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Properdin contributes to allergic airway inflammation through local C3a generation[1]

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Abstract

Complement is implicated in asthma pathogenesis but its mechanism of action in this disease remains incompletely understood. Here we studied the role of properdin (P), a positive alternative pathway complement regulator, in allergen-induced airway inflammation. Allergen challenge stimulated P release into the airways of asthmatic patients and P levels positively correlated with proinflammatory cytokines in human bronchoalveolar lavage (BAL). High levels of P were also detected in the BAL of OVA-sensitized and challenged but not naïve mice. Compared with wild-type mice, P-deficient ($P^{-/-}$) mice had markedly reduced total and eosinophil cell counts in BAL and significantly attenuated airway hyperresponsiveness to methacholine. Antibody blocking of P at both sensitization and challenge phases or at challenge phase alone, but not at sensitization phase alone, reduced airway inflammation. Conversely, intranasal reconstitution of P to $P^{-/-}$ mice at the challenge phase restored airway inflammation to wild-type levels. Notably, C3a levels in the BAL of OVA-challenged $P^{-/-}$ mice were significantly lower than in wild-type mice, and intranasal co-administration of an anti-C3a mAb with P to $P^{-/-}$ mice prevented restoration of airway inflammation. These results show that P plays a key role in allergen-induced airway inflammation and represents a potential therapeutic target for human asthma.

Keywords

Complement; properdin; asthma; airway inflammation

Introduction

Allergic asthma is a chronic inflammatory disease characterized by pulmonary eosinophilia, increased serum IgE levels, airway hyperreactivity, mucus hypersecretion and structural

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remodeling of airways (1). Current treatment for asthma such as systemic corticosteroids and inhaled beta agonists are far from optimal and there is a need to develop novel therapeutic approaches, particularly for those patients with severe asthma (2). Asthma is believed to be driven by inappropriate Th2-dominated immune responses to environmental allergens (3–6). In addition, the role of innate immunity in asthma has also attracted interest of investigators (7–10). Complement is a key component of the host innate immunity, and previous clinical and laboratory studies have suggested an important role of the complement system in this disease (11–20). A better understanding of the activation mechanisms and effectors of complement in asthma would aid the development of novel anti-complement therapies.

Properdin (P) is a plasma glycoprotein and the only known positive regulator of the complement cascade (21). It binds to and stabilizes the alternative pathway (AP) C3 convertase C3bBb (21, 22), and in some cases, may also serve as a platform to form new C3bBb convertases on the cell surface (23). P is best known as a promoter of AP complement activation in the context of host defense, and its deficiency in humans increases the risk of *Neisseria Meningitidis* infection (24–27). There is considerable evidence to suggest that P may also play a critical role in AP complement-mediated tissue injury, e.g. in the setting of ischemia reperfusion injury or inflammatory joint destruction (28, 29). On the other hand, P deficiency or inhibition in a murine model of fH-related C3 glomerulopathy exacerbated glomerular disease (30), suggesting that the role of P in AP complement-mediated diseases may be complex and potentially context-specific. Previous studies have found the AP complement and anaphylatoxin receptors to be involved in murine models of asthma, but whether and how P might play a role in this disease is not known. Here we tested the hypothesis that P contributes to the pathogenesis of allergen-induced airway inflammation and that targeting P dampens Th2 and Th17 immune responses. Our study provides proof of concept for therapeutic targeting of P in allergic asthma.

Materials and Methods

Human patient samples

All subjects gave their informed consent and the study was approved by the IRB of the Thomas Jefferson Medical College. De-identified BAL samples were obtained from study subjects as described before (31). Briefly, healthy subjects without asthma and subjects with allergic asthma and rhinitis were recruited for the study and screened to assess suitability. Screening consisted of medical history and physical examination, followed by skin testing for allergy to common common aeroallergens. All subjects were non-smokers and were not taking any chronic medications. Asthmatics met the National Institute of Health/National Heart, Lung, and Blood Institute expert panel criteria for the diagnosis of asthma, and the diagnosis was confirmed by spirometry and responsiveness to methacholine (32). In an effort to reduce variability only a single allergen was used, ragweed antigen E (Amb a I)), and patients were studied outside of ragweed season. The concentration of the lung delivered dose of ragweed antigen was determined by serial intradermal skin testing, and was 100-fold higher than that required to cause a minimum positive skin wheal, based on our established protocol (31). Briefly: on Day 1 the subject presented between the hours of

7:00 and 9:00 A.M. and underwent bronchoalveolar lavage (BAL) with 150 ml saline in 50-ml aliquots in a lingular segment. This was immediately followed by antigen instillation into a right middle lobe segmental bronchus. For safety reasons, a 10-fold test dose preceded instillation of the full challenge dose. Both test and challenge volumes were 5 ml. On Day 2 the challenged segment was lavaged in the same way as on day 1. For the present study, paired BAL samples from an individuals before and after allergen challenge were available from asthmatic patients only

Animals

WT C57BL/6 mice were obtained from the Jackson Laboratory; $P^{-/-}$ mice with B6/129J mixed background were generated by gene targeting as previously described (33). Homozygous $P^{-/-}$ mice were screened from pups from heterozygotes breeding pairs; WT littermates from the same breeding pairs were used as controls. Mice were used at 6–8 weeks of age and housed in a specific pathogen-free facility. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Regents and antibodies

Chicken ovalbumin (OVA, Grade V) was obtained from Sigma-Aldrich; the adjuvant aluminum hydroxide (Alum imject) was purchased from Pierce; antibodies and protein standards for mouse IL-4 (capture: purified rat anti-mouse IL-4, clone 11B11, 554434; detection: biotinylated rat anti-mouse IL-4, clone BVD6-24G2, 554390; standard: recombinant mouse IL-4, 550067), IL-5 (capture: purified rat anti-mouse IL-5, clone TRFK5, 554393; detection: biotinylated rat anti mouse IL-5, clone TRFK4, 554397; standard: recombinant mouse IL-5, 554581), IL-17A (capture: purified rat anti-mouse IL-17A, clone TC11-18H10, 555068; detection: biotinylated rat anti mouse IL-17A, clone TC11-8H4, 555067), INF-γ (capture: purified rat anti-mouse INF-γ, clone R4-6A2, 551216; detection: biotinylated rat anti mouse INF-γ, clone XMG1.2, 554410; standard: recombinant mouse INF-γ, 554587), IgE (capture: purified rat anti-mouse IgE, clone R35-92, 553416; detection: biotinylated rat anti-mouse IgE, clone R35-72, 553414; standard: purified mouse IgE κ Isotype control, 557079), C5a (capture: purified rat anti-mouse C5a, clone I52-1486, 558027; detection: biotinylated-rat anti-mouse C5a, clone I52-278, 558028; standard: purified mouse C5a, 622597) and C3a (capture: purified rat anti-mouse C3a, clone I87-1162, 558250; detection: biotin- rat anti-mouse C3a, clone I87-419, 558251; standard: purified mouse C3a, 558618) ELISA detection and streptavidin HRP (554066) were purchased from BD Biosciences; recombinant mouse IL-17A protein purchased from eBioscience (14-8171-62) served as mouse IL-17A ELISA standard, mouse anti-human P and anti-mouse P mAbs and control mAbs, MOPC-31C were produced in house (28). A neutralizing rat anti-mouse C3a mAb, clone 3/11, was purchased from Hycult biotech. Human C3a ELISA Kit, 550499 was from BD Biosciences; human IL-4 (D4050) and IL-5 (D5000B) Quantikine ELISA kits were purchased from R&D Systems; human IL-13 ELISA Kit (EHIL13) was from Thermo Scientific.

Mouse allergic asthma

Murine allergic asthma model was developed as described before (34, 35), mice were sensitized with 10 μg OVA mixed with 2 mg alum by i.p. injection on day 0 and day 14.

From day 21, they were challenged daily for 5 consecutive days with 30 minutes exposure to aerosolized OVA (1% wt/vol) in a closed chamber. 24 hours after the last challenge, mice were sacrificed for analysis (Fig. 1C). Mice that were sensitized and challenged with vehicle (PBS) served as negative controls ('naïve' mice). To block endogenous P at both sensitization and challenge phases, a mouse anti-mouse P mAb (29) was administrated to WT mice (50 μg/g body weight/mouse, i.p.) on day −1, 6, 13 and 20 of the experimental protocol (Fig. 3A). To block P at sensitization phase alone, the anti-P mAb was administrated to WT mice on day −1, 6 and 13 (Fig. 4A). To block P at challenge phase alone, a single dose of the anti-P mAb was administrated to WT mice one day before OVA challenge on day 20 (Fig. 5A). To block P locally in the airways, 30 μg/mouse anti-P mAb in 20 μl PBS was intranasally instilled to WT mice immediately prior to each OVA challenge (Fig. 6A). For reconstitution of P in $P^{-/-}$ mice at the challenge phase, 10 μg/mouse recombinant P in 20 μl PBS was delivered intranasally to $P^{-/-}$ mice immediately before each OVA challenge (Fig. 7A). $P^{-/-}$ mice receiving recombinant P pre-mixed with the anti-P mAb were used as controls. To evaluate the role of C3a, a rat anti-mouse C3a mAb (5 μg/mouse) was included with recombinant P in some of the reconstitution experiments (Fig. 8C).

