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C5 Modulates Airway Hyperreactivity and Pulmonary Eosinophilia during Enhanced Respiratory Syncytial Virus Disease by Decreasing C3a Receptor Expression▽

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Enhanced respiratory syncytial virus disease, a serious pulmonary disorder that affected recipients of an inactivated vaccine against respiratory syncytial virus in the 1960s, has delayed the development of vaccines against the virus. The enhanced disease was characterized by immune complex-mediated airway hyperreactivity and a severe pneumonia associated with pulmonary eosinophilia. In this paper, we show that complement factors contribute to enhanced-disease phenotypes. Mice with a targeted disruption of complement component C5 affected by the enhanced disease displayed enhanced airway reactivity, lung eosinophilia, and mucus production compared to wild-type mice and C5-deficient mice reconstituted with C5. C3aR expression in bronchial epithelial and smooth muscle cells in the lungs of C5-deficient mice was enhanced compared to that in wild-type and reconstituted rodents. Treatment of C5-deficient mice with a C3aR antagonist significantly attenuated airway reactivity, eosinophilia, and mucus production. These results indicate that C5 plays a crucial role in modulating the enhanced-disease phenotype, by affecting expression of C3aR in the lungs. These findings reveal a novel autoregulatory mechanism for the complement cascade that affects the innate and adaptive immune responses.

Respiratory syncytial virus (RSV) is the leading cause of severe viral respiratory infections in infants worldwide (7). In the 1960s, a formalin-inactivated RSV vaccine (FIRSV) was administered to infants in the United States (6, 13, 25, 27). Subsequent exposure of vaccinated children to RSV resulted in increased morbidity and mortality. The mechanism of illness was never clarified, hampering the development of safe vaccines against the virus. Four decades later, there is still no licensed vaccine against RSV.

Recently, a mouse model of enhanced RSV disease (ERD) that uses airway hyperreactivity (AHR) and pneumonia, characteristic manifestations of ERD in children (6, 13, 25, 27), as primary correlates of disease enhancement (34) was established. Using this model and postmortem lung sections from affected children, deposition of immune complexes that fix complement in the lungs was shown to play a critical role in AHR during ERD (34).

The complement components associated with AHR during ERD have not been characterized, but a role for C3a in AHR has been described for rodent models of asthma (3, 19). Conversely, C5aR signaling has been reported to decrease susceptibility to asthma, presumptively by promoting interleukin-12 (IL-12), IL-23, and IL-27 production and enhancing production of IL-4 and IL-13 (12, 17, 26). However, conflicting data suggest that C5a may decrease IL-12 production in certain models and enhance AHR (5, 17, 26, 32, 46). In fact, a recent paper described a dual role for C5a in allergic asthma during allergen sensitization (protective) and in an established inflammatory response (proinflammatory) (28). It is conceivable that C3a and C5 may modulate each other in the lungs, but no direct regulatory role between them in AHR and airway inflammation has been described.

Interestingly, while complement components determine AHR, ERD pneumonia has been attributed to cytokine release by $CD4^+$ Th2 cells (8, 9, 31, 39, 44). Often, pneumonia in mice has been characterized by the presence of a peribronchiolar and perivascular inflammatory infiltration (8, 9, 27, 34) associated with abundant pulmonary eosinophils in bronchoalveo-

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lar lavage (BAL) fluid (18, 21, 24, 31, 37). However, both hallmark signs of ERD, AHR and pneumonia, are thought to occur in parallel, and there is no evidence that the complement and T-cell-mediated processes are interrelated (9, 34).

To elucidate the complement components associated with AHR and examine whether complement affects the severity of pneumonia or eosinophilia during ERD, we characterized the AHR and lung histopathologies of mice with functional deficiencies in the complement cascade. In this paper, we demonstrate that C3a is critical for AHR during ERD and that AHR is negatively regulated by C5, which modulates the levels of C3aR expression in the lungs of mice. We also show that severity of lung eosinophilia, long regarded as a surrogate marker of ERD pneumonia (18, 21, 24, 37), does not correlate with severity of lung inflammation, suggesting that these are independent manifestations of the disease. Furthermore, lung eosinophilia and mucus production correlate with AHR and are modulated by the effect of C5 on C3aR expression.

MATERIALS AND METHODS

Mice. Four- to 8-week-old C3a receptor-deficient ($C3aR^{-/-}$) and control (WT) mice and B10.D2/0Sn C5-deficient ($C5^{-/-}$) and B10.D2/NSn WT mice (The Jackson Laboratory, Bal Harbor, ME) were used for these experiments. The $C3aR^{-/-}$ and control mice were described previously (19) and backcrossed eight generations onto the BALB/c background. $C5^{-/-}$ mice received 1 mg/kg of purified human C5 complement factor (The Binding Site, CA), 1 mg/kg of SB290157 C3aR antagonist (EMD Bioscience, CA), or 300 μg/mouse of IL-13Ra2-Fc (R&D) intraperitoneally on days 0, 4, 6, and 7 after RSV challenge. All experimentation was approved by the Johns Hopkins Medical Institutions.

Preparation of vaccines and immunization. FIRSV was prepared using the RSV A2 strain, as described previously (34). Mice received the equivalent of two doses of 10^5 PFU of FIRSV intramuscularly (days 0 and 7) and were challenged intranasally 28 days after the second dose of FIRSV with 10^6 PFU of RSV A2. Mice inoculated with lipopolysaccharide (LPS) received 5 μg intranasally 24 h. before evaluation.

