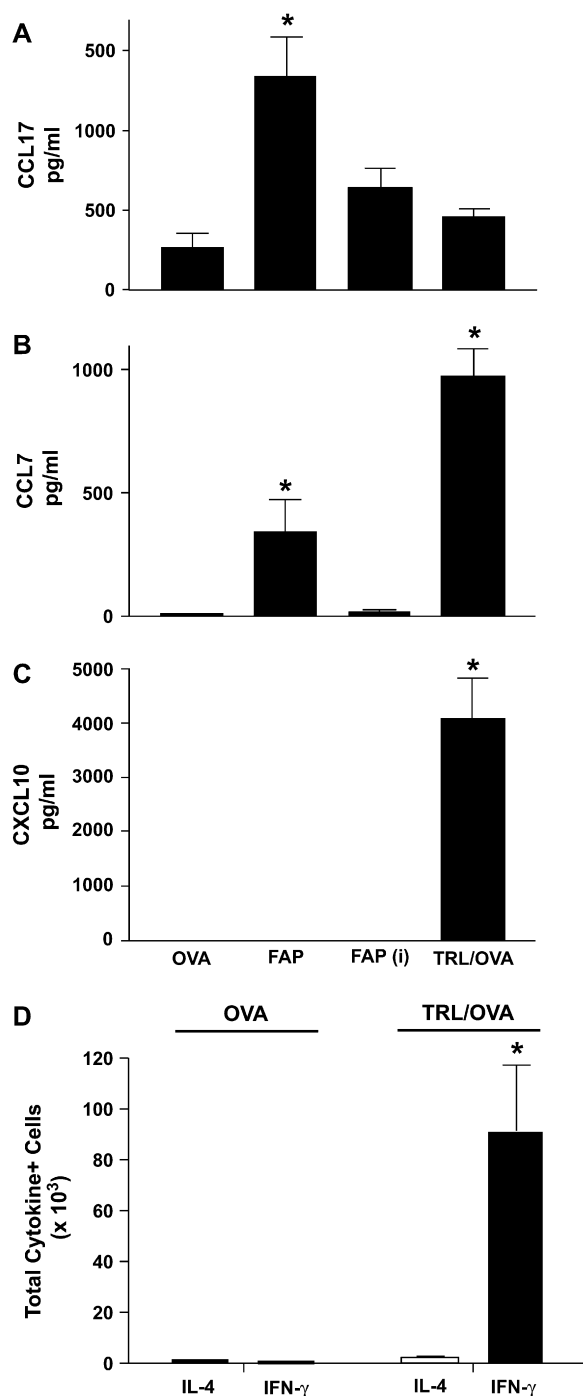


FIG 2. Proteinase activity, but not endotoxin, is required for allergic chemokine secretion and inflammation. RAG-1^{-/-} mice were challenged intranasally once with OVA, FAP, proteinase-inactive FAP (FAP [i]), or a combination of TLR ligands (*E coli* LPS and CpG motif-containing deoxyligonucleotides) prepared with OVA (TRL/OVA). CCL17 (A), CCL7 (B), and CXCL10 (C) levels were measured by means of ELISA from bronchoalveolar lavage fluid 18 hours later. D, Total lung IL-4- and IFN- γ -secreting cells were enumerated by means of ELISPOT from whole lungs of mice challenged with either OVA or TRL/OVA.

results were obtained in mice selectively deficient in TLR4 (D. Corry, data not shown). Together, these findings demonstrate that neither endotoxin nor other TLR ligands are required for allergic chemokine secretion and innate allergic inflammation. Instead, the effect of TLR ligands was to enhance neutrophil recruitment and suppress FAP-dependent innate allergic chemokine and eosinophil responses.