Collection of bronchoalveolar lavage (BAL) and cell counts

After serum collection, mice were sacrificed and lungs were lavaged as previously described (34). Briefly, the left lung was ligated, trachea was cannulated and the right lung was lavaged with 500 μl PBS 3 times. Cell counting was performed on cytospin preparations stained with May-Grünwald-Giemsa (Merck) and at least 200 cells were classified in blinded samples by an independent investigator using standard morphologic criteria. BAL was stored at −80 °C until analysis. Cytokines and C3a/C5a concentration in BAL was determined by ELISA using commercial antibodies and standards (BD Biosciences and eBioscience) according to the products instruction.

Measurement of P concentration in BAL and serum

Mouse P concentrations in BAL and serum were measured using sandwich ELISA. Plates were coated with the anti-P mAb clone 14E1 (28). BAL was diluted with PBS containing 1% BSA, and serum was diluted with PBS containing 1% BSA and 20 mM EDTA. Recombinant mouse P was used as a standard. Plate-bound P was detected by biotinylated anti-mouse P mAb clone 5A6 generated in house. To measure P in human BAL samples, two in-house generated mouse anti-human P mAbs, clone 8.1 and 24.2, were used as capture and detection antibodies, respectively. Human P purified from plasma was used as a standard to generate standard curves.

Preparation and culture of BAL and lung cells

Naïve, OVA sensitized and challenged, and OVA challenged but not pre-sensitized mice were sacrificed; lungs were lavaged with 1 ml PBS 3 times; Infiltrated cells in BAL were collected by centrifuge and counted. After BAL collection, whole lungs were then dissected, minced by razor blade, and enzymatically digested for 1hour at 37°C with a PBS containing 2 mg/ml collagenase A (Roche) and 40 U/ml of DNase I (Roche). Lung digests were then filtered (70-μm cell strainer; BD Falcon) to collect total lung cells, and the latter were

washed twice in PBS+ 2% fetal bovine serum. BAL and total lung cells were cultured with RPMI-1640 medium containing 10% fetal bovine serum for 2 days in the presence or absence of OVA (50 ng/ml) at a density of 10^6 cells/ml, and the supernatant was collected for mouse P detection using ELISA.

Lung Histopathology

Immediately after collection of lavage fluid, lungs were incised and treated with ice-cold 10% formalin before processing for histology. Paraffin sections were cut at 5 μm thickness, mounted on positively charged slides, and stained with hematoxylin and eosin (H&E) or periodic acid/Schiff reagent (PAS). PAS+ airways of lung was quantitated by counting both PAS+ and PAS- airways using light microscopy for a total of 3 lung sections per animal, and the percentages of PAS+ airways was calculated based on airways counted on all sections in a given experimental group.

Serum IgE detection

Serum was separated by centrifugation and stored at −80 °C until analysis. Total IgE levels were determined using ELISA. Purified rat anti-mouse IgE capture mAb (R35-72, BD Biosciences) was used to coat the plates. Purified mouse IgE (C38-2, BD Biosciences) served as a standard, and biotinylated rat anti-mouse IgE (R35-92, BD Biosciences) was used for IgE detection.

Mediastinal lymph node (MLN) cell culture and stimulation

After mice were sacrificed, MLN cells were isolated, prepared as single cell suspension and seeded on 96-well plates. Cells were re-stimulated with OVA (50 ng/ml) or vehicle (PBS) for 72 hours, and the supernatant was collected for cytokines detection using ELISA.

Determination of airway hyperresponsiveness (AHR)

24 hours after the last OVA challenge, mice were anesthetized and intubated for measurement of lung resistance (R_L) as described before (36). Briefly, mice were challenged with increasing concentrations of nebulized methacholine (0, 12.5, 25, 50 and 100 mg/ml) for 10 s (60 breaths per min, 0.5 ml of tidal volume) and were mechanically ventilated by 160 breaths per min, tidal volume of 0.15 ml, positive end-expiratory pressure of 2–4 cm H_2O. The peak value for R_L was measured before and after inhalation of PBS and after each concentration of methacholine, and cumulative concentration-response curves were constructed. Responses are presented as increases in R_L above baseline.

Assay of serum AP complement activity *in vitro*

Serum AP complement activity of anti-P mAb-treated mice was determined using an LPS-dependent ELISA assay as previously described (33). Briefly, 96-well plates were pre-coated with LPS. Serum samples were serially diluted with GVB buffer containing EGTA and Mg^{2+} and incubated on the plates for 1 hour at 37 °C. After the reaction was stopped by EDTA, a HRP conjugated goat anti-mouse C3 Ab (55557, MP Biomedicals) was used to detect surface-bound activated C3b.

Statistics

For experiments containing 2 groups, difference between the groups was calculated using unpaired t test. For data with nonparametric distributions, Mann-Witney test was used. For experiments containing 3 or more groups, ANOVA was used to determine the levels of difference and Bonferroni was used to perform the post-test of all pairs of data. P-values less than 0.05 were considered to be significant. Data are expressed as mean ± S.E.M.

Results

Properdin is released into the bronchoalveolar lavage (BAL) of allergen-challenged asthmatic patients and experimental mice

In both humans and mice, P is primarily produced and released by leukocytes (37–40). To evaluate if P might be secreted by inflammatory cells infiltrating the asthmatic airways where it promotes AP complement activation, we measured P levels in BAL samples of 7 individuals with allergic asthma and rhinitis collected before and 24 hours after an allergen challenge. As shown in Fig 1A, although the absolute level of P varied considerably between patients, it was consistently higher in BAL collected after allergen challenge in these individuals. Furthermore, P levels correlated significantly with that of IL-5, IL-4 and IL-13 in post allergen challenged BAL (Fig 1B), suggesting that P release into BAL is a significant marker of the allergic response in patients. To further study this phenomenon and experimentally test the role of P in asthma pathogenesis and therapy, we induced airway inflammation in mice by chicken ovalbumin (OVA) sensitization and airway challenge. Mice were immunized (day 0, 14) with OVA and alum, and challenged on 5 consecutive days (day 21–25) by exposure to aerosolized OVA followed by examination of airway inflammation and immune responses on day 26 (Fig 1C). Consistent with data from asthmatic patients, we found a dramatic 6-fold elevation of P level in the BAL of OVA sensitized and challenged mice compared with naïve mice (Fig 1C). As expected, no P was detected in BAL samples of either naïve or experimental P knockout ($P^{-/-}$) mice.

To investigate the mechanism of P secretion into BAL, we prepared BAL cells and total lung cells from naïve, OVA sensitized and challenged, and OVA challenged but not pre-sensitized mice and cultured them for 2 days with or without OVA stimulation. Examination of the cell culture medium revealed that high levels of P were secreted by BAL and total lung cells from mice sensitized and challenged with OVA, irrespective of whether the cells in culture were stimulated with OVA or not (Supplemental Fig 1A). Furthermore, the level of P and number of inflammatory cells in BALof mice sensitized and challenged with OVA were closely correlated and each was increased with successive OVA challenges (Supplemental Fig 1, B and C). These data suggested that P secretion into BAL was related to airway inflammation arising from allergen sensitization and challenge rather than from non-specific allergen challenge alone and that inflammatory cells in the lung were a major source of P detected in BAL.

Properdin deficiency or systemic inhibition in mice ameliorates allergen-induced airway inflammation and Th2 immune responses

To determine if P contributes to allergen-induced airway inflammation and immune responses, we subjected wild-type (WT) and $P^{-/-}$ mice to OVA sensitization and challenge and compared their phenotypes. We assessed airway inflammation and tissue damage by counting the number of inflammatory cells in BAL and by examining lung histology. As shown in Fig 2A and 2B, we detected significantly fewer numbers of total inflammatory cells, eosinophils and lymphocytes in $P^{-/-}$ mouse BAL, as well as less pulmonary inflammation and mucus secretion in the mutant mouse lungs by H&E and PAS staining, respectively. Notably, P deficiency did not significantly affect BAL macrophage and neutrophil numbers (Fig 2A) or serum IgE levels (Fig 2C). We also evaluated airway hyperresponsiveness (AHR) to methacholine challenge of the experimental mice, and found $P^{-/-}$ mice to demonstrate significantly reduced AHR compared with WT mice (Fig. 2D).

