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Complement-mediated regulation of the interleukin 17A axis is a central genetic determinant of the severity of experimental allergic asthma

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Abstract

Severe asthma is associated with interleukin 17A (IL-17A) production. The exact role of IL-17A in severe asthma and the factors driving its production are unknown. Here we have demonstrated that IL-17A mediated severe airway hyperresponsiveness (AHR) in susceptible strains of mice by enhancing IL-13-driven responses. Mechanistically, we have demonstrated that IL-17A and AHR were regulated by allergen-driven production of anaphylatoxins, as complement factor 5 (C5) and C5aR-deficient strains mounted robust IL-17A responses, while C3aR-deficient mice had reduced T_H17 cells and AHR following allergen challenge. The opposing effects of C3a and C5a were mediated through their reciprocal regulation of IL-23 production. These data demonstrate a critical role for complement-mediated regulation of the IL-23–T_H17 axis in severe asthma.

Keywords

airway hyperresponsiveness; asthma; IL-17A; anaphylatoxins; IL-23

Introduction

Recent decades have seen substantial increases in the prevalence and severity of asthma, a complex inflammatory disorder of the airways. Although the unifying pathophysiological feature of asthma is airway hyperresponsiveness (AHR) leading to dynamic airflow obstruction, the clinical expression of disease is heterogeneous. For instance, a large

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Author contribution

S.L. and I.P.L. initiated, designed and performed experiments, analyzed data and wrote the manuscript; K.D., A.S., J.C., and Y.S. conducted experiments; A.B. provided critical materials; and M.W-K. directed the research, designed experiments, and wrote the manuscript.

percentage of asthmatics are atopic, with elevated serum immunoglobulin E (IgE); others have no evidence of atopy. Moreover, whereas the airway granulocytic infiltrate of most asthmatics is dominated by eosinophils, neutrophils dominate in patients at the severe end of the disease spectrum. Current models suggest asthma arises as a result of the development of aberrant T_H2 immune responses to innocuous environmental antigens in susceptible individuals. However, the T_H2 paradigm alone does not seem to explain the full spectrum of asthma, as severe disease is not associated with an exclusive bias towards T_H2 cytokine production1–3. Thus, additional factors besides T_H2 cytokines are likely to be playing a role in disease pathogenesis, underscoring the need for a better understanding of the molecular and cellular mechanisms underlying the diverse clinical expression of asthma to improve disease management and develop effective therapeutics.

Given that individuals with severe asthma display not only severe AHR, but also robust neutrophilia and increased IL-17A production1–3, it has been postulated that aberrant IL-17A production may drive severe forms of the disease. However, the role of IL-17A in severe asthma and the factors driving the production of IL-17A in susceptible individuals are not well understood. Studies of the role of IL-17A in experimental mouse models of asthma have yielded variable results. Some studies suggest that IL-17A drives increased neutrophil recruitment and AHR4, while others report that IL-17A plays no role in ovalbumin (Ova)-induced AHR or airway inflammation5. Lastly, IL-17A has been reported to have both a deleterious and a protective role depending on whether IL-17A was present during sensitization or challenge, respectively6. The mechanisms underlying the variable results obtained in these studies are currently unknown.

To determine the factors driving aberrant IL-17A production and subsequent development of the severe asthma phenotype, we utilized a well-characterized mouse model of differential susceptibility to severe asthma. Our data suggests that strains of mice that develop severe AHR (A/J) produced both elevated IL-17A and T_H2 cytokines, while strains manifesting less severe AHR (C3H/HeJ) produced only T_H2 cytokines and little to no IL-17A. Blockade of IL-17A in susceptible mice decreased severity, while reconstitution of IL-17A in C3H/HeJ mice exacerbated AHR. Consistent with the widely acknowledged importance of T_H2 cytokines in driving the development of allergen-induced AHR, our data show that IL-17A alone was unable to induce AHR. However, the simultaneous production of IL-17A and T_H2 cytokines exacerbated T_H2-driven pathology, leading to more severe disease. Mechanistically, the production of IL-17A and development of robust AHR was reciprocally regulated by the anaphylatoxins with C5a signaling limiting the frequency of T_H17 cells and AHR, while C3a signaling enhanced T_H17 responses and AHR. The opposing actions of C3 and C5 on IL-17A were mediated via reciprocal regulation of dendritic cell (DC) IL-23 production. The identification of a link between complement activation and the IL-23–T_H17 axis may provide insights into the underlying pathogenesis of a plethora of chronic inflammatory diseases in which IL-17A plays an important pathogenic role.

Results

Severe AHR is associated with both IL-17A and IL-13

To determine if the development of more severe AHR in asthma-susceptible strains of mice (A/J) as compared to C3H/HeJ mice was associated with differences in lung IL-17A following house dust mite (HDM) exposure, we treated mice with HDM intratracheally i.t. on days 0 and 14, and on day 17 we measured AHR, and collected lungs for analysis of cytokine concentrations by ELISA and flow cytometry (Supplementary Fig. 1**a**). As previously observed, airway responses to the cholinergic agonist, acetylcholine, were higher in A/J mice than in C3H/HeJ mice (Fig. 1**a**). HDM-induced T_H2 cytokine concentrations were equivalent in the two strains of mice, while IL-17A concentrations were higher in susceptible A/J mice than in resistant mice (Fig. 1**b**). In both strains, $CD4^+$ cells (likely $CD4^+$ T cells or natural killer T, cells) represented the only relevant source of IL-17A (as detected by intracellular flow cytometry), and both the frequency of IL-$17A^+CD4^+$ cells (Fig. 1**c**, Supplementary Fig. 2) and the intensity of IL-17A staining (as measured by IL-17A MFI) (Fig. 1d, Supplementary Fig. 2) were increased after HDM. However, HDM challenge induced greater increases in the frequency of IL-$17A^+CD4^+$ cells, and the intensity of IL-17A staining in susceptible A/J mice as compared to C3H/HeJ mice (Fig. 1**c,d**). Similar strain differences were seen in the frequency of lung IL-$17F^+CD4^+$ cells (Fig. 1**e**, Supplementary Fig. 2). In contrast, the intensity of IL-17F staining (MFI) was similar between both strains following HDM exposure (Fig. 1**f**, Supplementary Fig. 2). As IL-21 and IL-22 have been preferentially associated with T_H17 cells, we also measured the frequency of IL-21^+ and IL-22^+CD4^+ T cells in the lungs after HDM challenge. The frequency of both IL-21^+CD4^+ and IL-22^+CD4^+ cells were increased following allergen challenge in both strains of mice (Supplementary Fig. 2,3). Similar to IL-$17A^+CD4^+$ T cells, the percentage of lung $CD4^+$ cells expressing IL-22 was higher in A/J versus C3H/HeJ, however the frequency of IL-21^+ cells did not differ in A/J and C3H/HeJ mice. Taken together, these data suggest that the susceptibility of A/J mice to allergen-induced AHR is strongly correlated with a greater frequency of lung T_H17 cells expressing IL-17A, IL-17F, and IL-22 as compared to the less susceptible C3H/HeJ strain.

IL-17A exacerbates allergen-induced AHR

To determine whether IL-17A played a functional role in susceptibility to allergen-driven AHR, we examined the effects of *in vivo* blockade of IL-17A by i.p. injection of HDM-treated A/J mice with either isotype control or an IL-17A antibodies (250 μg) in a protocol spanning sensitization and challenge periods (Supplementary Fig. 1**a**). As expected, HDM exposure induced robust AHR compared to PBS treatment (Fig. 2**a**). In comparison to mice receiving HDM plus the control antibody, those that received anti-IL-17A had significantly reduced AHR. However, IL-17A blockade did not completely abrogate HDM-induced AHR, suggesting that other factors, such as T_H2 cytokines, drive the remaining AHR. The decreased AHR in anti-mIL-17A treated mice was accompanied by significantly decreased bronchoalveolar lavage (BAL) neutrophilia, with no substantial changes in macrophage or eosinophil numbers (Fig. 2**b**). No reduction in HDM-induced increases in PAS^+ airway cells was observed following IL-17A blockade (Fig. 2**c**) or in total serum IgE titers (Supplementary Fig. 4). As reported elsewhere7, IL-17A blockade enhanced production of

the T_H2 cytokines in HDM-restimulated lung cells *in vitro* (Fig. 2**d–f**). Lung cells secreted elevated T_H2 cytokines in the media without the need for further HDM recall. A/J and C3H/HeJ produced vastly different amounts of IL-17A but were equivalent for T_H2 cytokines, we asked whether exogenous delivery of rIL-17A during allergen challenge would enhance the responsiveness of C3H/HeJ mice. Indeed, co-exposure to rIL-17A and HDM during the last allergen challenge (Supplementary Fig. 1**b**) rendered C3H/HeJ mice more susceptible to HDM-induced AHR (Fig. 2**g**). Collectively, these data suggest that IL-17A plays a pathogenic role in asthma, enhancing the severity of allergen-induced AHR.