Airway hyperresponsiveness. Seven days after challenge, animals were anesthetized with sodium pentobarbital (90 mg/kg) and ventilated (120 breaths/min; 0.2 ml). Carbamylcholine was given intravenously (200 μg/kg) and the dynamic airway pressure measured for 3 min. In addition, airway pressure time index (APTI) determinations were confirmed by analysis of lung resistance to aerosolized methacholine (0.01 to 3 mg/kg).

Lung histopathology. Lungs from mice were removed 7 days after challenge. Immunohistochemistry was performed on paraffin-embedded sections using affinity-purified chicken anti-mouse C3aR antibody (1/300 dilution; Aves Laboratories), an isotype control, and a secondary anti-chicken antibody (1/2,000 dilution) (Promega, Madison, WI).

Pneumonia severity score. A previously described severity scoring system was used by blinded observers to characterize the degrees of pulmonary infiltration (8, 9). Briefly, for ERD pneumonia, the vessels and bronchi were initially scored as 1 when no or few infiltrating cells were present, 2 when focal aggregates of infiltrating cells were present or the structure was cuffed by one definite layer of infiltrating cells, and 3 when two or more definite layers of infiltrating cells with or without focal aggregates were present. Subsequently, ERD pneumonia was categorized as mild (>60% of vessels and bronchi with scores of 1 and 0% with scores of 3), moderate (>30% with scores of 2 and/or <20% with scores of 3), or severe (>20% with scores of 3). The degrees of pulmonary eosinophilia were estimated using a hematoxylin-Congo red stain to enumerate eosinophils and expressed as numbers of eosinophils present per 40× field. Mucus production was scored using a hematoxylin–periodic acid-Schiff (PAS) stain as previously described (45). In addition, percent eosinophils in cells from BAL fluids was estimated by Giemsa stains.

C3aR western blot analysis. Lungs of mice belonging to different experimental groups were rinsed with phosphate-buffered saline (PBS), extracted from the animals, and immediately frozen using a bath of dry ice in 100% ethanol. Subsequently, the frozen tissue was mechanically homogenized in the presence of ice-cold extraction buffer (0.1% Nonidet P-40, 50 mM HEPES [pH 7.4], 1 mM EDTA, 150 mM NaCl, 2 mM MgCl, 500 μM dithiothreitol, 100 μM NaF) supplemented with a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml benzamidine-HCl, and 1 μg/ml pepstatin A). Tissue debris and nuclei of lysed cells were removed by centrifugation, and a portion of the supernatant, containing cellular cytosolic and membrane fractions, was subjected to a standard bicinchoninic acid assay (Pierce) to determine protein concentration. The remainder of the supernatant was diluted in loading buffer and boiled for 5 min in preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Equivalent amounts of protein were loaded in 10% sodium dodecyl sulfate-polyacrylamide gels, separated by electrophoresis, and transferred onto nitrocellulose membranes (Millipore, Bedford, MA). Membranes were then blocked with blocking buffer (5% milk in PBS-T [1× PBS, 0.1% Tween 20]), and C3aR was detected using a chicken anti-mouse polyclonal C3aRc antibody (GlaxoSmithKline) diluted 1:1,000 in blocking buffer, followed by a secondary horseradish peroxidase-conjugated anti-chicken immunoglobulin Y (IgY; Promega) diluted 1/1,000 in the same buffer. A protein of approximately 62 to 65 kDa, consistent with the reported molecular mass of the C3a receptor (4), was detected upon incubation with the Super Signal Pico chemiluminescent substrate (catalog no. 34080; Pierce).

RT-PCR analysis. Quantitation of mouse C3aR expression with amplification of mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA as an endogenous control was performed with an ABI PRISM 7700 sequence detector system. One hundred nanograms of total RNA/sample (prepared using Ambion [Austin, TX] RNAqueous) was subjected to real-time (RT)-PCR (SYBR green PCR Master Mix kit; ABI, Foster City, CA), using the following primer sequences: mC3aR forward, 5′-CAGGCAAGGGATTACTTTGG-3′; mC3aR reverse, 5′-TGTGAGGACATTAGGAGGCTTTCC-3′; mGAPDH forward, 5′-TGCCCAGAACATCATCCCTG-3′; mGAPDH reverse, 5′-ATCCACGACGG ACACATTGG-3′. IL-13 (Mm00434204_m1) and GAPDH (Mm99999915_g1) mRNA determinations in lung homogenates were performed using primers from Applied Biosystems. Experiments were performed in duplicate for each standard and lung sample.

Virus titration in lung tissue and antibody assays. Lungs from mice were removed aseptically 4 days after RSV challenge and virus titers estimated as previously described (35). Sera were tested for antibodies to RSV 7 days after challenge by 60% plaque reduction neutralization titer (PRNT) analysis and for RSV F and G using protein-specific immunoassays (34).

Immunospot assay. Nitrocellulose-based 96-well microtiter plates (Millititer HA; Millipore, Bedford, MA) were coated overnight at room temperature with 10 μg/ml of anti-gamma interferon (IFN-γ; clone R4-6A2; Pharmingen) or anti-IL-5 monoclonal antibody (clone TRFK5; Pharmingen). Lung mononuclear cells were stimulated for 18 h with concanavalin A (ConA; Sigma). IFN-γ-producing cells were detected with biotinylated anti-IFN-γ (clone XMG1.2), followed by streptavidin peroxidase and 3,3′-diaminobenzidine tetrahydrochloride dehydrate (Sigma). A biotynilated anti-IL-5 monoclonal antibody (clone XMG1.2) was used to detect IL-5. All assays were performed in triplicate.