Since allergic asthma is a typical Th2-driven disease and recent studies have also implicated Th17 cells in the disease (41–43), we next investigated if P deficiency might affect OVA-specific T cell immune responses. We collected the mediastinal lymph nodes (MLN) from OVA sensitized and challenged mice, prepared single cell suspensions and then re-stimulated the cells with OVA in culture for 3 days. By ELISA measurement of cytokines in the cell culture medium, we found that re-stimulated $P^{-/-}$ mouse lymphocytes produced dramatically reduced amounts of IL-4, IL-5 and IL-17A but much higher IFN-γ (Fig 2E). Separately, we measured IL-4, IL-5, IL-17A and IFN-γ levels in the BAL but did not observe any differences between the two groups of mice (Fig 2F). Thus, attenuated airway inflammation and AHR in $P^{-/-}$ mice were accompanied by a suppressed Th2 and Th17 immune responses by lymph node cells but not associated with cytokine changes in the BAL.

To confirm the above data from $P^{-/-}$ mice, we blocked P function in WT mice with a mouse anti-mouse P mAb (α-P mAb). Based on previously determined pharmacodynamics of the mAb (28), we treated WT mice weekly with either the α-P or a control mAb (50 μg/g body weight), starting at 1 day (day −1) before OVA immunization (Fig 3A). The efficacy of the α-P mAb was confirmed by an LPS-dependent ELISA assay which showed a single injection to effectively suppress serum AP complement activity in WT mice for up to a week (Fig 3B). Thus, weekly α-P mAb treatment was expected to continuously block plasma P function in WT mice during the 26-day experimental period (Fig 3A). Furthermore, the level of immuno-reactive P in the BAL of α-P mAb-treated mice was also diminished (Fig 3C). Thus, both systemic (in plasma) and local (in lung) P function was blocked under this experimental protocol. We found that compared with control mAb-treated animals, WT mice treated with α-P mAb displayed less airway eosinophilia (Fig 3D) and less pulmonary inflammation and mucus secretion in the lung (Fig 3E); their MLN cells produced lower amounts of the Th2 cytokines IL-4 and IL-5 but higher amount of IFN-γ after OVA re-stimulation *in vitro* (Fig 3F). As in $P^{-/-}$ mice, we observed no difference in serum IgE (Fig 3G) or BAL cytokines levels (Fig 3H) in α-P mAb-treated mice compared with control mAb-treated mice. Thus, blocking P function during allergen sensitization and challenge phases recapitulated the phenotype observed in $P^{-/-}$ mice.

Systemic inhibition of properdin during the challenge but not the sensitization phase reduces allergen-induced airway inflammation, Th2 and Th17 immune responses

Previous studies of the C5a/C5aR pathway have shown that complement mediators may play different roles in the allergen sensitization and challenge phases of asthma pathogenesis (15, 44, 45). To determine the stage at which properdin contributed to OVA-induced airway inflammation, we blocked P function in WT mice with α-P mAb at different time points of the experimental protocol. To inhibit P function during OVA sensitization only, WT mice were treated three times at weekly intervals with α-P mAb (Fig 4A). Functional assay confirmed that serum P activity was blocked in the treated mice but it recovered by the time of first OVA challenge on day 21 and the level of immuno-reactive P in the BAL of OVA-challenged mice was high (data not shown). Interestingly, we found no difference in BAL inflammatory cell counts, serum IgE levels, pulmonary inflammation and mucus secretion, BAL cytokine levels, or IL-4, IL-5 and IL-17A production by re-stimulated MLN cells between α-P mAb-treated and control mAb-treated mice (Fig 4, B-F). On the other hand, IFN-γ production by re-stimulated MLNs was significantly increased in α-P mAb-treated mice (Fig 4E).

To address the role of P during the challenge phase of OVA-induced airway inflammation, we treated OVA-sensitized WT mice with α-P mAb one day before OVA challenge (Fig 5A). This single intraperitoneal α-P mAb injection reduced the level of immuno-reactive P in BAL to naïve mouse level (Fig 5B). Intriguingly, blocking P function prior to OVA challenge alone was sufficient to ameliorate airway inflammation and injury, and reduce IL-4, IL-5 and IL-17A production by re-stimulated MLN cells (Fig 5, C-E). It also significantly decreased serum IgE levels (Fig 5F), an unexpected outcome considering that no change in serum IgE levels was observed in either $P^{-/-}$ mice (Fig 2) or WT mice continuously treated with α-P mAb during disease induction (Fig 3). Blocking P function during OVA challenge did not significantly alter BAL cytokine levels (Fig 5G), nor did it affect IFN-γ production by re-stimulated MLN cells (Fig 5E). The latter result contrasted with the finding of increased IFN-γ production by re-stimulated MLN cells when P had been inhibited during the sensitization phase (Fig 4E).

Local inhibition of properdin during allergen challenge ameliorates airway inflammation

Since P was found to be released abundantly into BAL in OVA-challenged mice and blocking P systemically at the challenge phase reduced airway inflammation, we investigated if local inhibition of P in the airways of WT mice before OVA challenge might be effective in reducing lung inflammation. We sensitized WT mice by OVA immunization as usual, and before each daily OVA challenge administered α-P or a control mAb by intranasal instillation (Fig 6A). As shown in Fig 6B and 6C, intranasal delivery of the α-P mAb markedly diminished immuno-reactive P level in BAL but had no impact on serum P in the treated mice. Furthermore, it significantly reduced airway inflammation, AHR, and pulmonary inflammation and mucus production (Fig 6, D-F), as well as decreased IL-17A and increased IFN-γ production by re-stimulated MLN cells (Fig 6G). On the other hand, local inhibition of P by intranasal route had no effect on serum IgE (Fig 6H) and BAL cytokine levels (Fig 6I), nor did it significantly alter IL-4 and IL-5 production by re-stimulated MLN cells (Fig 6G). Thus, local inhibition of P in the airways of pre-sensitized

mice was sufficient and potentially therapeutic in alleviating lung inflammation and AHR upon allergen re-exposure.

Airway reconstitution of P in $P^{-/-}$ mice restores sensitivity to lung inflammation and injury

To confirm the pathogenic effect of P in the airways, we performed the reverse experiment, i.e. by reconstituting OVA-sensitized $P^{-/-}$ mice with recombinant P locally in the airways through intranasal instillation prior to each OVA challenge (Fig 7A). To ensure that any effect of the treatment was attributable to P reconstitution *per se* rather than introduction of potential impurities in the recombinant P preparation, we intranasally reconstituted a second group of $P^{-/-}$ mice with the same recombinant P which had been pre-mixed with α-P mAb. As shown in Fig 7B and 7C, we found that intranasal administration of P, but not P pre-treated with the α-P mAb, to $P^{-/-}$ mice before each OVA challenge restored airway inflammation and mucus secretion to a similar or more severe degree than that observed in WT mice. Likewise, IL-4, IL-5 and IL-17A production by re-stimulated MLN cells was significantly higher in $P^{-/-}$ mice reconstituted with P alone than in mice reconstituted with P mixed with α-P mAb (Fig 7D). Notably, airway reconstitution of P at the challenge phase did not alter IFN-γ production by re-stimulated MLN cells (Fig 7D) but reduced IFN-γ level in BAL (Fig 7E). Other BAL cytokines including IL-4, IL-5 and IL-17A showed no significant difference between the groups (Fig 7E). It is also interesting that although P deficiency did not affect serum IgE levels, intranasal instillation of P at the OVA challenge phase markedly elevated serum IgE (Fig 7F). By ELISA assay, we confirmed that intranasal P reconstitution led to detection of abundant P in BAL but not in sera of $P^{-/-}$ mice (Fig 7G). Thus, intranasally reconstituted P must have acted locally in the airways of the mutant mice to cause the observed phenotype changes.

Properdin-dependent C3a production contributes to airway inflammation at the allergen challenge phase

Consistent with previous studies showing a pathogenic role of the complement C3a/C3aR pathway in murine models of asthma (11, 13, 14, 46, 47), we observed a positive correlation between C3a levels and total inflammatory cell counts in WT mouse BAL (Fig 8A) and between BAL C3a and P levels in allergen-challenged human asthmatic patients (Supplemental Fig 2A) as well as between human C3a and Th2 cytokines in asthma patient's BAL (Supplemental Fig 2B). Furthermore, of several complement gene mutant mouse strains (C3aR, C3, C4, fD) tested in the current model, we found C3aR gene deletion to have the largest protective effect on airway inflammation (Supplemental Fig 3 and data not shown). Unlike C3a levels, C5a levels in BAL of WT asthmatic mice are not strongly correlated with infiltrated inflammatory cell counts (Supplemental Fig 4A). Although, compared with that of asthmatic WT mice, there was a trend of reduced C5a level in the BAL of asthmatic P deficient mice and this reduction in BAL C5a was reversed by intranasal reconstitution of P, the change in BAL C5a levels, in each case, was not statistically significant (Supplemental Fig 4B). Based on these observations, we hypothesized that as a positive regulator of the AP complement activation, P may have contributed to airway inflammation and injury by promoting local C3a production in the lung. Indeed, ELISA assay revealed a significant reduction in C3a levels in the BAL of experimental $P^{-/-}$ mice compared with WT mice (Fig. 8B). To further evaluate the

intermediacy of C3a in the mechanism of action of P in OVA-induced airway inflammation, we co-administered a control or anti-C3a mAb in the P reconstitution experiment of $P^{-/-}$ mice (Fig 8C). As shown in Fig 8, while intranasal reconstitution of P together with a control mAb restored sensitivity to airway inflammation and mucus secretion in $P^{-/-}$ mice (Fig 8, D and E), and elevated serum IgE levels (Fig 8F), concurrent intranasal administration of an anti-C3a mAb significantly blunted the effect of P reconstitution (Fig 8, D-F). Anti-C3a treatment completely inhibited the enhancing effect of P reconstitution on IL-17A production, and partially but significantly on IL-5 production, by OVA re-stimulated MLN cells but did not alter IL-4 or IFN-γ production by re-stimulated MLN cells (Fig 8G), nor did it have an effect on BAL cytokine levels (Fig 8H).Thus, P contributed to OVA-induced airway inflammation in the effector phase of lung disease by promoting complement activation and its effect, in several respects, was mediated by the C3a/C3aR pathway.