IL-17A and IL-13 synergistically induce AHR

To define the individual contributions of IL-17A and IL-13 to the development of AHR, we examined the effects of airway administration of rIL-17A (5 μg), rIL-13 (5 μg), or the combination of both on AHR in A/J mice (Supplementary Fig. 1**c**). Similar to previous findings8, 9 we found that IL-13 induced increases in AHR, whereas IL-17A alone had no effect (Fig. 3**a**). Treatment with both IL-17A and IL-13 induced a significantly greater increase in AHR than that observed with IL-13 alone,suggesting that these two cytokines work synergistically to enhance AHR. As such, the production of both of these cytokines in the A/J mouse may explain their profound susceptibility to AHR.

To gain insight into the mechanisms underlying the synergy between IL-17A and IL-13, we evaluated the expression of the IL-13 receptor subunits in whole lung from mice following cytokine treatment (Fig. 3**b–d**). Neither IL-13 nor IL-17A altered *Il4ra* or *Il13ra1* expression. IL-13 significantly upregulated *Il13ra2* expression, while IL-17A had no effect. The presence of IL-17A synergistically enhanced IL-13-driven *Il13ra2* expression. Similarly, we observed a synergistic enhancement of the expression of several genes (*Tff2*, *Arg1* and *C3*) typically induced by IL-13 when IL-17A was also present (Fig. 3**e–g**). In contrast, IL-13 was inhibitory to the expression of the downstream IL-17A target gene, *Cebpb* (CCAAT/enhancer-binding protein beta) (Fig. 3**h**). Thus, the combination of IL-17A and IL-13 may lead to the enhancement of downstream IL-13 signals and suppression of IL-17A-driven genes.

A/J mice display greater IL-17A responsiveness

To determine whether the susceptibility of A/J mice may also be due to altered sensitivity to IL-17R stimulation, we compared IL-17A responsiveness in A/J and C3H/HeJ mice by treating lung cells from naïve A/J and C3H/HeJ mice with IL-17A and assessing phosphorylation of kinases Erk1 and Erk2, key IL-17A signaling intermediates10, 11. We observed Erk1/2 phosphorylation of $CD11b^+$ cells after IL-17A stimulation in both strains. A/J cells were more sensitive to IL-17A and showed greater amounts of phosphoErk1/2 as compared to cells from C3H/HeJ mice (Fig. 4**a**). In addition, compared to bone marrow-derived DCs (BMDCs) from C3H/HeJ mice, IL-17A induced greater costimulatory molecule and MHC class II expression on A/J BMDCs (Fig. 4**b**). To demonstrate that enhanced responsiveness was not limited to hematopoietic cells, we show that expression of *Cebpd* (CCAAT/enhancer-protein delta), *Cebpb*, and *Cxcl1*, as well as CXCL1 protein abundance, factors downstream of IL-17A, were greater in A/J lung fibroblasts treated with rIL-17A (Fig. 4**c–f**). To determine whether the increased responsiveness of A/J cells to

IL-17A was due to higher amounts of the IL-17A receptor (IL-17RA), we measured *Il17ra* mRNA abundance in BMDCs, and lung fibroblasts from both strains. We found that A/J cells expressed more *Il17ra* than C3H/HeJ cells (Fig. 4**g**). Collectively, our findings suggest that excessive IL-17A production in A/J mice is compounded by an enhanced sensitivity to IL-17A.

Link between genetic deficiencies in C5, IL-17A and AHR

To identify potential mechanisms responsible for differential IL-17A production in A/J and C3H/HeJ mice, we focused on known genetic differences between the two strains. It is known that C3H/HeJ mice possess a natural mutation in *Tlr4*, rendering them unresponsive to lipopolysaccharide (LPS)12. However, the failure of C3H/HeJ mice to develop either AHR, or an IL-17A response was not a result of defective LPS signaling, as their *Tlr4*-sufficient congenic strain C3H/HeN mice, displayed similarly less AHR and IL-17A production (Supplementary Fig. 5). This result is consistent with our previous report demonstrating that the *Tlr4* genotype did not co-segregate with airway responses in (A/JxC3H/HeJ)F1 × A/J backcross mice13.

A/J mice also have a two base pair deletion in the C5 gene that results in the production of a non-functional protein14 and C5 deficiency of A/J mice is strongly associated with allergen-driven AHR13. To examine the association between C5a and IL-17A more broadly, we measured C5a serum concentrations and IL-17A-producing T cells in the lungs of several commonly used mouse strains. We observed a linear inverse relationship between absolute amounts of serum C5a and the frequency of T_H17 cells in the lungs ($r^2 = 0.6915$, $P = 0.0105$) and IL-17A staining intensity ($r^2 = 0.8525$, $P = 0.0011$) after HDM challenge (Fig. 5**a, b**). In contrast, we observed no correlation between serum C5a concentrations and frequency of lung $CD4^+IL\text{-}13^+$ or $CD4^+Foxp3^+$ cells (data not shown).

To directly demonstrate that C5 deficiency plays a role in IL-17A regulation and development of severe AHR, we blocked the C5aR in the commonly used C5-sufficient BALB/c mice using C5aR mAb or isotype control mAbs 24 h prior to each HDM exposure. When C5aR was blocked, we observed a significant exacerbation of AHR (Fig. 5**c**) compared to that seen in isotype mAb-treated HDM-exposed mice and IL-17A blockade completely reversed the enhancement of AHR in anti-C5aR-treated mice. Further, we show that blockade of the C5aR pathway resulted in elevated numbers of lung T_H17 cells (Fig. 5**d**). Interestingly, unlike in the C5-deficient A/J mice, HDM challenge in C5-sufficient BALB/c mice induced few $CD4^+IL\text{-}17A^+$ cells (see Fig. 5**a,b,d**), and therefore IL-17A blockade alone had no impact on HDM-induced AHR in the absence of C5aR blockade (Fig. 5**c**). Confirming these findings, we show that the influx of T_H17 cells into the lungs after HDM treatment is greater in mice deficient in *C5ar* (BALB/c background) as compared to their wild-type controls (Supplementary Fig. 6**a**) and they produce more IL-17A per cell (Supplementary Fig. 6**b**) similar to the association of C5 deficiency and HDM-induced increases in pulmonary $CD4^+IL\text{-}17A^+$ cells observed in A/J mice. These results demonstrate that regardless of overall genetic background, C5a concentration is strongly associated with IL-17A production in the lung following allergen exposure, and with susceptibility to the development of allergen-induced AHR in mice.

To dissect the mechanism by which C5a regulates IL-17A production, we examined the production of DC cytokines (IL-6, IL-1β, IL-23) that are known to drive T_H17 differentiation and proliferation. We found that compared to BMDCs from wild-type BALB/c controls, those from *C5ar* deficient mice produced more HDM-driven IL-23 (Fig. 5**e**), with no significant differences in IL-6 and IL-1β concentrations (Fig. 5**f,g**). Similarly, we found that BMDCs from A/J mice produce more IL-23 than in C3H/HeJ mice, while only small differences were seen in IL-6 and IL-1β production (Fig. 5**h–j**). Taken together these results suggest that C5a signaling controls IL-17A by limiting IL-23 production.

C3 signaling promotes DC IL-23 production

It has recently been shown that C5a and C3a play reciprocal roles in the regulation of allergic inflammation15. Thus, to determine whether C3a had an impact on the IL-23–T_H17 axis, we determined whether C3a altered DC production of IL-23 by comparing the effects of HDM on IL-23 production in *C3ar* deficient mice and their BALB/c wild-type controls. The lack of C3aR signaling reduced HDM-induced IL-23 (Fig. 6**a**) and IL-6 (Fig. 6**b**) production. In contrast, HDM-induced IL-1β secretion was enhanced in *C3ar*-deficient cells (Fig. 6**c**). *C3ar* KO mice demonstrated decreased induction of AHR, fewer $CD4^+IL17A^+$ T cells in the lung and decreased IL-17A MFI compared to BALB/c wild-type mice (Fig 6**d–f**). We next examined the kinetics of complement expression following allergen exposure in A/J and C3H/HeJ mice. We found that HDM enhanced *C3* and *C3ar* mRNA in the lungs of both strains, but to a greater extent in C5-deficient A/J (Fig. 6**g,h**). Collectively, these results suggest that C3a mediates IL-23 production, IL-17A production and susceptibility to AHR, while C5a production suppresses DC production of IL-23 and IL-17A production limiting the magnitude of allergen-driven AHR.

Positive feedback regulation of C3–C3aR by IL-17A

As it was shown that IL-17A induces C3 expression in skin fibroblasts16, we determined whether IL-17A induced C3 production in A/J lung cells. We found that IL-17A induced C3 gene expression in mouse tracheal epithelial cells and that compared to cells from C3H/HeJ mice, the production of C3 was higher in A/J cells (Fig. 7**a**). To confirm a role for IL-17A regulation of the C3 cleavage product C3a *in vivo*, we measured BAL C3a concentrations from A/J mice treated with HDM following IL-17A blockade. We found that the C3a concentration was increased after allergen challenge, and was reduced by IL-17A blockade (Fig. 7**b**). In addition, *in vitro* treatment of BMDCs with IL-17A also induced C3a (Fig. 7**c**), and intratracheal administration of rIL-17A to A/J mice lead to elevated BAL C3a (Fig. 7**d**). Taken together these findings suggest that IL-17A induces its own production through a positive feedback mechanism that involves IL-17A production of C3, which in turn, drives greater DC IL-23 leading to further IL-17A production from T_H17 cells.