Statistical analysis. Comparisons among airway responses were made using analysis of variance for parametric cases. The Kruskal-Wallis and Mann-Whitney U test were used for nonparametric comparisons where appropriate. A P value of <0.05 was considered significant.

RESULTS

AHR during ERD is decreased by complement component C5. We initially determined whether C5 plays a role in AHR during ERD. For this purpose, we elicited ERD by immunizing $C5^{-/-}$ and WT mice with FIRSV and challenging them with RSV (Fig. 1a). As previously reported for other murine strains (34), this strategy elicited enhanced AHR in both groups compared to what was found for WT and $C5^{-/-}$ mice immunized with a placebo and infected with RSV (Fig. 1a), naïve control WT and $C5^{-/-}$ mice, and mice immunized with inactivated vaccine and challenged with a placebo (not shown). Among mice with ERD, AHR reactivity to acetylcholine was significantly greater in $C5^{-/-}$ than in WT mice (Fig. 1a). To confirm the role of C5 in modulating AHR during ERD, we reconstituted $C5^{-/-}$ mice with purified C5 (hereafter referred to as C5H mice) (Fig. 1a). AHR levels were not different in C5H and WT mice.

Subsequently, we confirmed these observations by measur-

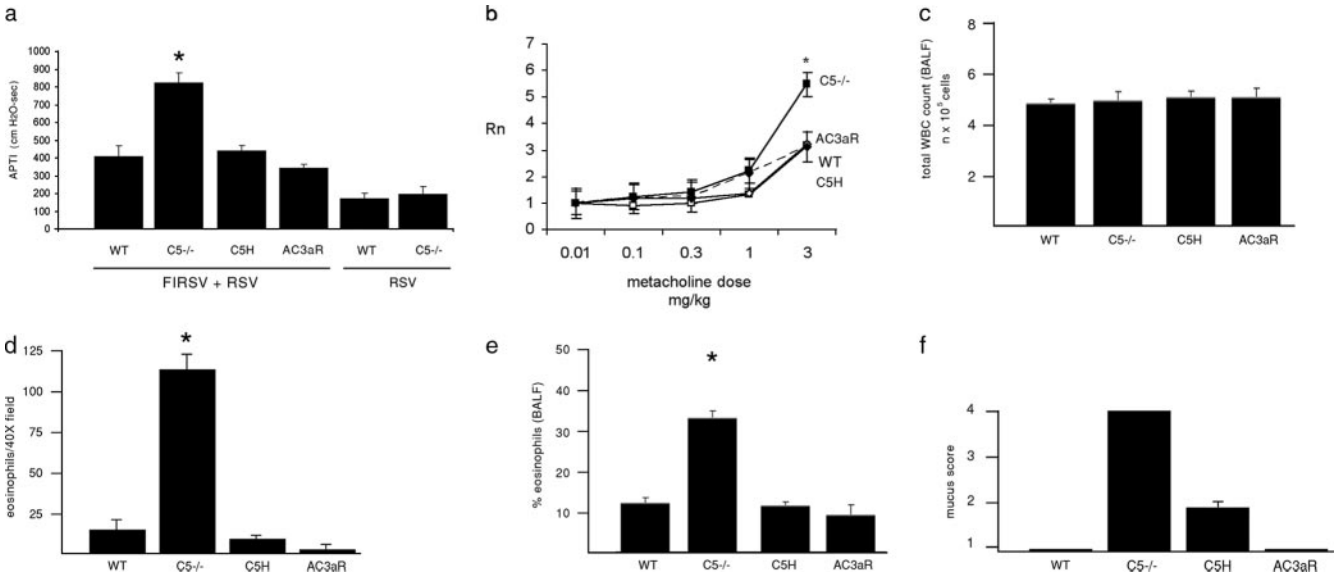

FIG. 1. Modulatory role of C5 in ERD 7 days after RSV challenge. (a) Airway reactivities to acetylcholine challenge in FIRSV-immunized WT mice and C5-deficient ($C5^{-/-}$) mice with or without treatment, using human C5 (C5H) or a C3aR antagonist (AC3aR) and placebo-immunized WT and $C5^{-/-}$ mice. Reactivity was defined by the time-integrated rise in peak airway pressure (APTI) (values are means ± standard errors of the mean for six to eight animals/group and are representative of two independent experiments). *, $P < 0.001$. (b) Airway resistance (Rn) in response to methacholine inhalation 7 days after RSV challenge in mice with ERD (values are means ± standard errors of the means for five to seven animals/group and are representative of two independent experiments). *, $P < 0.01$. (c) Total white blood cell counts in BAL fluids from mice with ERD (P values not significant). (d) Numbers of lung eosinophils per 40× field in mice with ERD. *, $P < 0.001$. (e) Percentages of eosinophils in cells from bronchoalveolar lavage fluids from mice with ERD. *, $P < 0.001$. (f) Scores for mucus production in mice with ERD.

ing airway resistance to increasing concentrations of aerosolized methacholine (Fig. 1b). Methacholine responses were higher in $C5^{-/-}$ mice than in WT and C5H mice with ERD ($P < 0.01$). Resistance levels in C5H and WT mice were similar (Fig. 1b).