Discussion

We describe here that properdin, the only known positive regulator of complement activation, plays a significant pathogenic role in allergen-induced airway inflammation and immune responses. We showed that P is released into the BAL of human asthmatic patients in response to airway exposure of allergens and its level correlated with key Th2 cytokines. Similarly, we detected abundant P in the BAL of mice subjected to OVA-induced experimental asthma. These findings suggested that in pre-sensitized human individuals and mice P might have been released by inflammatory cells infiltrating the airway during allergen challenge, a hypothesis in line with current understanding of P biosynthesis by leukocytes in humans and mice (37–40). Indeed, in a separate experiment, we showed that BAL cells or total lung cells from OVA-immunized and -challenged mice, but not from naïve mice or mice challenged with OVA without prior sensitization, autonomously secreted P in culture independent of further OVA stimulation. Furthermore, kinetic analysis showed P level in BAL to be closely correlated with the number of inflammatory cells in BAL during consecutive days of OVA challenge. It is likely that inflammatory cells infiltrated into lung tissues were present in the total lung cell preparation and contributed to P secretion in culture but we cannot rule out the possibility that lung epithelial cells from asthmatic mice may also have produced and contributed to P secretion into BAL. By using $P^{-/-}$ mice and a function-blocking mouse anti-P mAb in WT mice, we further demonstrated that P deficiency protected mice from airway inflammation and attenuated Th2 and Th17 immune responses. These data showed that P plays an important role in promoting AP complement activation in asthma and blocking P may be an effective way to ameliorate the known pathogenic effect of complement in this disease.

Complement is a danger-sensing component of the innate immune system which is critical for host defense (48, 49). However, inappropriately regulated complement, particularly the alternative pathway, can cause severe inflammatory and allergic reactions and damage host tissues (50–53). Previous animal studies have shown that complement can regulate Th2 and Th17 immune responses, AHR and pulmonary inflammation and remodeling (11–18, 47, 54, 55). Some of the earlier studies focused on receptors of the complement anaphylatoxins C3a and C5a, and used either gene targeted mice or pharmacological reagents to block C3aR

and/or C5aR signaling (11–15, 46, 47, 54–56). These studies demonstrated that the C3a/C3aR pathway promoted allergic asthma both at allergen sensitization (47) and challenge phases (54, 55) and regulated IL-17 production (47, 54, 55). In contrast, the role of the C5a/C5aR pathway was found to be more complex, and blocking C5aR activity at the sensitization or challenge phase produced opposing results (15, 44, 57, 58). Other studies examined the roles of C3, fB, C5 and fH in asthma (16, 57, 59–62). While these works have collectively supported the importance of complement in allergic asthma, questions remain on the regulatory mechanisms of complement activation and on the most optimal and promising target for anti-complement therapy in asthma.

A major finding of the current study is that P played a pathogenic role in airway inflammation primarily at the effector phase of disease induction. Using a function-blocking anti-P mAb, we were able to selectively inhibit P activity at either the allergen sensitization or challenge phase or at both phases. Blocking P activity in WT mice at both phases recapitulated data obtained from $P^{-/-}$ mice, i.e. mice developed attenuated asthmatic lung injury. Interestingly, blocking P activity at the allergen sensitization phase alone had no effect on airway inflammation, Th2 and Th17 cytokine production by re-stimulated MLN cells, whereas blocking P activity at the allergen challenge phase only was sufficient to ameliorate lung inflammation and Th2, Th17 immune responses. The only effect that we were able to detect from blocking P activity at the allergen sensitization phase was augmented IFN-γ production by re-stimulated MLN cells. The fact that intranasally delivering anti-P mAb prior to OVA challenge had similar therapeutic efficacy to systemically administered anti-P mAb suggests that P plays a pathogenic role locally in the airways. This conclusion was further supported by local P reconstitution experiments and by diminished level of immuno-reactive P in the BAL of anti-P mAb-treated mice. We showed that intranasal delivery of recombinant P to $P^{-/-}$ mice prior to OVA challenge restored sensitivity of these mice to airway inflammation and increased Th2 and Th17 immune responses by re-stimulated MLN cells.

Mechanistically, locally released P in the airways appeared to have contributed to eosinophilia and lung injury primarily through increased C3a production. We detected a significant reduction in C3a levels in the BAL of $P^{-/-}$ mice compared with WT mice subjected to the same OVA-dependent airway disease induction, and there was a positive correlation between P and C3a levels in the BAL of allergen-challenged human asthmatic patients. Furthermore, blocking C3a function in the airways with a neutralizing mAb largely abrogated the pathogenic effect of intranasally reconstituted P protein in $P^{-/-}$ mice. While we cannot entirely rule out the relevance of the C5a/C5aR pathway in the mechanism of action of P in this model, P deficiency seemed to have less of an effect on local C5a production in the airways. We found C5a levels in the BAL of experimental $P^{-/-}$ mice to be moderately reduced compared with that of WT mice, and conversely intranasal administration of P to $P^{-/-}$ mice increased BAL C5a levels (Supplemental Fig 4). However, the differences between these groups did not reach statistical significance. It is possible that non-specific protease activities contributed more significantly to local C5a production in the airways whereas C3a generation was more dependent on P-facilitated AP activation.

Whether P acted as a trigger or facilitator of AP complement activation in the airways has not been determined in the current study. Recent studies have shown that surface-bound P can act as a platform to assemble new C3 convertases and initiate AP complement activation (23, 33, 63–66). Although direct binding of plasma P to host cells in the absence of deposited C3b has not been demonstrated, newly released P from activated neutrophils was found to be capable of binding to apoptotic cells and trigger AP complement activation (40, 67). Thus, it is possible that locally released P from initially migrated leukocytes may bind to airway epithelial cells and trigger AP complement activation, amplifying inflammatory lung injury. Alternatively, P may have primarily acted as a stabilizer of the AP C3 convertase formed via a tick-over mechanism on asthmatic airway surfaces where further AP amplification may subsequently occur due to loss or reduction of negative complement regulatory activities. Regardless, there is little doubt that AP complement is activated in asthmatic airways as has been demonstrated also by studies of $fB^{-/-}$ mice (59). The balance between positive and negative regulators of AP may critically determine how much complement-mediated injury ensues (30). For example, a previous report has demonstrated the protective role of fH as a negative regulator of AP complement in a murine model of asthma and provided proof of concept favoring therapeutically increasing fH activity in the treatment of asthma (62). Our studies here in turn have revealed the pathogenic role of P as a positive regulator of AP complement in asthma and demonstrated the therapeutic efficacy of blocking P in the effector phase of the disease.

The effect of blocking P activity before allergen challenge on Th2 and Th17 immune responses and on serum IgE levels is quite notable. Although we did not observe any effect on Th2 or IL-17A cytokine change in the BAL of experimental mice in association with P inhibition, IL-4, IL-5 and IL-17A production by OVA re-stimulated MLN cells was invariably affected when P activity was manipulated at the effector phase. It is possible that properdin-dependent local C3a production in the airways regulated the migration, proliferation, differentiation and survival of memory Th2 and Th17 cells in MLN but not in the airways. The dissociation between airway inflammation and Th2 and IL-17A levels in the BAL of $P^{-/-}$ mice suggested that these cytokines may not be the sole mediators responsible for pulmonary inflammation. Conversely, inflammatory infiltrates that showed an increase in the BAL of $P^{-/-}$ mice may not be the only source of Th2 and IL-17A cytokine production. Another prominent finding was that blocking P at the allergen challenge phase strikingly reduced total serum IgE levels. In contrast, we observed no serum IgE changes when P was blocked at the allergen sensitization phase and, intriguingly, when P was inhibited at both allergen sensitization and challenge phases, nor did we see a change in serum IgE in $P^{-/-}$ mice. Thus, therapeutically blocking P activity at the effector phase only may be more desirable than blocking at both phases in ameliorating the adverse allergic immune reactions. The link between serum IgE level and P activity in the airways during allergen exposure remains an observational finding for now and more studies are required to elucidate the underlying mechanisms. It did appear however that the regulating effect of P on serum IgE was mediated also by C3a since reconstitution of $P^{-/-}$ mice at the effector phase increased serum IgE as expected and co-administration of an anti-C3a mAb revered this effect.