FAP-mediated allergic chemokine secretion requires neither the C3a receptor nor STAT6

Wild-type mice and mice deficient in STAT6, C3, and the C3a anaphylatoxin receptor were challenged once intranasally with FAP to determine the requirement of these molecules for innate allergic inflammation. All of these mice showed robust induction of CCL7 and CCL17, but neither vehicle nor OVA induced these chemokines, regardless of genetic background (Fig 3, A and B, and data not shown). Analysis of bronchoalveolar lavage fluid from C3^{-/-} and wild-type mice challenged with FAP or vehicle demonstrated similar degrees of neutrophilia induced by FAP (Fig 3, C). Although greater numbers of eosinophils were recruited to bronchoalveolar lavage fluid in wild-type relative to C3^{-/-} mice, eosinophilia was nonetheless specific to challenge with FAP. Thus FAP induces the secretion of allergy-specific chemokines through a mechanism distinct from known allergy-related signaling pathways. Moreover, although C3 is required for airway eosinophilia after prolonged allergen challenge,¹ it is specifically not required for eosinophilia after a single challenge.

Although STAT6 is required for OVA-dependent recruitment of T_H2 cells to the lung and allergic inflammation,^{6,18} the preceding findings suggested that T_H2 cells and eosinophils could be recruited to the lung through the unique FAP-activated signaling mechanism independently of STAT6. To confirm this, we adoptively transferred fluorescently labeled FAP-specific CD4⁺ T cells into both wild-type and STAT6^{-/-} mice that then received either FAP or vehicle intranasally. From the lungs of these mice, we enumerated total CFSE⁺ T_H cells by using bead-enhanced flow cytometry (Fig 4, A). This analysis revealed equivalent numbers of CFSE⁺ T_H cells recruited to the lung after 3 days of allergen challenge, irrespective of genotype (Fig 4, B). The CFSE mean fluorescence intensities of cells from wild-type and STAT6^{-/-} mice were identical (212 $\pm$ 4 vs 214 $\pm$ 2.5, respectively), discounting the possibility that the different host environments resulted in different rates of mitosis in the transferred cells, an effect that could potentially interfere with the enumeration of labeled cells.

We further quantitated bronchoalveolar lavage fluid eosinophils and lung IL-4- and IFN- γ -producing cells from allergen- and vehicle-challenged, T cell- and sham-reconstituted mice (Fig 4, C-E). Eosinophils were recruited to the airways to a similar degree in FAP-challenged wild-type and STAT6^{-/-} mice but in a manner that did not require exogenous T_H cells, only FAP (Fig 4, C). Recruitment to the lung of exogenous T_H cells