Complement regulation of IL-23 is IL-10 dependent

To delineate the mechanisms whereby C5a and C3a regulate IL-23 production, we explored the possibility that IL-10, was responsible for C5a-mediated inhibition of IL-23 production. HDM alone induced IL-10 production in BMDCs from BALB/c mice, addition of C5a enhanced IL-10 production, while C3a inhibited IL-10 secretion (Fig. 8**a**). Blocking IL-10 in

HDM-stimulated cultures enhanced IL-23 production (Fig. 8**b**), suggesting that IL-10 negatively regulates IL-23 production. Moreover, blockade of IL-10 in cultures stimulated with HDM plus C5a completely abrogated the ability of C5a to limit IL-23 production, thus suggesting that IL-10 is critical for C5a-mediated inhibition of IL-23 production.

To identify the intracellular signaling pathways through which HDM and C5a regulate the production of IL-23 production, we stimulated DCs with HDM, or HDM+C5a in the presence of inhibitors to a variety of signaling pathways. Treatment of DCs with the c-Jun N-terminal kinase (Jnk) inhibitor SP600125 did not impact HDM-induced IL-23 production, while inhibition of IKK2 with AS60286817 completely abrogated IL-23 production suggesting that NF-kB was absolutely required for allergen-induced IL-23 (Fig. 8**c**). In addition, like the previously identified inhibitory role for mTOR in regulating LPS-induced IL-23 production18, we find that inhibition of mTOR signaling with rapamycin enhanced HDM-induced IL-23 (Fig. 8**c**). In contrast, blockade of Jnk signaling completely abrogated the impact of C5a on IL-23 production, while inhibition of either NF-kB or mTOR had no effect (Fig. 8**c**) This finding suggests that Jnk is a critical pathway by which C5a regulates HDM-induced IL-23 production in DCs. Consistent with an important role for IL-10 in the ability of C5a to limit IL-23 production, blockade of Jnk decreased both C5a+HDM-induced and HDM-induced IL-10 production from DCs (Fig. 8**d**). while rapamycin decreased production of IL-10, further supporting the role of IL-10 in negative regulation of IL-23 (Fig. 8**d**). Additionally, stimulation of RAW 264.7 cells with rC5a for 30 min induced phosphorylation (Ser63) of c-Jun (Fig. 8**e**), suggesting that C5a can directly activate that pathway.

Jnk can activate several downstream molecules including c-Jun, a canonical component of AP-1. Thus, to test the importance of AP-1 in C5a-mediated inhibition of HDM-stimulated IL-23 production, we transfected RAW 264.7 macrophages with an AP-1 reporter construct and observed that while AP-1 activity was not affected by HDM alone, AP-1 activity was increased ~25% in the presence of C5a, and this effect was abolished by blockade of Jnk by SP600125 (Fig. 8**f**). To demonstrate a role for C5a-induced IL-10 in regulating AP-1, cells receiving HDM plus C5a in the presence of anti-IL-10 had reduced AP-1 activity compared to cells treated in the presence of a control IgG1 antibody, while adding rIL-10 to HDM-treated cells also upregulated AP-1 (Fig. 8**g**). To conclusively show that AP-1 activity decreases IL-23 expression, we transfected RAW 264.7 macrophages with an A/J *Il23a* promoter fragment fused to luciferase, and observed that HDM-induced *Il23a* promoter activity was substantially reduced by overexpression of the canonical AP-1 components c-Jun and c-Fos (Fig. 8**h**). To confirm that these AP-1 components were differentially expressed in C5-deficient A/J and C5-sufficient C3H/HeJ BMDCs, we looked at *Jun* and *Fos* message and found that *Jun* mRNA was more abundant in the C5-sufficient strain as compared to the C5-deficient A/Js (Fig. 8**i**). Taken together these data show the critical importance of C5a-induced IL-10 in regulating IL-23 output from dendritic cells and the central role of AP-1 in mediating this effect.

Discussion

Although asthma has long been thought to be a T_H2-driven disease, recent studies suggests that elevated pulmonary T_H17 cytokine expression1–3 is correlated with elevated airway hyperresponsiveness3 and disease severity in humans1, 2. Despite the association between T_H17 and severe asthma, very little is known about the regulation of T_H17 production in asthma or the mechanisms by which it drives severe disease. Consistent with the role of T_H17 in human disease, we found that susceptibility to severe AHR is associated with elevated production of T_H17-associated cytokines (IL-17A, IL-17F, IL-22), whereas mild AHR is associated with abundant T_H2 cytokine production, but little to no T_H17 cytokine production. We have shown that IL-17A drives the severe phenotype as blockade of IL-17A reduced AHR in susceptible mice, while reconstitution in resistant strains enhanced AHR. Although the mechanisms responsible for IL-17A-mediated exacerbation of AHR are unclear, the reduction of AHR in IL-17A blockade was associated with reduced BAL neutrophils. Although neutrophils likely play a role in driving asthma severity, it has been shown that although they contribute, they are not sufficient to drive AHR19. Our observation that administration of IL-17A alone cannot induce airway responses supports this concept. These findings are consistent with marked increases in airway neutrophilia in severe asthma patients and in those undergoing acute severe exacerbations20–22. Although it has been previously noted that T_H17 cells and T_H2 cells may be directly antagonistic23, 24, we observed that the dominant effect of IL-17A *in vivo* in promoting severe AHR occurs downstream of the T cell, through enhancement of IL-13 signaling19, 25, 26. Thus, our data demonstrate that excessive IL-17A production drives the severe asthma phenotype by enhancing T_H2-driven pathology.

As we have previously identified C5 as a susceptibility gene for asthma13, we explored the possibility that alterations in the C5 gene may underlie excessive T_H17 production in susceptible strains of mice. Here we have reported a previously unrecognized role for C5 in the regulation of T_H17 immune responses and severe asthma. We have demonstrated a direct link between C5aR signaling, IL-17A production, and severe AHR in that the enhancement of AHR severity observed in mice following C5aR blockade is completely reversed by coincident IL-17A blockade. Notably, the inverse association between C5 and T_H17 held up across a wide spectrum of mouse strains. These findings provide a mechanistic explanation for the long unexplained observation that C5 provided protection against the development of AHR13, 27, 28 and support the general tenet that the complement system plays an important immunoregulatory role at the interface of innate and acquired immunity.

Although several factors have been shown to be important in T_H17 differentiation, our data demonstrated that C5a regulates T_H17 cytokine production through specific regulation of IL-23 production, as it does not affect the production of other Th17-promoting cytokines in the context of allergen exposure (IL-6, IL-1β, TGF-β). The regulation of IL-23 by C5aR signaling occurs through Jnk-dependent induction of IL-10 production. IL-10, in turn, increases AP-1 activation (c-Jun, c-Fos) that directly limits *Il23a* promoter activity. C5a's ability to stimulate IL-10 production likely explains its ability to suppress IL-12p40, IL-12p7029, 30 and IL-23p19, and thus T_H1 and T_H17 responses, respectively and supports our general tenet that C5a regulates the maintenance of tolerance at the mucosal surface.

Consistent with our previous report showing that C5a and C3a reciprocally regulate several features of the adaptive immune response15, we demonstrate that in contrast to C5a, C3a promotes AHR, IL-23 production and T_H17 responses by suppressing IL-10 synthesis by DCs. Consistent with a pro-allergic role for C3a, several triggers of asthma (HDM, cigarette smoke, viruses, pollutants) both activate C331–35 and drive AHR in a C3-dependent manner in mouse models. These findings are relevant to human disease as elevated plasma concentrations of C3a have been found in individuals experiencing acute asthma exacerbations, more so, in patients, which require hospitalization than those that do not36. Moreover, polymorphisms in the *C3* and *C3ar* gene are positively associated with human asthma37, 38. In contrast, polymorphisms in the *C5* gene have been associated with protection from asthma in humans38. Collectively, these studies suggest that C5, normally present at the mucosal surface, plays a role in maintaining a tolerogenic environment through the promotion of IL-10 production. Following exposure to allergenic triggers, C3 is activated and shifts the balance towards the activation of the IL-23-T_H17 axis driving more severe AHR. Once produced, IL-17A alone or synergistically with IL-13, initiates an amplification loop by directly inducing more C3 production by pulmonary cells enhancing IL-23 production and thereby perpetuating the response.

We have ruled out a role for the traditional TLR pathway in regulation of IL-17A and AHR in our model, in that the *Tlr4*-deficient (C3H/HeJ) strain and its *Tlr4*-sufficient congenic (C3H/HeN) strain both develop less severe AHR and produce low amounts of IL-17A following HDM exposure. Although TLR pathways may play important roles in driving T_H2 responses and milder AHR responses as we39 and others40 have recently shown, it does not appear to be important in regulating the T_H17 response to allergen exposure.

In summary, we have demonstrated that the severe asthma phenotype is driven by dysregulated anaphylatoxin control of the IL-23-T_H17 axis leading to excessive IL-17A production. This aberrant T_H17 response occurs as a result of a shift from C5a-driven tolerance towards C3a-driven T_H17 responses at the airway surface. Disruption of this delicate balance may occur as a result of either genetic alterations in complement genes or activation of the C3a pathway by environmental triggers of asthma. As severe forms of asthma have proven difficult to treat with existing therapies, modulation of anaphylatoxins may hold promise for the treatment of this ever-increasing disease. Our findings have implications beyond the context of asthma, as in both mice and humans, the dysregulation of complement pathways have been linked to a plethora of chronic inflammatory human diseases in which IL-17A plays an important role41–44.