Pulmonary eosinophilia and mucus production, but not the severity of ERD pneumonia, are decreased by C5. An excess of eosinophils in the lungs of mice has often been considered a surrogate marker for ERD pneumonia (18, 21, 23, 24, 37), and the association between lung eosinophilia and AHR has been studied with certain murine models of asthma (20, 30). Therefore, we examined whether C5 modified the severity or histological distribution of the inflammatory pneumonia during ERD and/or affected the degree of lung eosinophilia (Fig. 1c to e and 2). Since pneumonia in mice during ERD has a characteristic peribronchiolar and perivascular pattern and is typically distributed unevenly over the lung parenchyma (8, 9), we chose to grade its severity using two different strategies: a modified version of a well-characterized, disease-specific severity score (8, 9) and a quantification of the total white blood cell (WBC) counts in BAL fluids from different groups (17). According to the disease-specific severity score (detailed in Materials and Methods), the lungs of $C5^{-/-}$ mice with ERD had peribronchiolar and perivascular cellular infiltration levels similar to those for WT and C5H mice (Fig. 2, left panel), and pneumonia in all groups was scored as severe. In consonance with this observation, the total WBC counts in BAL fluids did not differ among the three groups (Fig. 1c).

Interestingly, the severity of pneumonia was dissociated from the degree of lung eosinophilia during ERD. Again, to estimate the degree of lung eosinophilia, we used two different approaches: counting eosinophils in hematoxylin-Congo red-stained lung sections (Fig. 1d) and establishing percent eosinophils in inflammatory cells from BAL fluids by using Giemsa stains (Fig. 1e). Eosinophil accumulation in the lungs ($P < 0.01$) and percent eosinophils in inflammatory cells from BAL fluids ($P < 0.01$) were significantly greater in $C5^{-/-}$ mice than in WT and C5H mice with ERD (Fig. 1d and e and 2, middle panel). Eosinophils were rare to undetectable in WT and $C5^{-/-}$ mice immunized with the placebo and challenged with RSV (not shown). To further characterize the modulatory role of C5, we examined lung sections for mucus production using a PAS stain and a well-characterized score (Fig. 1f and 2, right panel) (45). In agreement with the observations on AHR and eosinophilia, mucus production in $C5^{-/-}$ mice with ERD was enhanced compared to that in WT and C5H mice.

C5 levels do not affect neutralizing antibody responses or RSV titers after challenge. We subsequently examined whether C5 contributed to neutralizing antibody-mediated control of RSV replication. If this were true, the greater severity of disease in $C5^{-/-}$ mice could be explained by higher titers of RSV in the lungs. We therefore measured RSV titers in the lungs of $C5^{-/-}$ and WT mice with ERD (Table 1). No difference in peak RSV titers on day 4 postchallenge was observed between both groups. Virus replication was undetectable in $C5^{-/-}$ and WT mice 7 days after challenge. In addition, neutralizing antibody titers were undetectable (as previously described for BALB/c mice [34]) and RSV glycoprotein-specific anti-fusion (F) and anti-attachment (G) IgG antibody levels were not different in $C5^{-/-}$ and WT controls with ERD (Table 1).

C5 regulates the expression of C3a receptor in the lungs of mice with ERD. Since we had previously described a critical