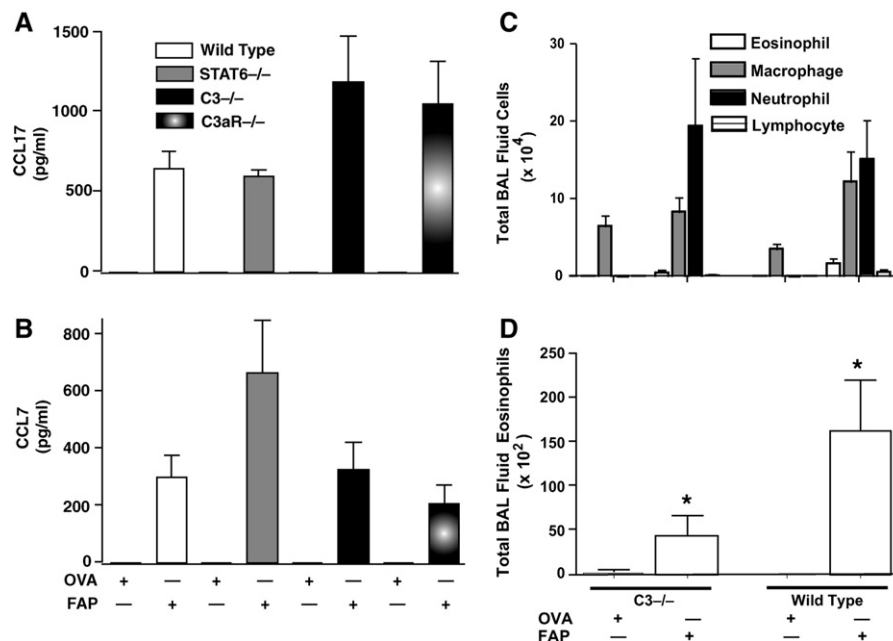


FIG 3. Allergic chemokine secretion without STAT6 and C3. Wild-type, STAT6^{-/-}, C3^{-/-}, and C3a anaphylatoxin receptor^{-/-} mice were challenged once intranasally with FAP or OVA, and CCL17 (**A**) and CCL7 (**B**) concentrations in bronchoalveolar lavage (BAL) were determined 18 hours later. BAL fluid was obtained from wild-type and C3^{-/-} mice challenged once intranasally with saline or FAP, and the total numbers of eosinophils, macrophages, neutrophils, and lymphocytes were determined (**C**). Eosinophil data are re-expressed in panel **D**. Vehicle (saline)-challenged animals showed induction of no chemokines or airway inflammation. Representative of 2 independent experiments (n = 3 or 4 mice per group).

corresponded with predominant IL-4 secretion from whole lungs of both wild-type and STAT6^{-/-} mice, indicating that the FAP induced equivalent and predominant recruitment of T_H2 over T_H1 cells, regardless of mouse genotype (**Fig 4, D and E**). Together, these findings demonstrate that a pathogen-specific factor, FAP, is sufficient to induce T_H2 cell recruitment to the lung and establish allergic lung inflammation in a manner entirely independent of STAT6.

DISCUSSION

We have explored the earliest immune events induced by a potent respiratory allergen to elucidate the fundamental cause of allergic lung diseases, such as asthma. Our studies confirm that allergic lung inflammation in response to a fungal allergen begins as an innate response before the development and recruitment of the T_H2 cells that control subsequent stages of disease. Furthermore, the innate allergic response to fungal allergen required intact proteinase activity, which transcriptionally controlled production of the chemokines necessary for recruitment of allergic inflammatory cells. The proteinase-driven signaling mechanism underlying innate allergic chemokine secretion was independent of the C3a receptor signaling pathway, which is generally required for allergic lung disease in response to this class of allergen,^{1,2} and MyD88-dependent signaling, which might participate in allergic reactions of the

lung under some conditions.^{7,22} Most remarkably, we have shown that the *A. oryzae* proteinase was sufficient to induce allergic chemokine secretion and recruitment of T_H2 cells and eosinophils to the lung independently of the STAT6 signaling pathway that was previously thought to be required for these responses.⁶ Our findings thus demonstrate how a unique pathogen-specific factor initiates allergic inflammation and links innate to adaptive allergic immune events. These findings have significant implications for the future management of allergic lung diseases, such as asthma.

These data are consistent with studies of helminth-challenged,⁹ but not OVA-challenged,⁸ mice, indicating that innate allergic inflammation is a physiologic, not aberrant, response to relevant allergens. Furthermore, our findings demonstrate that innate allergic inflammation induced by inhaled proteinase precedes the amplified allergic responses controlled by T_H2 cells that induce to airway obstruction.⁸ Our findings raise the possibility that innate allergic inflammation is required for T_H2 cell-driven allergic lung disease, an important concept that future studies will address in detail. Although OVA alone is capable of transiently eliciting strong lung allergic responses if peripheral immunization with this antigen precedes airway challenge, this is an implausible model for how allergen is acquired naturally, which is most likely through the inhalational route alone. Thus our findings are likely to be broadly applicable to diverse allergens implicated in respiratory diseases, such as asthma.