Methods

Mice

Male A/J, C3H/HeJ, C57BL/6J, BALB/cJ, DBA/1J, DBA/2J, AKR/J, FVB/NJ (Jackson Laboratories), C3H/HeN (Harlan Laboratories), *C3ar* KO (BALB/c) and *C5ar* KO (BALB/c) (gifts of C. Gerard, Harvard University) were housed in a specific pathogen-free facility. Procedures were approved by Cincinnati Children’s Hospital Medical Center IACUC.

Treatment protocols

Mice were treated with HDM (100 μg; Greer Laboratories) or PBS intratracheally (i.t.) on days 0 and 14 and sacrificed on day 17 (Supplementary Fig. 1**a**). Where indicated, A/J mice were treated intraperitoneally (i.p.) with 250 μg of rat anti-mouse IL-17A mAb (clone M210, Amgen) or IgG_{2a} (clone GL117, a gift of F. Finkelman, CCHMC) on days −2, 3, 6, 10, 13, and 16 (Supplementary Fig. 1**a**). Where indicated, C3H/HeJ mice were treated with HDM i.t. on days 0 and 14, and HDM or HDM + IL-17A (15 μg) on day 42, and sacrificed on day 45 (Supplementary Fig. 1**b**). A/J mice were treated i.t. with 5 μg of rIL-17A, rIL-13 or both on days 0, 3 and 6, and sacrificed on day 7 to measure AHR (Supplementary Fig. 1**c**). Alternatively, BALB/c mice were treated as described (Supplementary Fig. 1**a**), some mice received 120 μg rat anti-mouse IL-17A mAb or IgG_{2a} i.p. as described above, and 35 μg anti-C5aR (Clone 20/70, AbD Serotec) or IgG_{2b} (R&D Systems) i.t. on days −1 and 13.

Development of bone marrow derived-myeloid DCs

Bone marrow cells from A/J, C3H/HeJ, *C3ar* KO, *C5ar* KO, and BALB/c mice, were cultured in RPMI+10% FBS supplemented with GM-CSF (10 ng/ml, Peprotech) on days 0 and 3. BMDCs were harvested on day 6 and plated at $2.5 \times 10^5 – 5.0 \times 10^5$ cells/well in 96-well plates, and treated with HDM (100 μg/ml) alone or in the presence of 200 ng/ml of hC3a or mC5a (R&D Systems). Alternatively, serum-starved (0.1% FBS) BMDCs were treated with 10 μM AS602868 (gift from I.M. Adcock, Imperial College London), 5–10 μM SP600125 or 0.1 μM rapamycin (Calbiochem), 2̣ nḡ/ml rIL-10 (R&D Systems), 5–10 μg/ml IgG1 or anti-IL-10 (JES5-2A5, eBioscience).

Immunoblot

RAW 264.7 cells were treated with media or 200 ng/ml rC5a for 30 min. Protein lysates were immunoblotted for phospho c-Jun (Ser63) or total c-Jun (Cell Signal).

Transfection

5×10^4 RAW 264.7 cells were seeded per well of a 96-well dish and serum starved (0.1 % FBS) overnight. Cells were transfected with 0.1 μg per well of an AP-1.Luciferase (Stratagene) reporter vector using FuGENE HD (Roche). Some cells were transfected with a 1096 bp fragment of the A/J *Il23a* promoter region inserted into pGL3 (Promega) in combination with 50 ng of either empty pcDNA3.1 (Invitrogen), or human c-Jun cDNA (#SC118762, Origene), or mouse c-Fos cDNA (#MC203181, Origene). Cells were stimulated for 24 h (HDM used at 10 μg/ml) and lysed for luciferase assay (Promega).

Assessment of allergen induced AHR

To evaluate AHR, mice were anaesthetized, intubated and respirated at a rate of 120 breaths/minute with a constant tidal volume (0.2 ml) and paralyzed with decamethonium bromide (25 mg/kg) 72 h after final allergen challenge. After a stable baseline was achieved, acetylcholine (50 mg/kg) was injected into the inferior vena cava and dynamic airway pressure (cm $H_20 \times$ sec) was followed for 5 min. Serum and BALF were collected, processed and analyzed as previously described 45.

Lung cell isolation and in vitro culture

Lungs were excised, minced and placed in 6 ml of serum-free RPMI containing Liberase CI (0.5 mg/ml, Roche) and DNase I (0.5 mg/ml, Sigma) at 37 °C for 45 min. Lung cell cultures were performed as we previously described 45.

Lung fibroblast and tracheal epithelial cell culture

Cells from lung digests were cultured overnight, trypsinized (~5 min) and placed in a new T25 flask until confluent. Passage of cells 3–4 times removed macrophage or monocyte contaminants. Resulting fibroblasts (1×10^4 cells/cm^2) were plated in 24-well plates until ~90% confluence, then serum starved in DMEM (0.5% FBS) overnight. Mouse tracheal epithelial cells were isolated from tracheas digested in Ham's F12 + 1 mg/ml pronase (Roche) overnight at 4 °C. Cells were cultured on Primaria plates (Falcon) for 3 h to remove fibroblasts. Non-adherent cells were resuspended in DMEM with 10 μg/ml insulin, 5 μg/ml transferrin, 0.1 μg/ml cholera toxin, 25 ng/ml epidermal growth factor, 30 μg/ml bovine pituitary extract, 5 % FBS, and 0.01 μM retinoic acid. 7.5×10^4 cells were plated on transwell membranes (0.4 μm pores, Costar) coated with 0.1 mg/ml type-1 rat-tail collagen (BD Biosciences), and cultured under immersion. At confluence (resistance > 1000 ohms), cells were stimulated with media or 100 ng/ml mIL-17A (R&D Systems) for 24 h. Cells were lysed with TRIzol (Invitrogen) for RNA extraction.

Flow Cytometry

Cell stainings were performed at 4°C following incubation with FcBlock (mAb 2.4G2) for 30 min. 1×10^6 lung cells were stimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) for ~16h, then Brefeldin A and monensin (eBioscience) were added for 4 h. Cells were fixed, permeabilized and stained with anti-CD4-PE-Cy7 (RM4–5) and anti-IL-17A-Ax647 (eBio17b7) or anti-IL-17F-Ax488 (eBio18F10). Phosphoflow was performed as described elsewhere46, 47. Briefly, 1×10^6 lungs cells were cultured in 96-well plates and stimulated for 45 minutes with media or 100 ng/ml rmIL-17A (R&D Systems). Cells were fixed for 10 min with 1.6% paraformaldehyde, and permeabilized in 100 μl methanol for 30 min at 4 °C, or stored at −80 °C until staining. Cells were stained for 30 min with anti-CD11b-PE-Cy7 (M1/70) and anti-phosphoErk1/2-Ax647 (clone 20A, BD Biosciences).

Determination of Cytokine Concentration

BAL C3a, serum C5a were measured by ELISA (BD Biosciences). Cytokine concentrations were measured by ELISA [Pharmingen (IL-4, IL-5); R&D Systems (IL-10, IL-13, IL-17A, IL-23)].

Quantitative real-time RT-PCR

Gene expression was measured by real-time PCR. Primer pairs are described in Supplementary Table 1. Data were normalized to ribosomal protein *S14* expression.

Statistical Analysis

To determine differences between multiple groups, ANOVA, followed by the Tukey-Kramer test was used. For comparison between two groups, a Student's *t*-test was performed. Significance was assumed at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

AHR	airway hyperresponsiveness
HDM	house dust mite
DC	dendritic cells
APTI	airway pressure time index