In summary, we have shown in this study that properdin plays a pathogenic role in allergen-induced airway inflammation and abnormal Th2 and Th17 immune responses. By selectively inhibiting P activity with a function-blocking mAb at different phases of disease pathogenesis and by testing different routes of introduction of the mAb, we were able to determine that P contributed to lung injury locally during allergen challenge only. Furthermore, we demonstrated that P exerted its effect in allergen-induced airway disease primarily through the promotion of AP complement activation and C3a production. Collectively, our data suggest that therapeutic inhibition of P locally in the airways may protect susceptible asthmatic patients from allergen-induced lung inflammation and injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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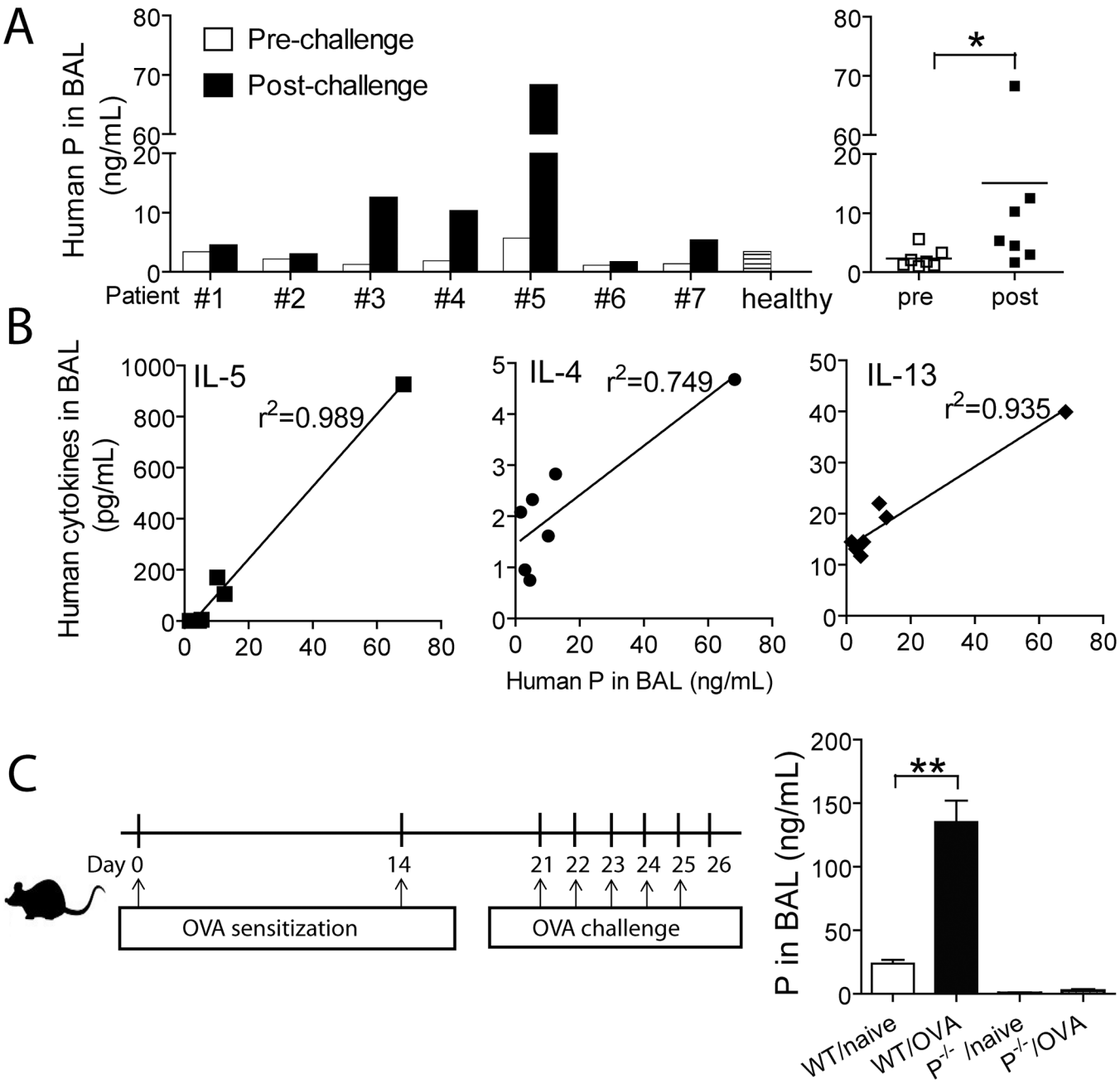

Fig. 1. Properdin is present in BAL of human asthmatic patients and OVA-sensitized and challenged mice

(A) P level in the BAL of human asthmatic patients was elevated after allergen challenge. P level in the BAL of an unchallenged healthy individual is shown by the hatched bar. (B) Levels of IL-4, IL-5 and IL-13 were positively correlated with P level in the BAL of asthmatic patients. (C) P level was markedly increased in the BAL of mice subjected to experimental asthma induction. Mice were immunized twice with OVA (sensitization) and then challenged daily with OVA for 5 consecutive days as indicated. They were sacrificed on day 26 for analysis. Compared with naïve mice (sham sensitization and challenge), mice subjected to OVA sensitization and challenge had markedly elevated levels of P in their

BAL. As expected, P was not detected in the BAL of $P^{-/-}$ mice. Values are expressed as mean ± SEM (n ≥ 5 mice in panel C). * $P<0.05$ and ** $P<0.01$.

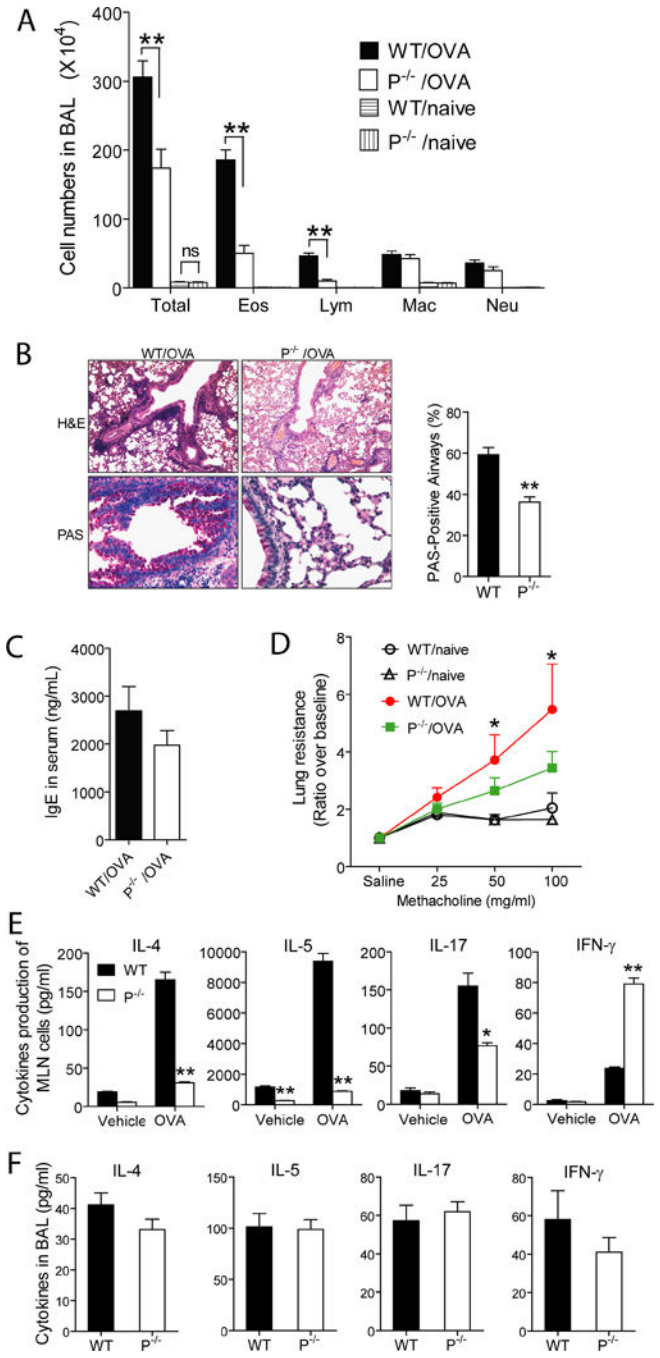

Fig. 2. Properdin deficiency protects mice from OVA-induced airway inflammation and alters Th2 and Th17 immune responses

(A) Differential cell counts in WT and $P^{-/-}$ mouse BAL show reduced total, eosinophil (Eos) and lymphocyte (Lym) cell counts but similar macrophage (Mac) and neutrophil (Neu) counts in $P^{-/-}$ mouse. (B) There was less pulmonary inflammation (upper panel) and mucus secretion (lower panel and right side plot showing quantification of PAS-positive airways) in $P^{-/-}$ mice than in WT mice. (C) Serum IgE level was not significantly different between WT and $P^{-/-}$ mice. (D) $P^{-/-}$ mice had lower AHR to methacholine compared with WT mice. (E) MLN cells were isolated and re-stimulated with OVA (50 ng/ml) or vehicle *in*

vitro for 3 days, and the supernatant was collected to determine cytokine (IL-4, IL-5, IL-17A and IFN-γ) levels by ELISA. Cells from $P^{-/-}$ mice produced significantly less IL-4, IL-5 and IL-17A but more IFN-γ. (F) BAL cytokine levels showed no difference between experimental WT and $P^{-/-}$ mice. Results shown are representative of 4 independent experiments. Values are expressed as mean ± SEM (n = 5 mice in naïve groups, n=17 mice in WT/OVA group and n=11 mice in $P^{-/-}$ /OVA group in panel A, C, D, E, F; n=4 mice in naïve groups and n=6 in OVA treated groups in panel B). * $P<0.05$ and ** $P<0.01$.