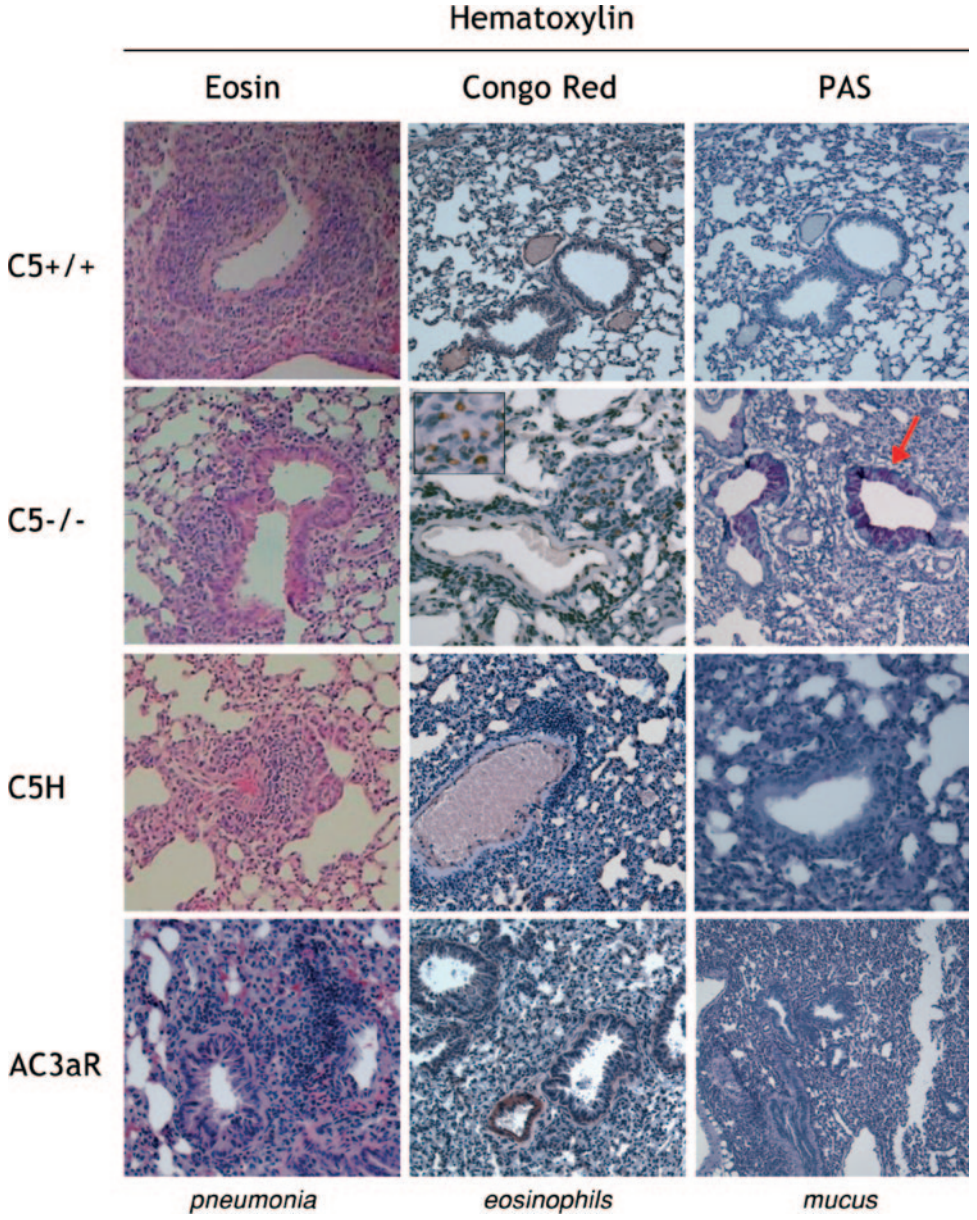

FIG. 2. Role of C5 in lung histopathologies of mice with ERD. Shown are pulmonary histopathologies (hematoxylin and eosin), eosinophil infiltration (hematoxylin-Congo red), and mucus production (hematoxylin-PAS) in WT, $C5^{-/-}$, C5H, and AC3aR mice preimmunized with FIRSV and challenged with RSV (7 days postchallenge). Red arrow, PAS-positive cells.

TABLE 1. Antibody and viral titers after RSV challenge in mice previously immunized with FIRSV[a]

Mouse group	$PRNT_{60}$	Antibody titer for indicated assay		RSV titer (log PFU/g)
		EIA-F	EIA-G	
$C3aR^{+/+}$	<10	683 ± 33	366 ± 26	3.2 ± 0.03
$C3aR^{-/-}$	<10	626 ± 42	366 ± 36	3.0 ± 0.01
$C5^{+/+}$	<10	403 ± 19	343 ± 38	2.2 ± 0.03
$C5^{-/-}$	<10	402 ± 16	350 ± 45	2.3 ± 0.03

[a] Antibody responses in mice were determined 7 days after RSV challenge and RSV pulmonary titers 4 days after challenge. $PRNT_{60}$, RSV-specific neutralization as measured by 60% complement-enhanced plaque reduction; EIA-F, immunoassay determination of IgG antibodies against RSV F protein (change in absorbance); EIA-G, immunoassay determination of IgG antibodies against RSV G protein (change in absorbance). Results are means ± standard errors of the means.

role for C3 in AHR during ERD using B6129F2 $C3^{-/-}$ mice (34), we hypothesized that the effects of C5 during ERD could be associated with modulation of C3aR expression. To test this hypothesis, we stained lung sections of $C5^{-/-}$ and WT mice with ERD using a polyclonal antibody against C3aR (Fig. 3A). C3aR expression in the bronchiolar epithelium and smooth muscle cells of $C5^{-/-}$ mice was enhanced compared to that in WT mice. Furthermore, expression of C3aR in C5H mice was not different than expression in WT mice with ERD. In addition, expression of C3aR in the lungs of naïve control mice by immunohistochemistry was equally low, regardless of the presence or absence of C5 (not shown).