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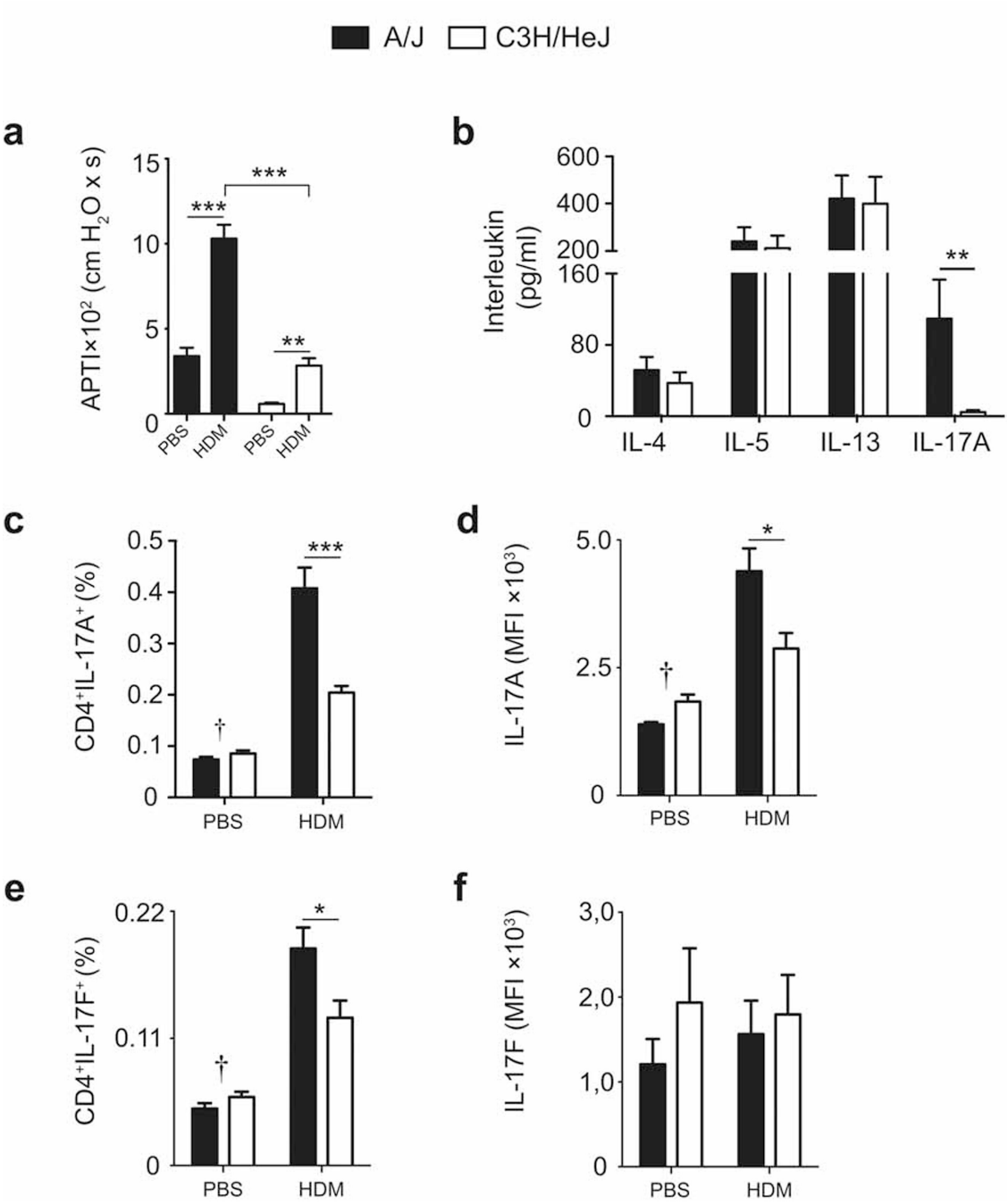

Figure 1. Susceptibility to allergen-driven AHR was associated a mixed T_H17-T_H2 immune response

Airway responsiveness (**a**) **P<0.01, ***P<0.001 (one-way ANOVA), cytokine production in HDM-restimulated lung cells (**b**), frequency of pulmonary $CD4^+IL\text{-}17A^+$ cells (**c**), IL-17A MFI (**d**), frequency of pulmonary $CD4^+IL\text{-}17F^+$ cells (**e**), and IL-17F MFI (**f**) were measured in HDM-challenged A/J and C3H/HeJ mice. *P<0.05, **P<0.01, ***P<0.001 comparing A/J vs. C3H/HeJ (one-way ANOVA), and †P<0.05, comparing PBS vs. HDM

(one-way ANOVA). Results are representative of three independent experiments (mean ± s.e.m. of 8–12 mice per group).

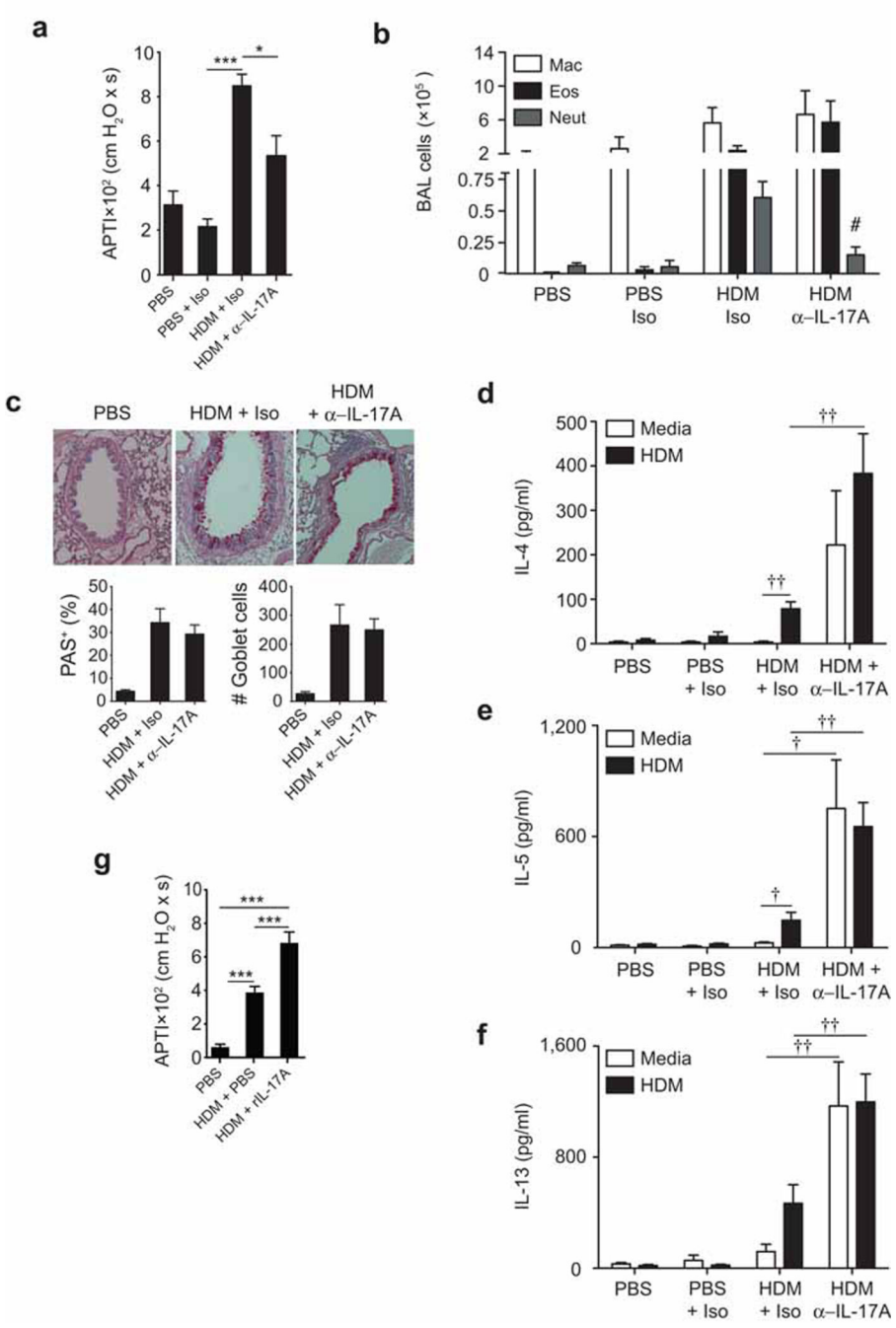

Figure 2. IL-17A blockade protects against allergen-induced AHR

A/J mice were treated with HDM or PBS and isotype or IL-17A blocking mAbs and airway function (**a**) (*P<0.05, ***P<0.001), BAL cellularity (**b**) (#P<0.05 versus HDM + Iso, one-way ANOVA) and mucus staining using periodic acid-Schiff (PAS) (**c**) were assessed. Supernatants from medium (open bars) and HDM-restimulated (closed bars) lung cell cultures were assayed for production of (**d**) IL-4, (**e**) IL-5, and (**f**) IL-13 by ELISA, (†P<0.05, ††P<0.01 [one-way ANOVA]). Airway reactivity was measured in C3H/HeJ mice treated with PBS, HDM or HDM + 15 μg rIL-17A, 3 days after the last allergen

challenge (**g**), ***P<0.001 (one-way ANOVA). Results are from two experiments [mean ± s.e.m. of 6–8 mice per group (**a–f**)] or representative of one experiment [mean ± s.e.m. of 4–8 mice per group (**g**)].