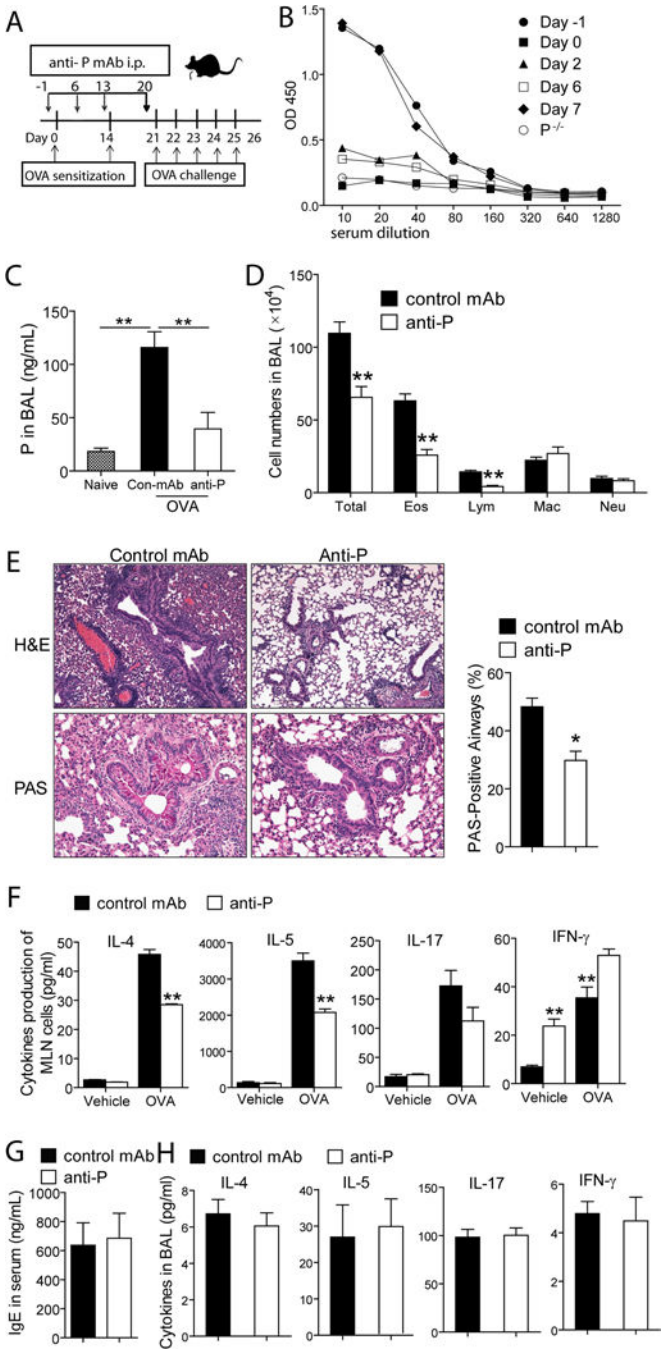

Fig. 3. Blockade of properdin in WT mice with mAb attenuates OVA-induced airway inflammation and alters Th2 immune responses

(A) WT mice were treated weekly at the indicated time points with an anti-P mAb to block P function during the whole experimental period. (B) ELISA based AP complement activity assay of sera from anti-P mAb-treated mice. A single dose of anti-P mAb blocked P activity for 7 days. (C) Level of immuno-reactive P in the BAL of anti-P mAb-treated mice was greatly reduced. (D) Differential cell counts in BAL of WT mice treated with anti-P or control mAb. Total, eosinophil (Eos) and lymphocyte (Lym) cell numbers were significantly less in anti-P mAb-treated mouse BAL than in control mAb-treated mouse BAL. Mac,

macrophages; Neu, neutrophils. (E) There was less pulmonary inflammation (upper panel) and mucus secretion (lower panel and right side plot showing quantification of PAS-positive airways) in anti-P mAb treated mice than in control mAb treated mice. (F) OVA (50 ng/ml) re-stimulated MLN cells from anti-P mAb-treated mice produced significantly less IL-4 and IL-5 but more IFN-γ than control mAb-treated mouse MLN cells. (G) There was no difference in serum IgE levels between control and anti-P mAb-treated mice. (H) Cytokines in BAL showed no difference between the two experimental groups. Results shown are representative of at least two independent experiemnts. Values are expressed as mean ± SEM (n = 7 mice in control mAb group and n=8 mice in anti-P mAb group). ** $P<0.01$.

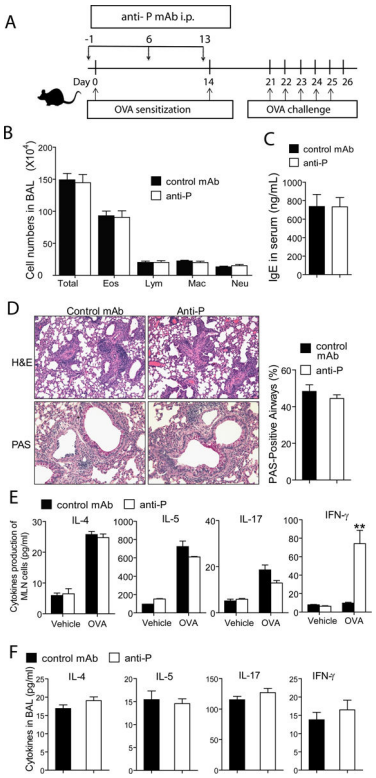

Fig. 4. Blocking properdin at OVA sensitization phase has no effect on airway inflammation, Th2 and Th17 immune responses

(A) WT mice received 3 weekly injections of anti-P mAb on indicated days. These treatments were expected to block P function during the OVA immunization phase but allow P activity to recover by day 21 when the first OVA challenge was performed. (B–F) There was no significant difference between control- and anti-P mAb-treated mice in differential cell counts in BAL (B), serum IgE levels (C), pulmonary inflammation and mucus secretion (D), IL-4, IL-5 and IL-17A production by OVA (50 ng/ml) re-stimulated MLN cells (E) or BAL cytokine levels (F). IFN-γ production by re-stimulated MLN cells was significantly increased in anti-P mAb-treated mice (E). Eos, eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils. Results shown are representative of at least two independent experiments. Values are expressed as mean ± SEM (n = 7 mice per group). ** P<0.01