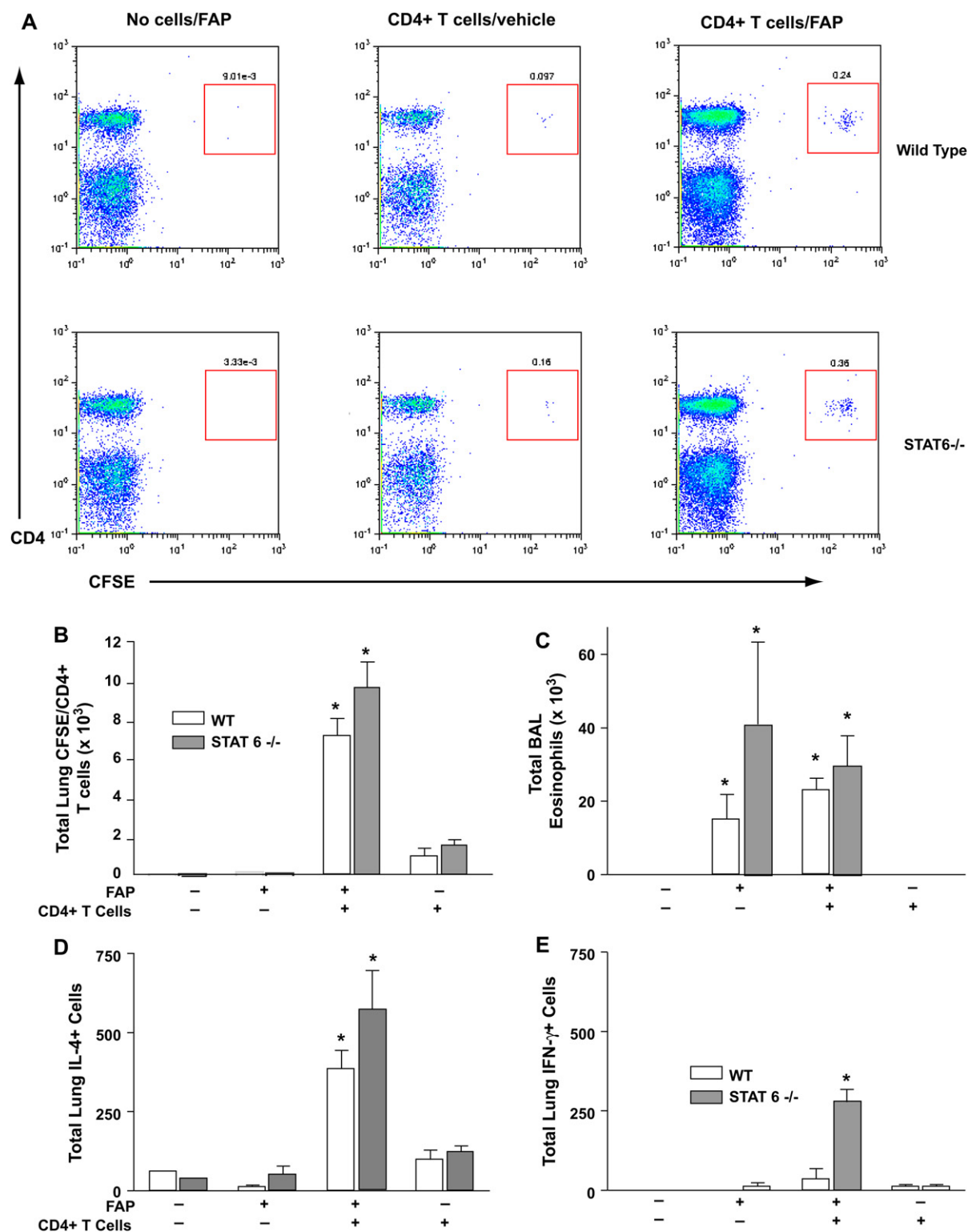


FIG 4. Allergic lung inflammation without STAT6. Wild-type and STAT6^{-/-} mice received CFSE-labeled T cells from FAP-sensitized wild-type mice or sham treatment and were challenged intranasally with FAP or vehicle daily for 3 days. **A**, Flow cytometric detection of labeled lung T cells from a representative mouse. Total lung CD4⁺/CFSE⁺ T cells (**B**), bronchoalveolar lavage eosinophils (**C**), and lung IL-4⁺ (**D**) and IFN- γ ⁺ (**E**) cells isolated from the indicated groups are shown. * $P < .05$ compared with mice receiving T cells and vehicle. Representative of 4 independent experiments ($n = 4-5$ mice per group).