Then, we compared C3aR mRNA levels by RT-PCR in the

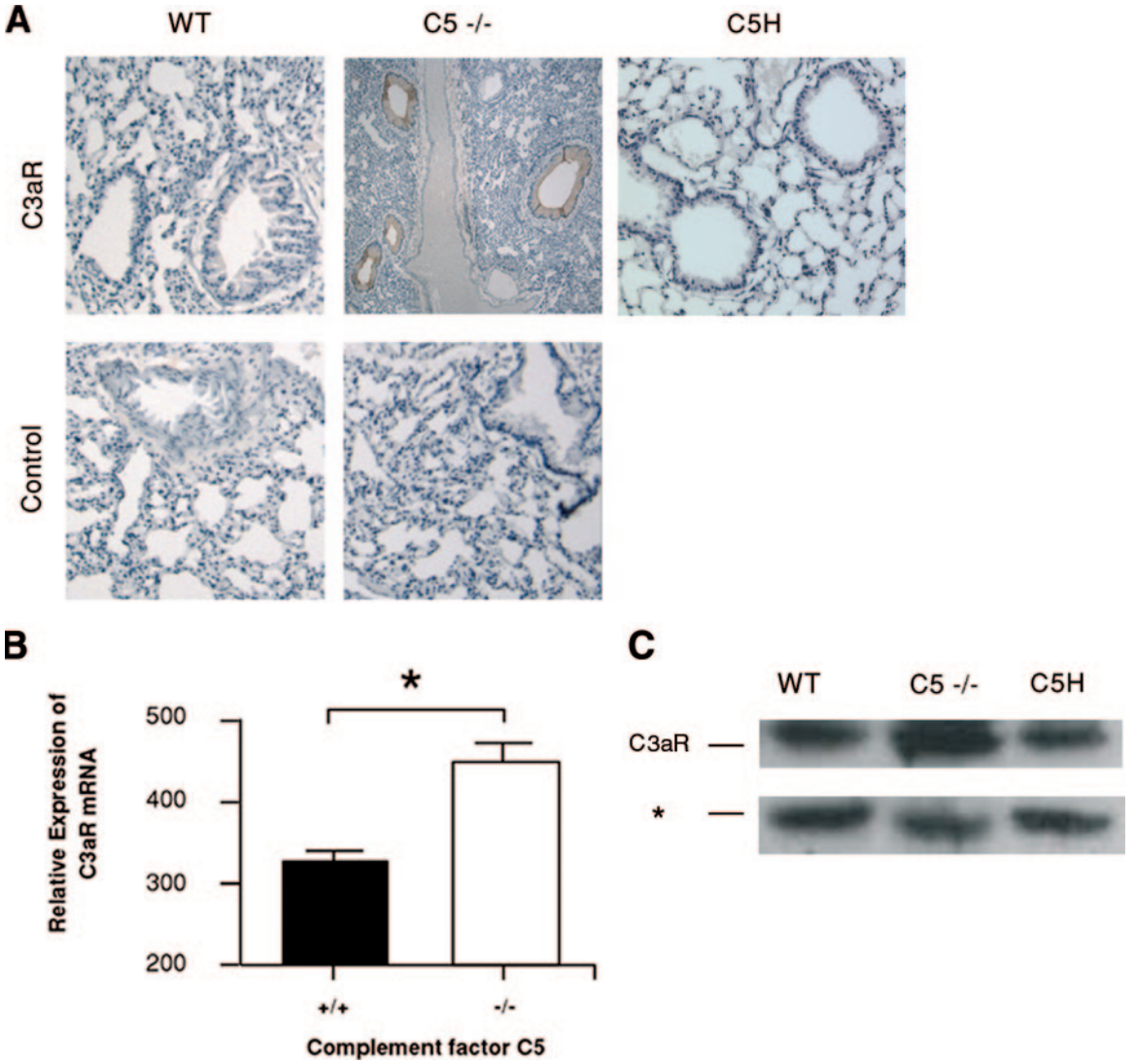

FIG. 3. Enhanced expression of C3aR in the lungs of $C5^{-/-}$ mice with ERD. (A) Expression of C3aR in the pulmonary tissue of WT, $C5^{-/-}$, and C5H mice preimmunized with FIRSV and challenged with RSV (7 days postchallenge). Immunohistochemistry staining for C3aR (see brown staining) and preimmune control sera are shown. (B) Expression of C3aR as measured by real-time PCR analysis. Results shown are the means ± standard errors of the means for mRNA C3aR expression relative to GAPDH expression. *, $P = 0.02$. (C) C3aR expression (50 kDa) by Western blot analysis (4) in the lung tissue of mice with ERD 7 days after RSV challenge (C3aR/actin ratios: WT, 0.82; $C5^{-/-}$, 1.33; C5H, 0.39). *, actin control.

lungs of WT and $C5^{-/-}$ mice with ERD (Fig. 3B). Expression of C3aR mRNA in $C5^{-/-}$ mice was enhanced compared to that in WT controls ($P = 0.02$).

Finally, to confirm the modulatory role of C5, we examined whether C3aR expression determined by Western blot analysis was also affected by lack of C5 during ERD (Fig. 3C). Again, the lungs of WT and C5H mice had lower levels of C3aR protein than those of $C5^{-/-}$ mice with ERD.

Since other effects of C5 on the immune response are cell and stimulus dependent (5, 17, 26, 46), we examined whether expression of C3aR was also enhanced in the lungs of naïve mice stimulated with an unrelated inflammatory agent. For this purpose, we inoculated mice intranasally with LPS. In contrast to the results obtained during ERD, C3aR expression following LPS inoculation in $C5^{-/-}$ mice was decreased compared to what was found for C5-deficient mice reconstituted with C5 (not shown).

The C3a anaphylatoxin is critical for AHR during ERD. Given the association between C5 and C3aR expression, we determined the role of C3a in ERD by immunizing $C3aR^{-/-}$ and WT mice with FIRSV and challenging them with RSV (Fig. 4A). This strategy, as previously reported, leads to enhanced AHR and pneumonia in WT BALB/c mice compared to what occurs for mice immunized with a placebo and challenged with RSV (34).