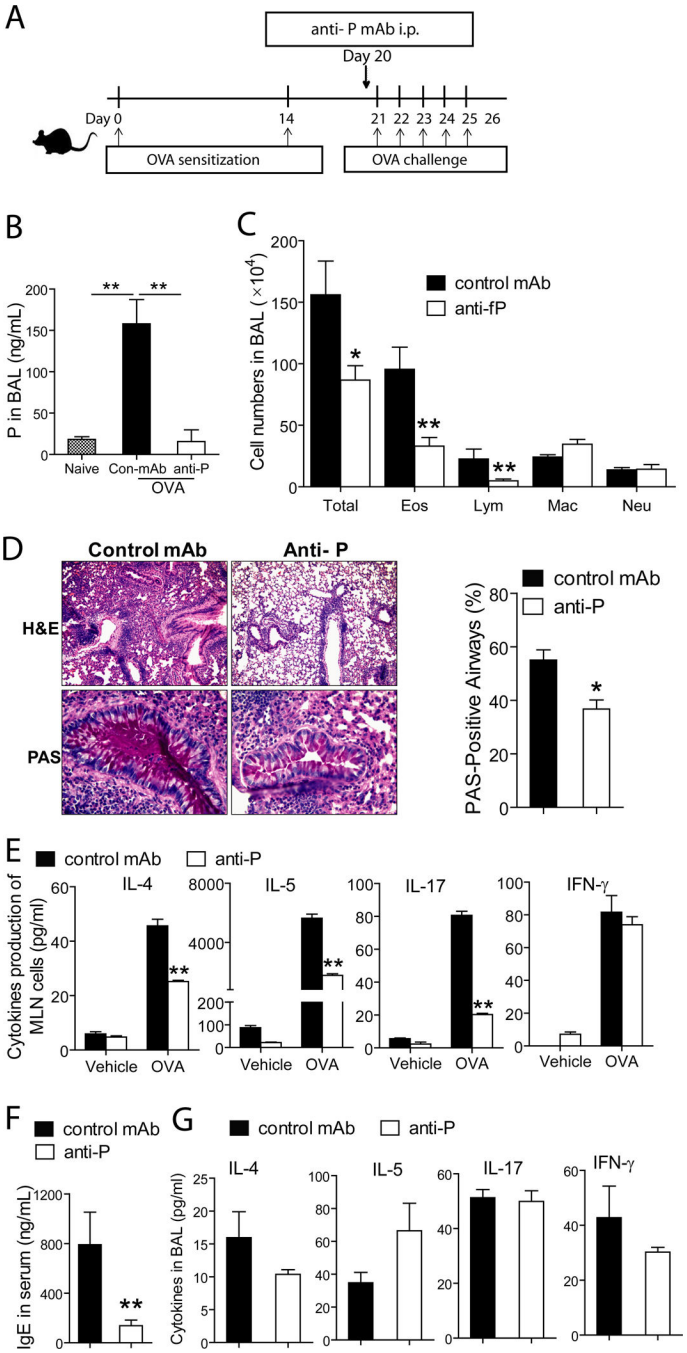

Fig. 5. Blocking properdin in WT mice at challenge phase alone reduces OVA-induced airway inflammation and alters Th2 and Th17 immune responses

(A) WT mice received a single injection of anti-P or a control mAb one day before OVA challenge as indicated. (B) Level of immuno-reactive P in the BAL of anti-P mAb-treated mice was reduced to naïve mouse level. (C) Differential cell counts in BAL show reduced numbers of total inflammatory cells, eosinophils (Eos) and lymphocytes (Lym) in anti-P mAb-treated mice than in control mAb-treated mice. Mac, macrophages; Neu, neutrophils. (D) Representative H&E and PAS staining shows reduced pulmonary inflammation (upper panel) and mucus secretion (lower panel and right side plot showing quantification of PAS-

positive airways) in anti-P mAb-treated mice. (E) MLN cells were isolated and re-stimulated with OVA (50 ng/ml) *in vitro* for 3 days, and the supernatant was collected to determine cytokine levels. IL-4, IL-5 and IL-17A but not IFN-γ production by anti-P mAb-treated mouse MLN cells was significantly decreased. (F) Serum IgE level was significantly reduced in anti-P mAb-treated mice than in control mAb-treated mice. (G) Cytokines in BAL showed no significant difference between different experimental groups. Results shown are representative of at least two independent experiments. Values are expressed as mean ± SEM (n = 5 mice per group). * $P<0.05$ and ** $P<0.01$.

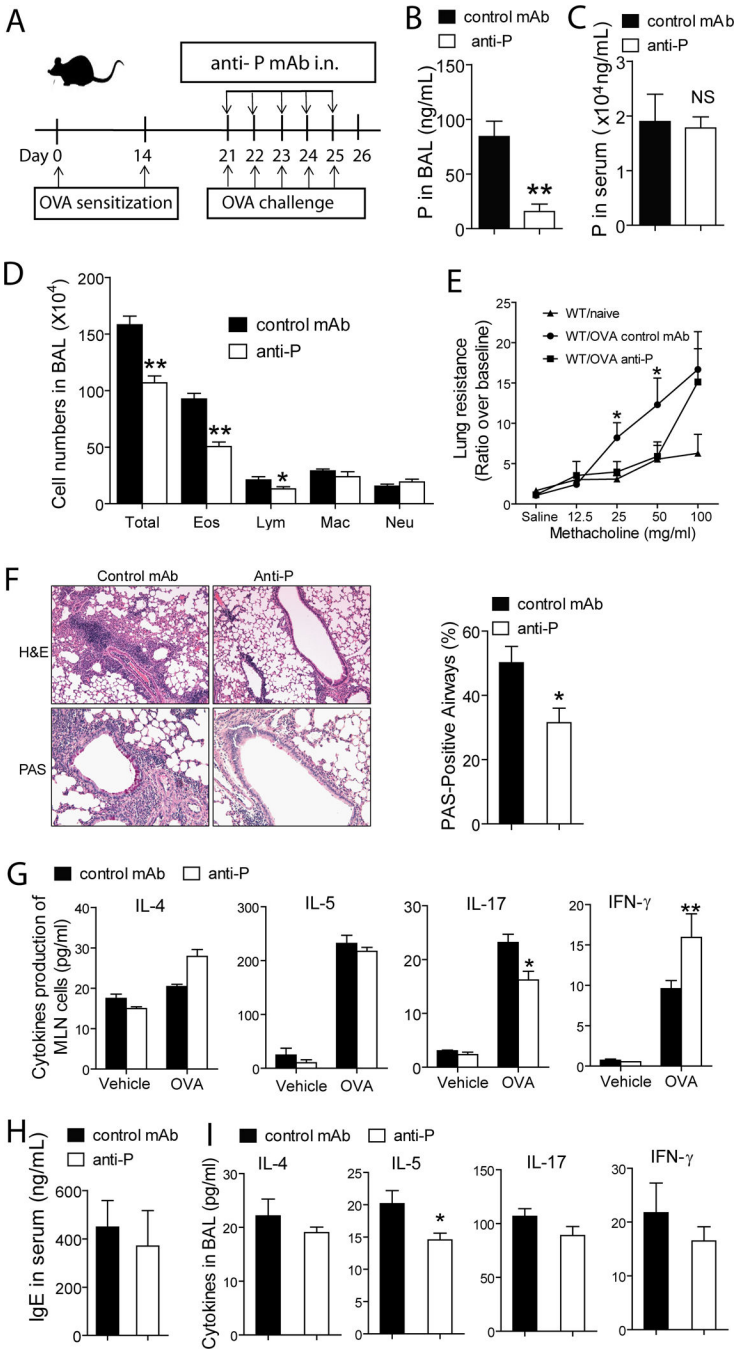

Fig. 6. Blocking properdin locally in the airways at challenge phase is sufficient to ameliorate lung inflammation

(A) WT mice were treated intranasally (i. n.) with anti-P or a control mAb prior to each OVA challenge as indicated. (B, C) Level of immune-reactive P in BAL but not serum was significantly lower in anti-P mAb-treated mice than in control mAb-treated mice. (D) Differential cell counts in BAL showing that mice treated with anti-P mAb had significantly less numbers of total inflammatory cells, eosinophiles (Eos) and lymphocytes (Lym) than control mAb-treated mice. Mac, macrophages; Neu, neutrophils. (E) Compared with mice treated with control mAb, mice treated with anti-P mAb had lower AHR in response to 25

and 50 mg/ml, but not 100 mg/ml, methacholine challenge. (F) There was less pulmonary inflammation (upper panel) and mucus secretion (lower panel and right side plot showing quantification of PAS-positive airways) in anti-P mAb-treated mice than in control mAb-treated mice. (G) MLN cells were isolated and re-stimulated with OVA (50 ng/ml) or vehicle in vitro for 3 days, and the supernatant was collected to determine cytokine (IL-4, IL-5, IL-17A and IFN-γ) levels by ELISA. Cells from anti-P mAb treated mice produced similar IL-4 and IL-5 but less IL-17A and more IFN-γ with OVA restimulation. (H) Serum IgE level was not significantly different between anti-P and control mAb- treated mice. (I) Except IL-5, there was no statistical difference in BAL cytokine levels between anti-P mAb-treated and control mAb-treated mice. Results shown are representative of at least two independent experiments. Values are expressed as mean ± SEM (n = 5 mice per group). * $P<0.05$ and ** $P<0.01$.