Our study indicates that 2 mechanisms for the recruitment of allergic inflammatory cells exist: a proteinase-activated, STAT6-independent pathway that initiates allergic inflammation innately, as shown here, and a subsequent STAT6-dependent pathway that serves to sustain the T_H2 -driven amplification of allergic disease.⁶ We propose that under physiologic conditions, allergic lung inflammation is the result of both signaling pathways operating in parallel, whereas only the STAT6 pathway controls allergic disease induced in the absence of adjuvant (ie, with OVA alone used as the allergen). T_H2 differentiation, allergic lung inflammation, and airway hyperresponsiveness all fail to develop in response to FAP in the absence of STAT6 (D. Corry and S. Susarla, manuscript in preparation), revealing that the requirement of STAT6 for allergic lung responses in wild-type mice, as established through OVA, cannot be escaped, regardless of the allergen used. Thus FAP and OVA differ primarily with respect to the innate immune pathways that they activate and that control the initial recruitment of allergic effector cells that might be critical for subsequent allergic inflammation.

Unexpectedly, TLR ligands, such as endotoxin, are also capable of inducing allergic airway responses in mice under some conditions,⁷ suggesting that endotoxin contaminating our fungal allergens could be responsible for the allergic inflammation that we have observed. However, proteinase inactivation of the FAP, which abrogates its allergenic activity,⁸ should not affect TLR4 (endotoxin) signaling. Nonetheless, proteinase-inactive FAP failed to induce chemokines in the airway, especially CCL7 and CXCL10, that are strongly induced by TLR ligands alone (Fig 2) or allergic lung inflammation.⁸ Furthermore, our studies are similar to many others that show that TLR signaling predominantly controls IFN- γ -related responses and antagonizes allergic inflammation (Fig 2).²³⁻²⁵

Additionally, FAP not only induced allergic lung inflammation and chemokine secretion, these responses were actually enhanced in the absence of a functional signaling pathway for endotoxin and other TLR ligands (see Fig E2, A and B, in the Online Repository at www.jacionline.org).²¹ Thus although endotoxin is capable of inducing allergic lung inflammation under some conditions,^{7,26} our findings demonstrate that endotoxin contaminating FAP is not required for innate allergic responses induced by a fungal proteinase.

Despite these findings, it remains widely perceived that allergic lung inflammation is a default (ie, nonadjuvant directed) immune response to inhaled antigen.^{22,23} However, the typical respiratory immune response to antigens devoid of adjuvant factors is not allergic but tolerogenic, in which allergic lung inflammation and antigen-specific IgE and T_H2 responses are suppressed.^{8,27,28} We have shown here that to bypass the tolerogenic response to inhaled antigen and achieve robust T_H2 responses and allergic inflammation, the respiratory tract must be specifically instructed. Thus rather than a default response, allergic lung inflammation caused by inhaled allergen is the result

of microbial-derived adjuvant factors, especially proteinases, acting through an innate signaling mechanism that specifically induces recruitment of allergic effector cells.

Our findings indicate the existence of proteinase-activated innate immune receptors in the mouse airway that are crucial for the initiation of allergic inflammation. Proteinase-activated receptor 2 is an endogenously activated receptor implicated in the regulation of airway caliber,²⁹ but mice deficient in proteinase-activated receptor 2 experience airway inflammation similar to that of wild-type mice after airway challenge with proteinase-active allergens (D. Corry, unpublished data). Furthermore, although prior studies emphasized the cleavage or manipulation by Der p 1 of molecules involved in adaptive immunity (IL-2R α chain [CD25],³⁰ low-affinity IgE receptor [CD23],³¹ and IL-4 and IFN- γ ³²), the current study reveals that FAP acts within the innate immune compartment, in which such molecules are less likely to play critical roles. Therefore it is likely that other molecules sensitive to exogenous proteinases are responsible for initiating allergic inflammation, as shown here. Identification of these and related genes will be important to further dissecting the innate control of allergic inflammation and in defining new opportunities for the prevention and treatment of diseases, such as allergic asthma.