AHR in WT mice was significantly enhanced compared to that in $C3aR^{-/-}$ mice with ERD ($P = 0.014$), demonstrating that C3a is critical for AHR during this illness. In addition, C3a did not affect the severity of the inflammatory infiltration in the lungs (the pneumonia score was "severe" for both groups)

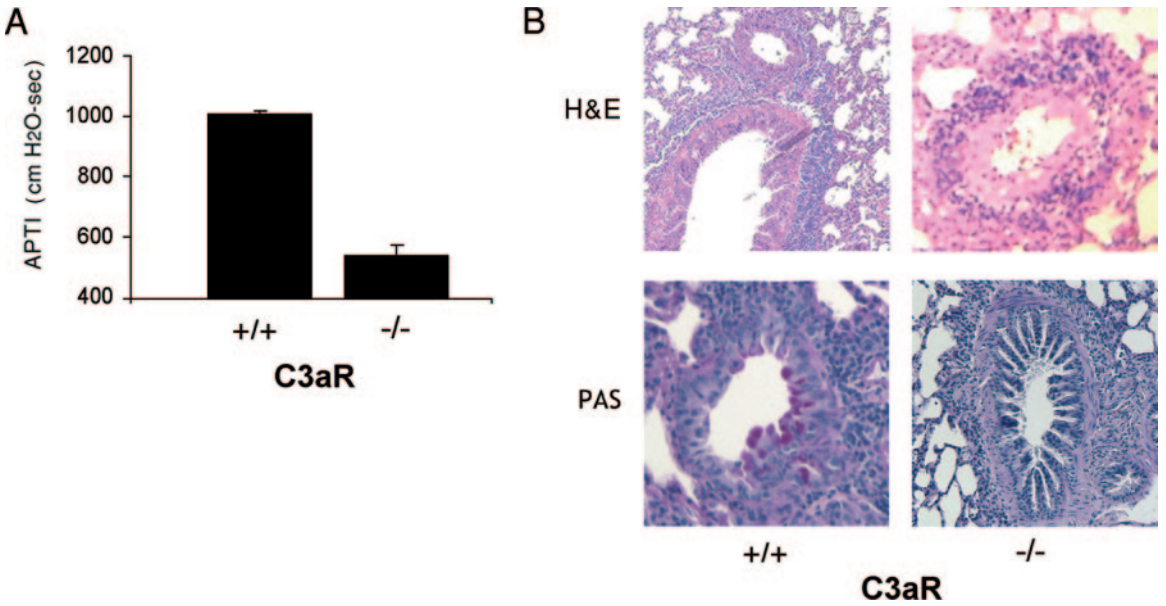

FIG. 4. A role for anaphylatoxin C3a in ERD 7 days after RSV challenge. (A) Airway reactivities to acetylcholine challenge in FIRSV-immunized control ($C3aR^{+/+}$) mice and C3aR-deficient ($C3aR^{-/-}$) mice. AHR was defined by the time-integrated rise in peak airway pressure (APTI). *, $P = 0.0014$. (B) Pulmonary histopathology (hematoxylin and eosin [H&E]) and mucus production (hematoxylin-PAS [PAS]) in $C3aR^{+/+}$ and $C3aR^{-/-}$ mice with ERD.

(Fig. 4B). Contrary to our observations in $C5^{-/-}$ mice, <10 infiltrating eosinophils per 40× field were detected in the lungs of three of five WT mice and one of five $C3aR^{-/-}$ mice. Mucus production (Fig. 4B) was detected in WT mice (score = 2) but not in $C3aR^{-/-}$ mice (score = 1) with ERD. Results similar to those for $C3aR^{-/-}$ mice were observed in WT mice treated with a C3aR antagonist (not shown).

Administration of a C3aR antagonist to $C5^{-/-}$ mice with ERD decreases hyperreactivity, eosinophilia, and mucus production. Subsequently, we examined whether there was an association between the modulatory effect of C5 on AHR during ERD and its effect on C3aR expression. For this purpose, we measured AHR in $C5^{-/-}$ mice with ERD treated with a C3aR antagonist (hereafter referred to as AC3aR mice) in comparison to that in WT, $C5^{-/-}$, and C5H mice (Fig. 1a). Treatment with a C3aR antagonist decreased airway reactivity in comparison to that in $C5^{-/-}$ mice with ERD. These results correlated with measurements of airway resistance to increasing concentrations of aerosolized methacholine, which were higher in $C5^{-/-}$ mice than in WT, C5H, and AC3aR mice ($P <$ 0.01). Again, the estimation of the severity of pneumonia using a disease-specific score (score for AC3aR mice = severe) (Fig. 2, left panel) and the total WBC counts in BAL fluids (Fig. 1c) were unaffected by treatment with the C3aR antagonist.

In addition to its role in AHR (Fig. 1a and b) and lack of effect on the severity of pneumonia (Fig. 1c and 2), we determined whether C3aR affected lung eosinophilia or mucus production. Interestingly, blocking C3aR in $C5^{-/-}$ mice significantly decreased eosinophil infiltration (as determined by histopathology and percent cells in BAL fluids) and mucus production during ERD (Fig. 1 and 2).

Increased IL-5 production is associated with eosinophilia during ERD. To determine whether enhanced eosinophilia correlated with high levels of IL-5 in the lungs of mice during ERD, we compared cytokine production in $C5^{-/-}$ and WT mice. $C5^{-/-}$ mice with ERD and eosinophilia had increased IL-5 production compared to WT mice ($P = 0.03$; Fig. 5A). This Th2 bias was also reflected in the decreased production of IFN-γ ($P = 0.004$) by pulmonary lymphocytes (Fig. 5A). Interestingly, and in agreement with the Th2 pulmonary lymphocytes and lung eosinophils detected in $C5^{-/-}$ mice, IgG subclass distribution differed between $C5^{-/-}$ and WT mice with ERD, when anti-G responses were compared. IgG1/IgG2a ratios were higher in $C5^{-/-}$ mice (Fig. 5B) ($P = 0.04$). No differences were observed in the distribution of IgG anti-F subclasses (Fig. 5B).