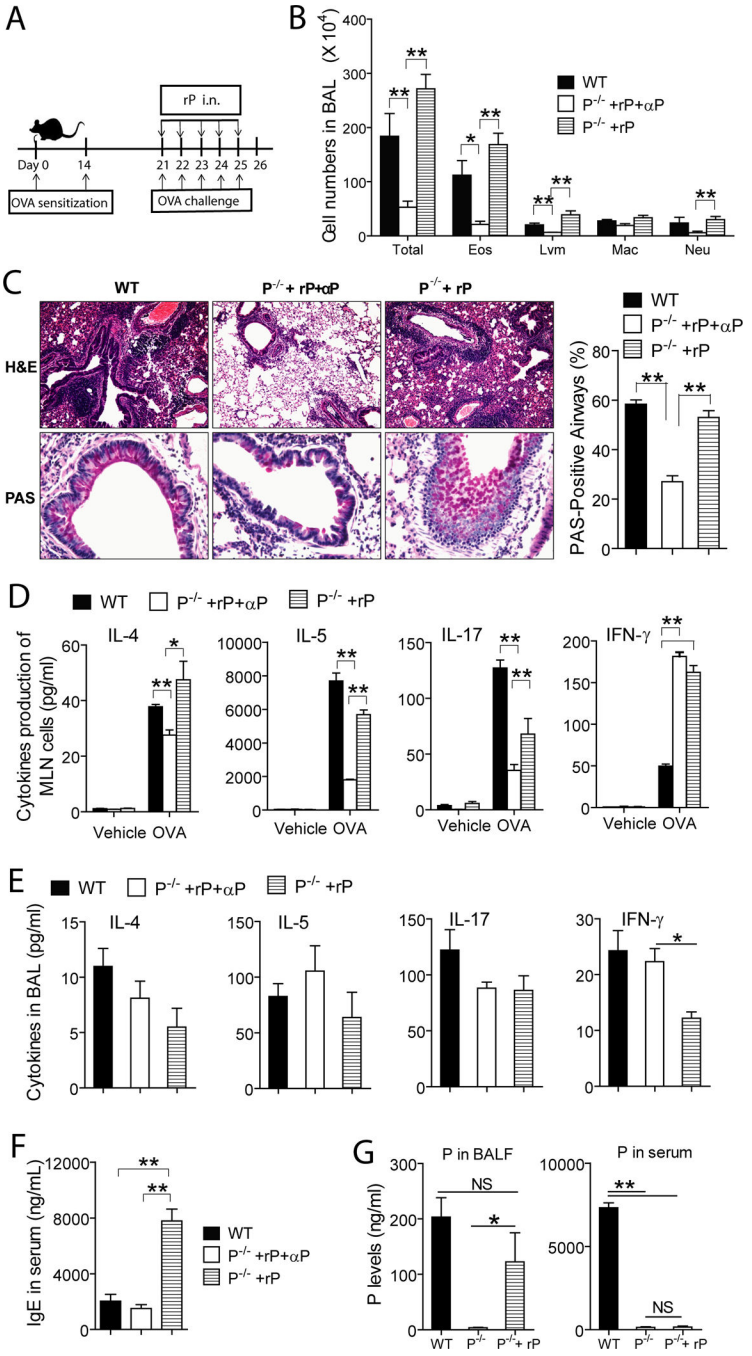

Fig. 7. Local reconstitution of properdin in the airways of $P^{-/-}$ mice restores their sensitivity to OVA-induced asthmatic lung inflammation and increases Th2 and Th17 immune responses

(A) $P^{-/-}$ mice were treated intranasally with recombinant P or recombinant P plus anti-P mAb (αP) prior to each OVA challenge as indicated. (B) Differential cell counts in BAL showing that $P^{-/-}$ mice reconstituted with recombinant P had much higher numbers of total inflammatory cells, eosinophils (Eos), lymphocytes (Lym) and neutrophils (Neu) than $P^{-/-}$ mice reconstituted with recombinant P plus anti-P mAb. The number of total cells, eosinophils and lymphocytes in P reconstituted $P^{-/-}$ mouse BAL were even higher than those of WT mice. Mac, macrophages. (C) Representative H&E and PAS staining showing

that pulmonary inflammation (upper panel) and mucus secretion (lower panel and right side plot showing quantification of PAS-positive airways) in P reconstituted $P^{-/-}$ mice were similar to WT mice, and both were more severe than $P^{-/-}$ reconstituted with P plus anti-P mAb. (D) MLN cells were isolated and re-stimulated with OVA (50 ng/ml) in vitro for 3 days, and the supernatant was collected to determine the cytokine levels. IL-4, IL-5 and IL-17A but not IFN-γ production in $P^{-/-}$ mice reconstituted with P only was significantly higher than that of $P^{-/-}$ mice reconstituted with P plus anti-P mAb. (E) Except IFN-γ, there was no statistical difference in BAL cytokine levels among the three experimental groups. (F) Serum IgE level was markedly higher in $P^{-/-}$ mice reconstituted with P than in either WT mice or $P^{-/-}$ mice reconstituted with P plus anti-P mAb. (G) Intranasal administration of recombinant P to $P^{-/-}$ mice before each OVA challenge led to a high level of P being detected in the BAL but not in serum. Results shown are representative of at least two independent experiments. Values are expressed as mean ± SEM (n = 5 mice per group). * $P<0.05$ and ** $P<0.01$.

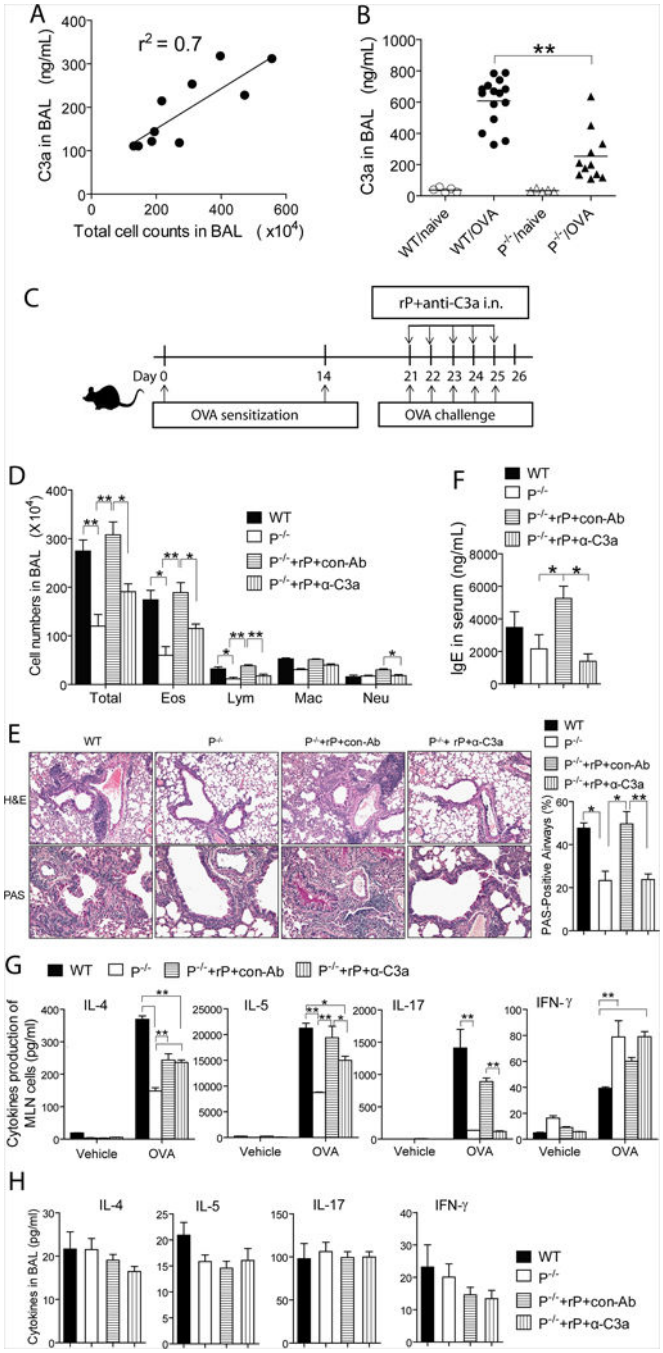

Fig. 8. Properdin plays a regulatory role at the effector phase of allergic asthma through the C3a/C3aR pathway

(A) C3a levels and total inflammatory cell counts in the BAL of experimental WT mice were positively correlated. (B) C3a levels in experimental $P^{-/-}$ mouse BAL were significantly lower than in experimental WT mouse BAL. (C) Experimental design to test the intermediacy of C3a in P activity in OVA-induced airway inflammation. $P^{-/-}$ mice were reconstituted intranasally with recombinant P pre-mixed with either a control or blocking anti-C3a mAb before each OVA challenge as indicated. (D) Differential cell counts in BAL showing that $P^{-/-}$ mice reconstituted with P plus anti-C3a mAb had significantly less

numbers of total inflammatory cells, eosinophils (Eos), lymphocytes (Lym) and neutrophils (Neu) than $P^{-/-}$ mice reconstituted with P plus control mAb. Mac, macrophages. (E) Representative H&E and PAS staining showing that pulmonary inflammation (upper panel) and mucus secretion (lower panel and right side plot showing quantification of PAS-positive airways) in $P^{-/-}$ mice reconstituted with P plus anti-C3a mAb were less severe than $P^{-/-}$ mice reconstituted with P plus control mAb. (F) $P^{-/-}$ mice reconstituted with P plus anti-C3a mAb also had significantly lower serum IgE levels than $P^{-/-}$ mice reconstituted with P plus control mAb. (G) IL-17A and IL-5 but not IL-4 or IFN-γ production by re-stimulated MLN cells was reduced in $P^{-/-}$ mice reconstituted with P plus anti-C3a mAb compared with the control mAb group. (H) BAL cytokine levels showed no difference between different experimental groups. Results shown are representative of at least two independent experiments. Values are expressed as mean ± SEM (n = 4 mice per group). * $P<0.05$ and ** $P<0.01$.