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METHODS

Affymetrix microarray analysis and quantitative real-time RT-PCR analysis

High-quality total lung RNA was isolated by using RNeasy Mini kit (Qiagen, Valencia, Calif). Total RNA was first reverse transcribed to first-strand cDNA. After second-strand cDNA synthesis and purification, biotinylated cRNA probes were generated by using *in vitro* transcription. The biotinylated cRNA was then cleaned and hybridized to the Affymetrix mouse Genome 430A 2.0 Array according to the manufacturer's protocol. The scanned images were aligned and analyzed by using the Affymetrix GeneChip software MAS 5.0 per the manufacturer's instruction. Signal intensity for each cDNA or expressed sequence tag entry was calculated as the difference of filtered Perfect Match probes minus Mismatch probes. The signal intensities were normalized to the mean intensity of all the genes represented on the array, and global scaling (scaling to all probe sets) was applied before comparison analysis.

For real-time RT-PCR analysis, oligonucleotide primers were selected by using Primer Express 2.0 (Applied Biosystems, Inc, Foster City, Calif) and synthesized by Invitrogen (Carlsbad, Calif). RNAs were first reverse transcribed into first-strand cDNA, and real-time quantitative PCR was performed with the ABI Prism 7900 (Applied Biosystems, Inc) sequence detection system by using Taqman reagents, according to the manufacturer's instructions. Equal amounts of each of the RNAs from the cells were used as

PCR templates in reactions to obtain the threshold cycle (C_t), and the C_t was then normalized by using the C_t from glyceraldehyde-3-phosphate dehydrogenase RNAs to obtain ΔC_t . To compare the relative levels of gene expression in different tissues/cells, $\Delta\Delta C_t$ values were calculated by using the ΔC_t values associated with the lowest expression levels as the bases. The $\Delta\Delta C_t$ values were then transformed to the real fold increase in expression by $2^{\Delta\Delta C_t}$.

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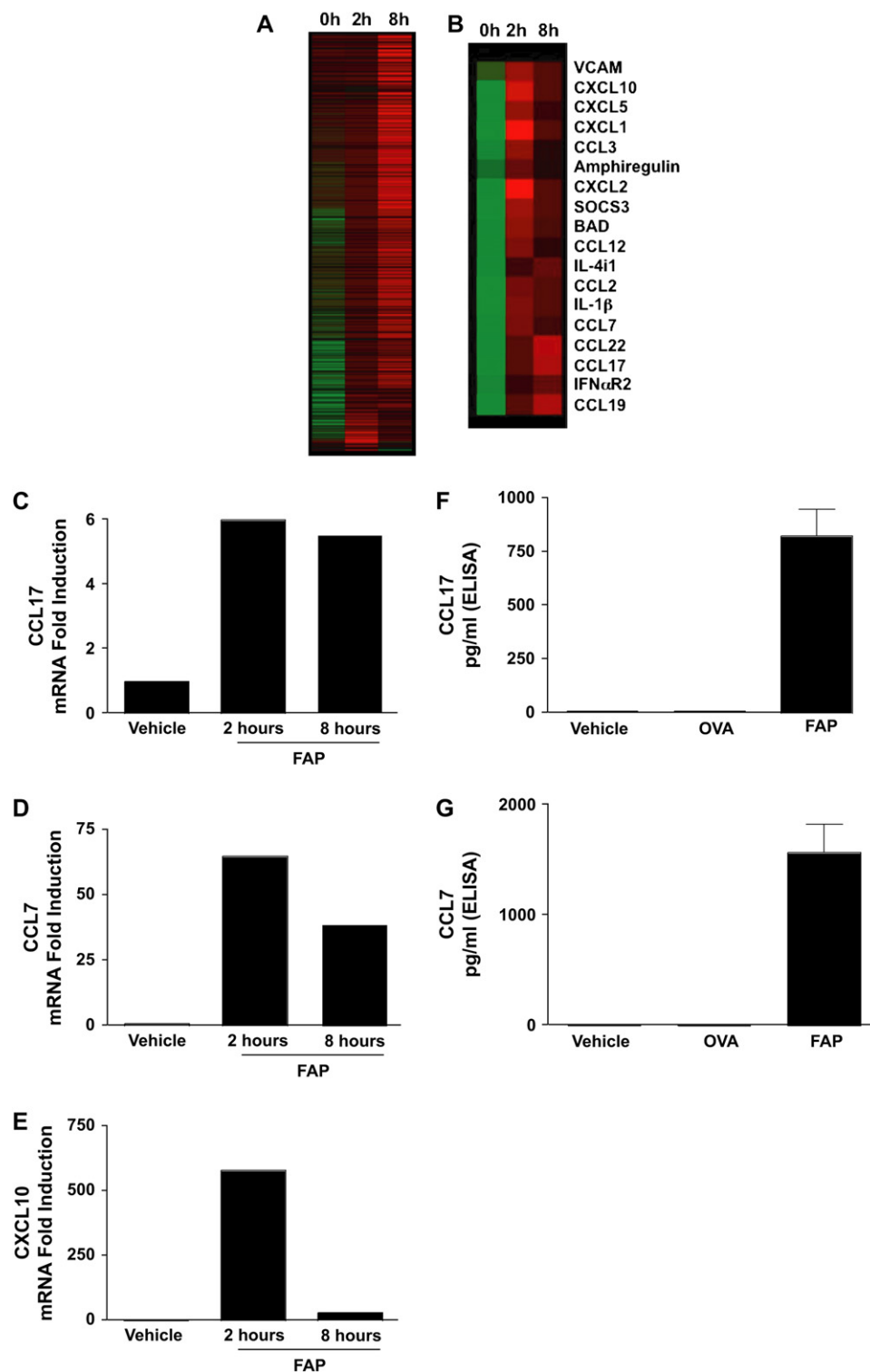