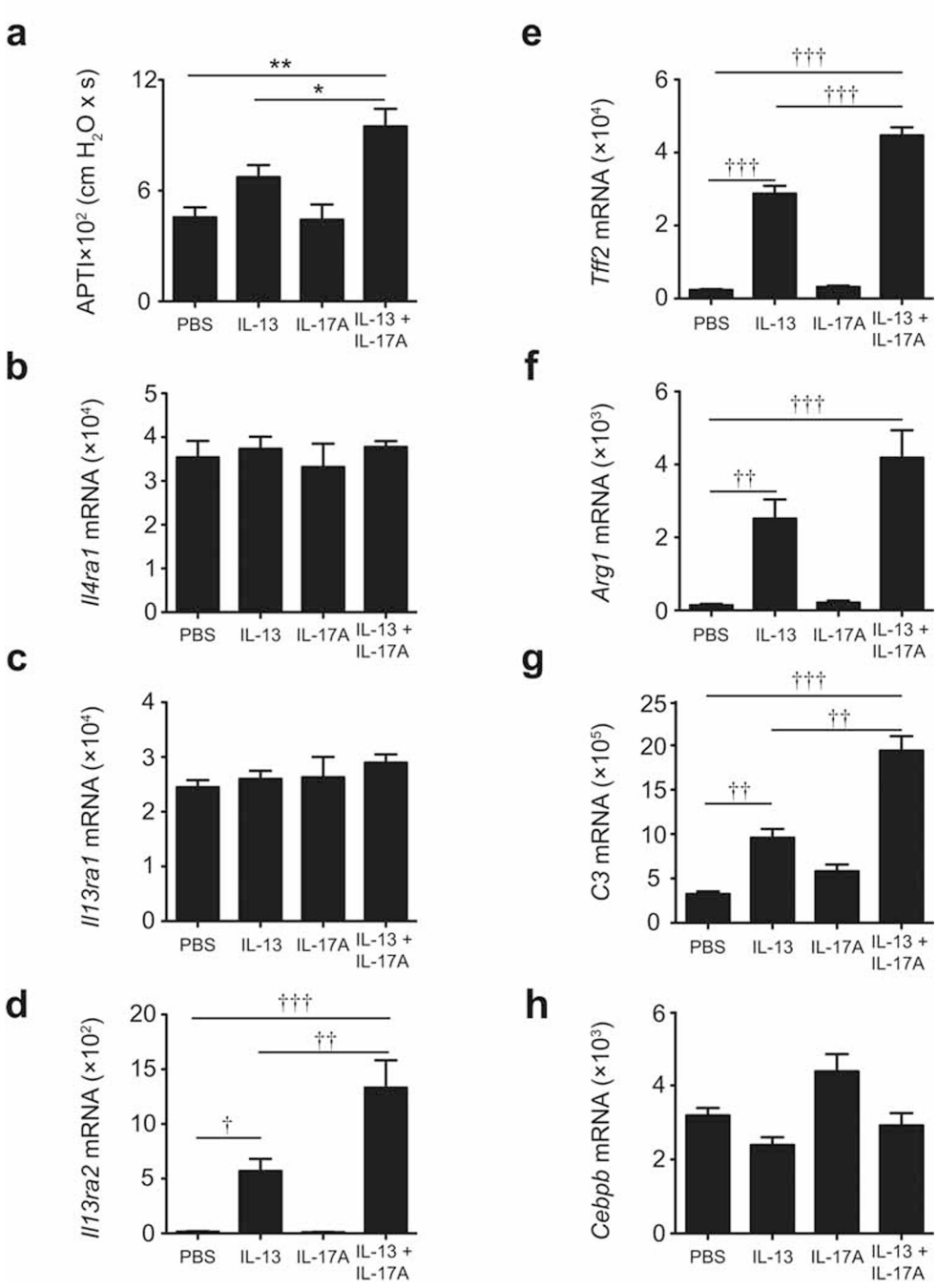

Figure 3. IL-17A and IL-13 synergistically induce AHR

A/J mice were treated with rmIL-13, rmIL-17A, or a combination of both and airway responsiveness was assessed (**a**) *P<0.05, **P<0.01 (one-way ANOVA). Lung expression of *Il4ra1* (**b**), *Il13ra1* (**c**), *Il13ra2* (**d**), *Tff2*, (**e**), *Arg1* (**f**), *C3* (**g**), and *Cebpb* (**h**) were measured by real-time PCR, †P<0.05, ††P<0.01, †††P<0.001, (one-way ANOVA). Results are representative of two independent experiments (mean ± s.e.m. of 8 mice per groups).

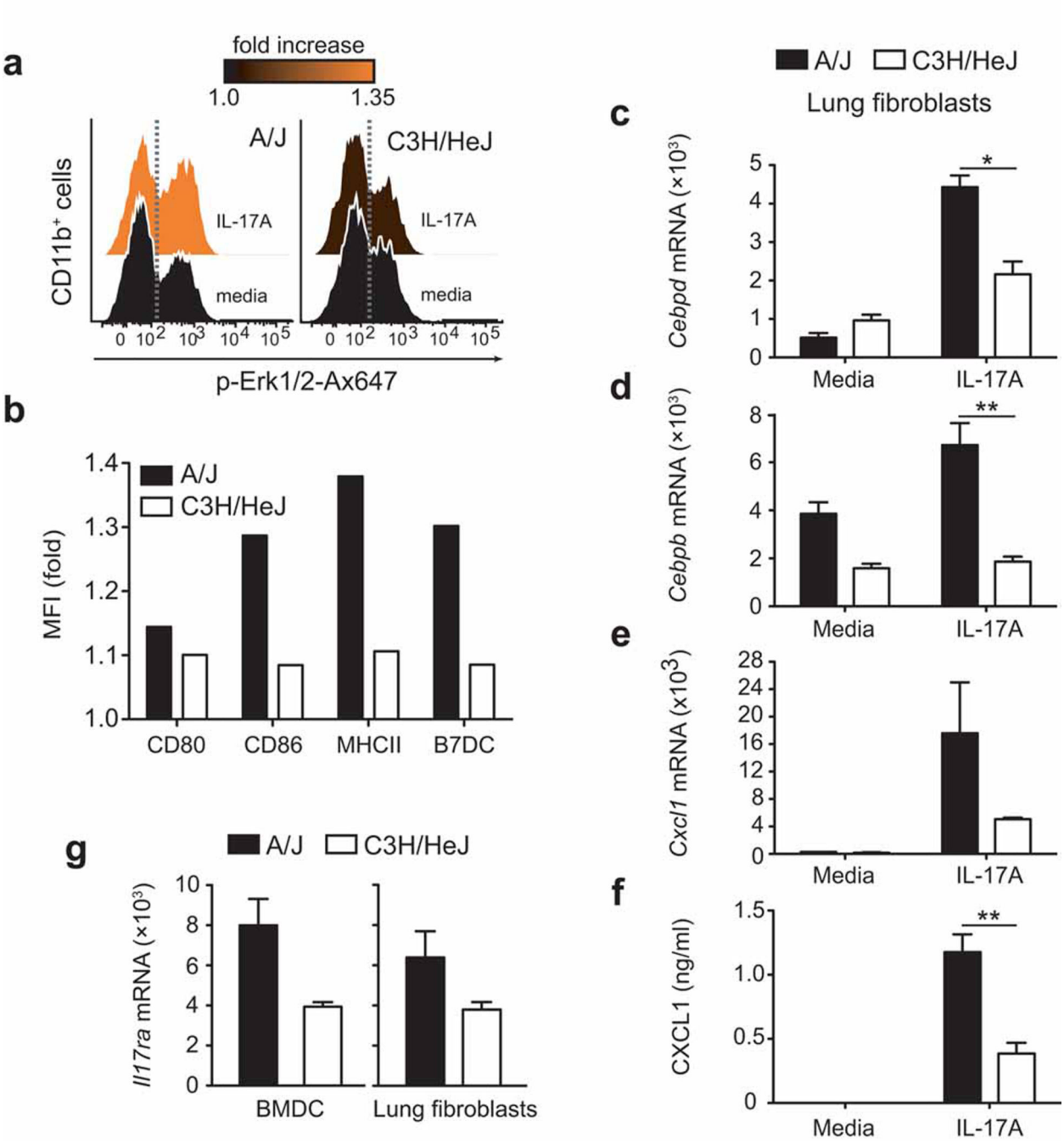

Figure 4. Enhanced responsiveness to IL-17A in susceptible A/J mice

Flow cytometric analysis of phosphoErk1/2 in IL-17A treated lung cells from naïve A/J and C3H/HeJ mice (**a**). Effect of IL-17A on expression of MHC class II and costimulatory molecules (CD80, CD86 and B7-DC) on BMDCs from naïve A/J and C3H/HeJ mice as analyzed by flow cytometry (shown as fold increase over media-treated cells) (**b**). Real-time PCR analysis of the IL-17A-responsive genes, *Cepbd* (**c**), *Cebpb* (**d**), and *Cxcl1* (**e**) and CXCL1 secretion (**f**) from media- or IL-17A-stimulated A/J and C3H/HeJ lung fibroblasts *P<0.05, **P<0.01 comparing A/J vs C3H/HeJ (two-tailed Student's *t*-test). *Il17ra* mRNA

in untreated A/J and C3H/HeJ BMDC or lung fibroblasts determined by real-time PCR (**g**). Results represent one to two separate experiments (mean ± s.e.m. of triplicate wells).