FIG E1. FAP induces allergy-associated chemokines and other genes. **A**, RNA extracted after 2 and 8 hours from RAG-1^{-/-} mice challenged once with FAP was hybridized to DNA microarrays and compared with lungs from vehicle-challenged animals (0 h). Only transcripts increasing by at least 2-fold in their expression (red color; total of 1475 genes) are shown. **B**, Expression of genes with known or putative function that were enhanced by allergen. Data are from mRNA pooled from 3 animals in each treatment group. **C-E**, RAG-1^{-/-} mice were challenged intranasally with vehicle or FAP as indicated. Whole-lung mRNA was collected after the indicated number of hours (2 hours later for vehicle-challenge animals) and subjected to quantitative real-time PCR analysis for the chemokines CCL17, CCL7, and CXCL10. Data are expressed as fold induction relative to vehicle-challenged animals and are from one of 2 independent analyses of mRNA pooled from 3 mice in each treatment group. **F** and **G**, Protein for CCL17 and CCL7 was measured by means of ELISA from the bronchoalveolar lavage fluid of OVA- and FAP-challenged animals 18 hours after a single challenge (CXCL10 was not detected for any treatment). Data are from one of 3 representative experiments with 4 mice in each treatment group. VCAM, Vascular cell adhesion molecule; SOCS3, suppressor of cytokine signaling 3; BAD, BCL2-antagonist of cell death; IL-4i1, IL-4-inducible gene of unknown function.

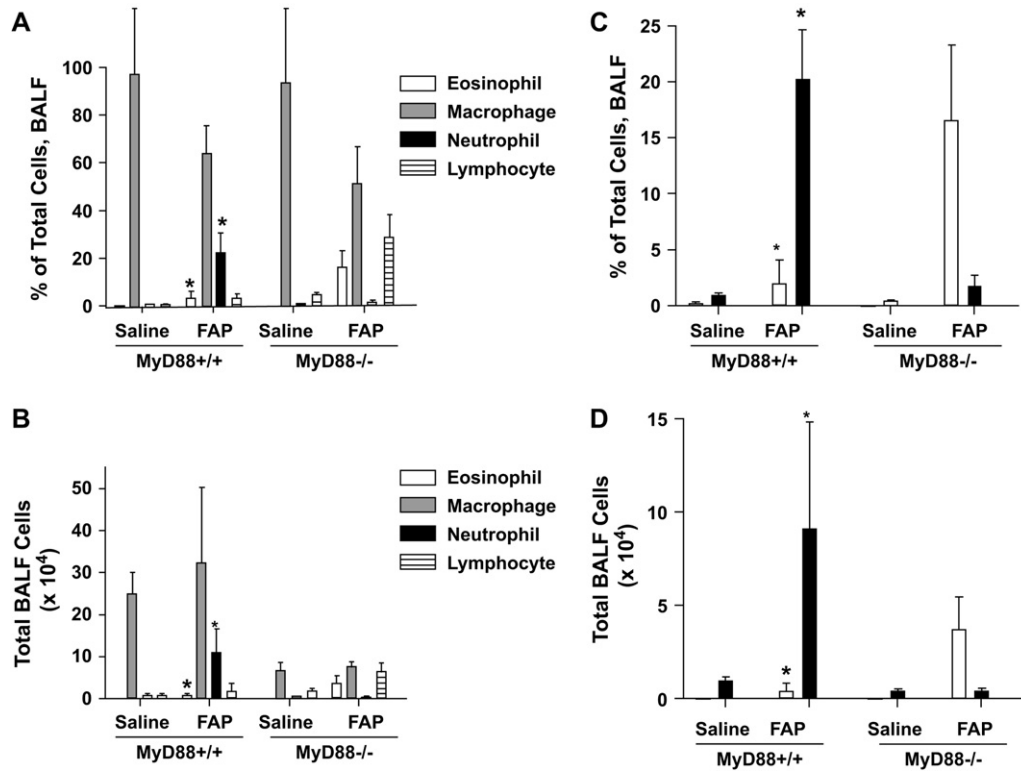


FIG E2. TLR signaling inhibits FAP-dependent innate allergic inflammation. **A-D**, Wild-type and otherwise genetically identical MyD88^{-/-} mice were challenged once intranasally with FAP, and the percentage abundance (Fig E2, **A**) and absolute number (Fig E2, **B**) of eosinophils, macrophages, neutrophils, and lymphocytes were determined 18 hours later. Fig E2, **C** and **D**, show eosinophil and neutrophil data excerpted from Fig 2, **A** and **B**. **P* < .05 compared with FAP-challenged Myd88^{-/-} mice. Data are from 5 mice in each group and are representative of 2 independent experiments. *BALF*, Bronchoalveolar lavage fluid.

TABLE E1. Selected gene microarray results based on lung mRNA extracted from RAG-1^{-/-} mice challenged with FAP or vehicle

Genes induced	Receptor or ligand	Function
CXCL1 (KC, Gro α)	CXCR1,2	Neutrophil chemokine
CXCL3 (Gro β)	CXCR1,2	Neutrophil chemokine
CXCL5 (ENA78)	CXCR2	Neutrophil chemokine
CCL3 (MIP-1 α)	CCR1,5	T _H 1 chemokine
CCL2 (MCP-1)	CCR2	T _H 1/T _H 2 chemokine
CCL7 (MCP-3)	CCR2	T _H 1/T _H 2 chemokine
MCP-5	CCR2	Chemokine for many cells
CCL17 (TARC)	CCR4	T _H 2 chemokine
CCL22 (MDC)	CCR4	T _H 2 chemokine
CCL19 (MIP-3 β)	CCR7	T-cell chemokine
CXCL10 (IP-10)	CXCR3	T _H 1 chemokine
Amphiregulin	EGF receptor	Wound (epithelial) healing and tumor suppressor ^{1,2}
IL-1 β	IL-1 β receptor	Growth factor favoring T _H 2 over T _H 1 cells ^{3,4}
IFN- α 2b	IFN- α receptor	Antiviral cytokine receptor
VCAM-1	CD29 integrins	Neutrophil and eosinophil homing
BAD		Regulation of apoptosis
SOCS3	IL-12 receptor	Suppressor of IL-12 signaling; promotes T _H 2 activity ⁵
IL4i1		IL-4-inducible protein of unknown function

MIP, macrophage inflammatory protein; *MCP*, monocyte chemoattractant protein; *TARC*, thymus and activation-regulated chemokine; *MDC*, macrophage derived chemokine; *IP*, interferon induced protein; *VCAM*, vascular cell adhesion molecule; *SOCS3*, suppressor of cytokine signaling 3.