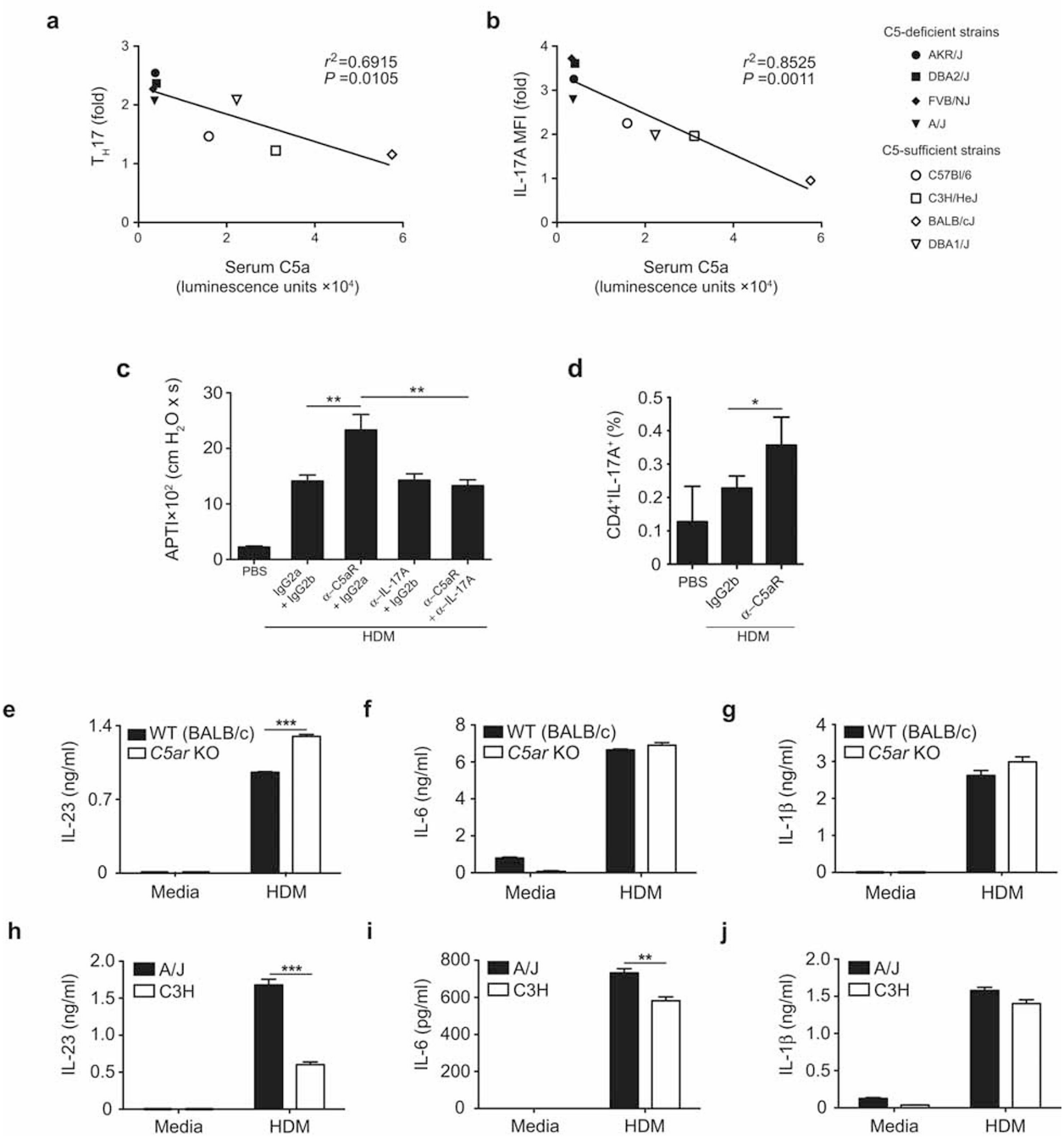

Figure 5. Link between C5 deficiency, IL-17A and IL-23 production

C5-sufficient and C5-deficient strains were treated with HDM as described in Methods. Frequency of lung T_H17 cells and IL-17A MFI were analyzed by flow cytometry and serum C5a was measured by ELISA (shown as fold change over PBS-treated mice) (**a, b**). BALB/c mice were treated with C5aR or IL-17A blocking antibodies as described in the Methods section. Airway responses (**c**), and the frequency of $CD4^+IL17A^+$ cells was determined by flow cytometry (**d**) *P<0.05; **P<0.01 (one-way ANOVA). BMDCs from BALB/c control or *C5ar*-deficient mice were treated with media or HDM (100 μg/ml) and supernatants were

analyzed for IL-23 (**e**), IL-6 (**f**), and IL-1β (**g**) production. Supernatants from HDM-treated BMDCs from A/J and C3H/HeJ mice were assayed for IL-23 (**h**), IL-6 (**i**) and IL-1β (**j**) production by ELISA **P<0.01; ***P<0.001 (Student's *t*-test). Results are representative of one [4 mice per strain - (**a,b**); 4–6 mice per group - (**c,d**)] or two to four independent experiments [mean ± s.e.m. of 4 individual samples, (**e–j**)].

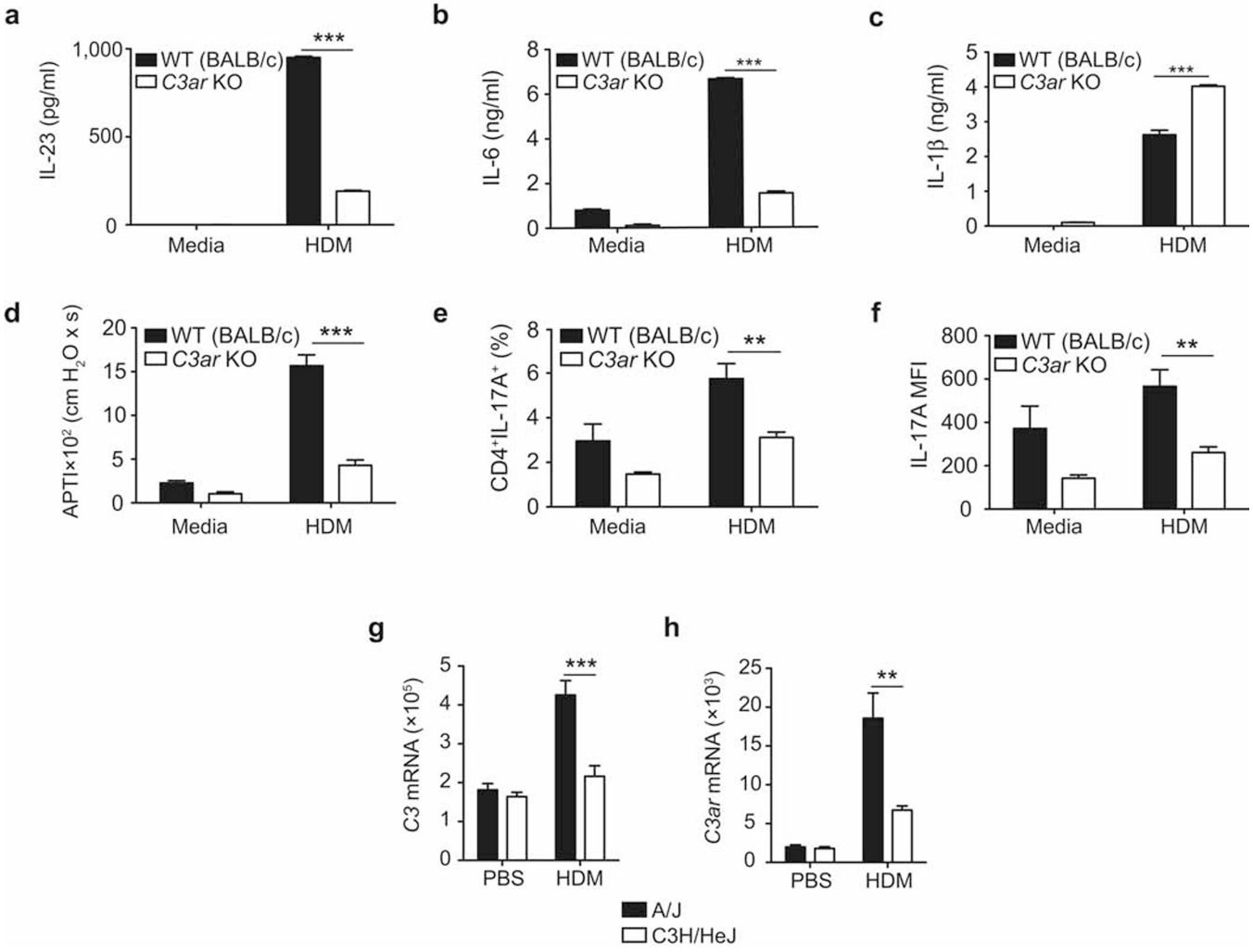

Figure 6. Complement factor 3 signaling promotes DC IL-23 production

BMDCs from wild-type (BALB/c) and *C3ar* KO mice were treated with media or HDM and supernatants were analyzed for IL-23 (**a**), IL-6 (**b**), and IL-1β (**c**) production, ***P<0.001 (two-tailed Student's *t*-test). BALB/c, and *C3ar* KO mice were exposed to HDM as described in the Methods section to assess airway function (**d**), and the frequency of lung T_H17 cells ($CD4^+IL\text{-}17A^+$) cells (**e**) and IL-17A MFI (**f**) by flow cytometry ††P<0.01, †††P<0.001 (two-tailed Student's *t*-test). A/J and C3H/HeJ mice were treated with a single exposure of PBS or HDM, and sacrificed 72h afterwards, lungs were harvested for RNA extraction, and gene expression of *C3* (**g**) and *C3ar* (**h**). ##P<0.01, ###P<0.001 (two-tailed Student's *t*-test). Results are representative of three independent experiments [mean ± s.e.m. of four individual samples (**a–c**)], one experiment with five to six mice per group (**d–e**) or three separate experiments [mean ± s.e.m. of eight mice per group (**g,h**)].

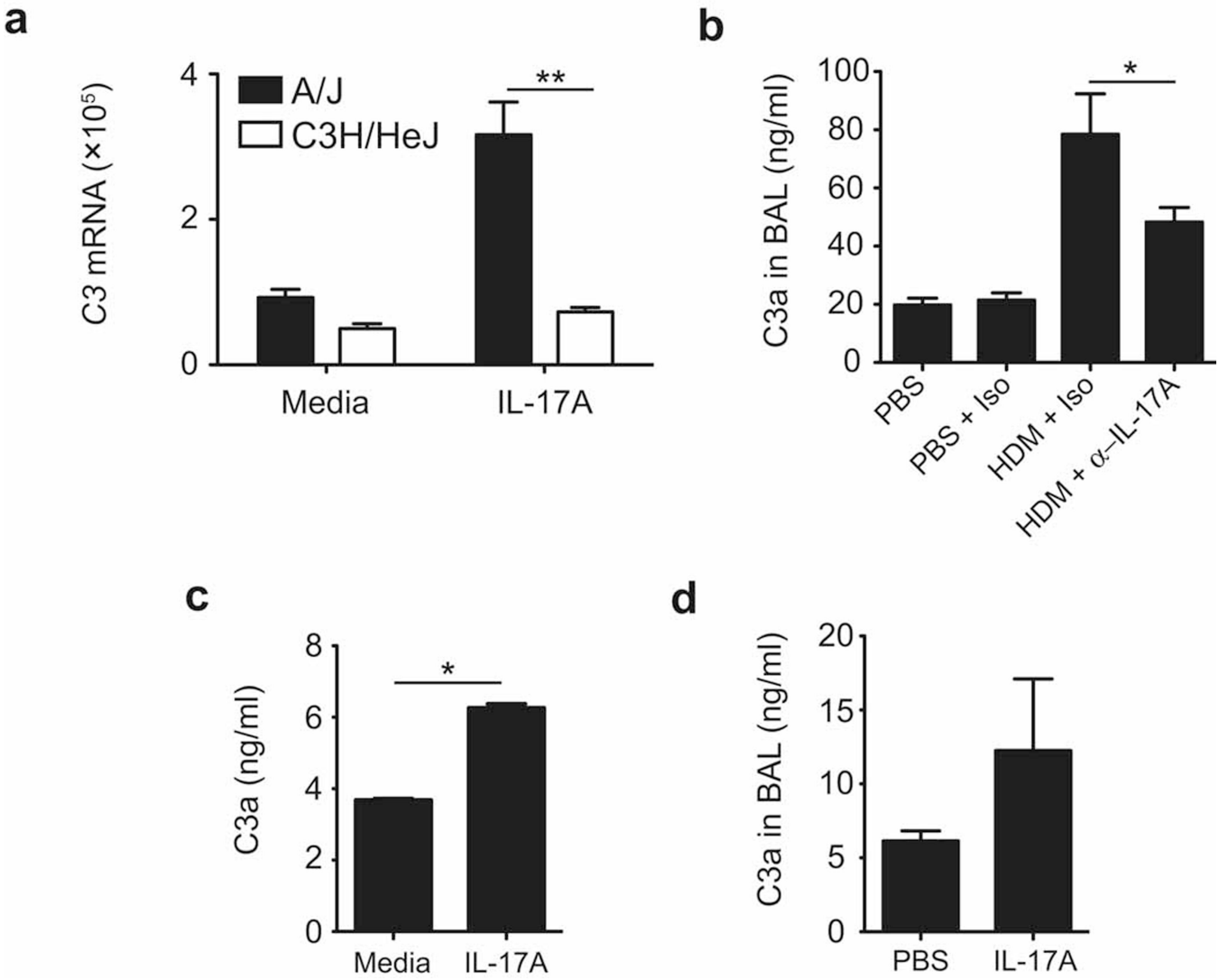

Figure 7. Positive feedback regulation of C3/C3aR by IL-17A

Primary mouse tracheal epithelial cells from A/J and C3H/HeJ mice were cultured in media or in 100 ng/ml mIL-17A and *C3* message was determined by real-time PCR (**a**), **P<0.01 (two-tailed Student's *t*-test). A/J mice were treated HDM or PBS and isotype or IL-17A blocking mAbs as described in Methods to quantify C3a in BAL by ELISA (**b**), †P<0.05 (two-tailed Student's *t*-test). C3a concentrations in the supernatants of BMDC (C57BL/6) treated with media or 100 ng/ml IL-17A (**c**), #P<0.05 (two-tailed Student's *t*-test) Concentrations of C3a in the BAL of A/J mice receiving rIL-17A (5 ug) intratracheally (**d**). Results are representative of one to two independent experiments (mean ± s.e.m. of three to eight individual samples). *P<0.05, **P<0.01, (two-tailed Student's *t*-test).

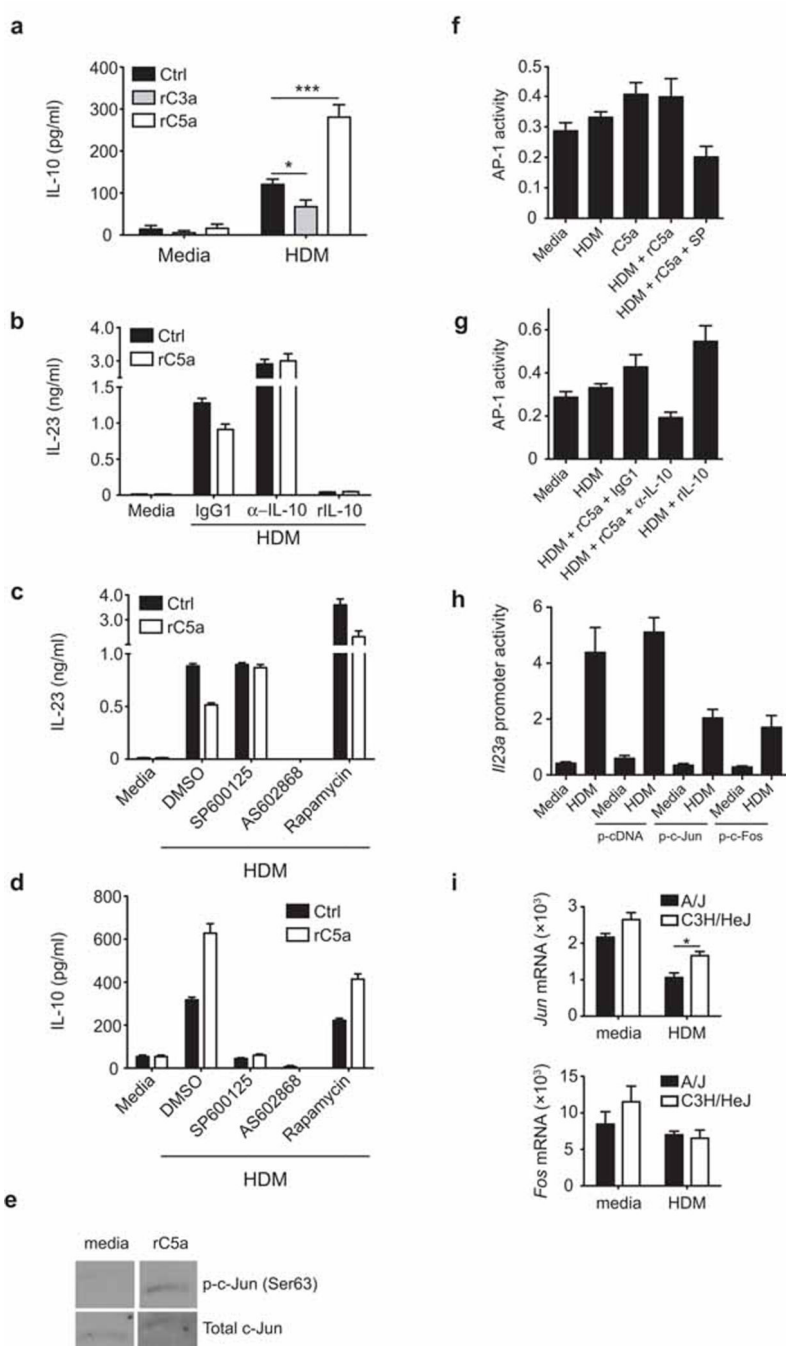

Figure 8. C5a-induced IL-10 regulates IL-23 production by engaging JNK/AP-1

IL-10 production in HDM-treated BMDCs (BALB/c) in the presence of medium, rC3a, or rC5a (**a**), *P<0.05, ***P<0.001 (one way ANOVA). IL-23 production by HDM-treated BMDCs (BALB/c) with or without rC5a in the presence of IgG1, anti-IL-10 or rIL-10 (**b**). IL-23 (**c**) and IL-10 (**d**) production by BMDCs (BALB/c) pre-treated (1 h) with DMSO, SP600125, AS602868, or rapamycin, then with HDM (100 μg/ml) in the presence or absence of rC5a. Phospho c-Jun and total c-Jun in media- or C5a-treated RAW 264.7 cells (**e**). AP-1 activity in RAW 264.7 cells were treated as indicated (SP = SP600125) (**f**). AP-1

activity in RAW 264.7 cells stimulated as indicated (**g**). *Il23a* promoter activity in RAW 264.7 cells co-transfected with p-cDNA3.1, or vectors containing c-Jun or c-Fos cDNAs (**h**). *Jun* and *Fos* mRNA was measured in A/J and C3H/HeJ BMDC (**i**). Results are representative of two to three independent experiments (mean ± s.e.m. from 4–8 individual samples).