Finally, we examined whether, as described for other disease models (45), AHR during ERD was mediated by IL-13. For this purpose, we first compared the mRNA and protein levels of IL-13 in $C5^{-/-}$, WT, C5H, and AC3aR mice with ERD. IL-13 determinations showed no differences in mRNA levels in lung homogenates or protein levels in BAL fluids between all groups (not shown). Subsequently, we targeted the IL-13 receptor at the time of RSV challenge in $C5^{-/-}$ mice previously immunized with FIRSV and measured airway resistance to increasing concentrations of aerosolized methacholine. No differences in resistance were observed during ERD between treated and untreated $C5^{-/-}$ mice (not shown), suggesting that AHR in this model is IL-13 independent.

DISCUSSION

This study describes a novel regulatory mechanism in the complement cascade that affects anaphylatoxin-mediated AHR and lung eosinophilia during ERD. ERD was associated with severe morbidity in the United States in 1967 (6, 13, 25, 27), but its pathogenesis remains unclear. A better understanding of

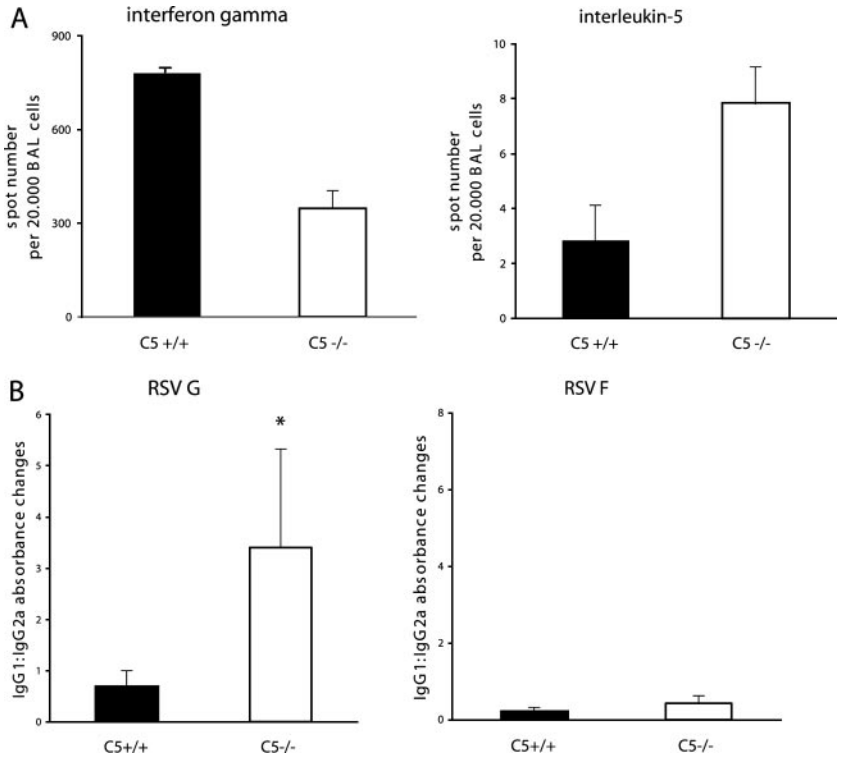

FIG. 5. Th2 bias in $C5^{-/-}$ mice with ERD. (A) IFN-γ and interleukin-5 production by pulmonary mononuclear cells in $C5^{-/-}$ and WT control mice preimmunized with FIRSV, determined 7 days after challenge with RSV. Results are means ± standard errors of the means (error bars) for four to six animals per group and are representative of two independent experiments. *P* was 0.004 for IFN-γ, and *P* was 0.03 for interleukin-5. (B) IgG subclasses against RSV glycoproteins in sera of $C5^{-/-}$ and WT control mice preimmunized with FIRSV, determined 7 days after challenge with RSV. Results are means ± standard errors of the means (error bars) for five to eight animals per group. *, *P* was 0.04 for responses against RSV G.

the mechanism of illness in ERD is critical for the development of safe vaccines to protect young infants against RSV.

Complement and AHR in ERD. Our results indicate that C5 modulates and C3a promotes airway reactivity in ERD. In fact, C3a is necessary for AHR in WT BALB/c mice with ERD compared to its role in $C3aR^{-/-}$ mice and can enhance AHR beyond the levels observed in WT mice when lung C3aR is overexpressed. This exaggerated response is observed in the absence of C5 and can be modulated by a C3aR antagonist or reconstitution with C5 factor (which reestablishes physiologic levels of C3aR).

The complement component C5 has been identified as a susceptibility locus for experimental allergic asthma (26). Production of IL-12 by human monocytes was blunted by blocking the C5a receptor and was decreased significantly in macrophages from C5-deficient mice (26). Since IL-12 can prevent and reverse experimental asthma in rodents, decreased IL-12 production was postulated as a possible mechanism for the role of C5 deficiency in reactive airway disease. In addition, C5 deficiency has been associated with increased AHR, IL-4-producing cells, and eosinophilia in allergic lung disease (10). But a recent report described a positive modulatory role for C5 in AHR, and C5a has been reported to decrease IL-12 production in macrophages (5, 17, 32). These apparently opposite observations may be explained by the different effects of C5a during allergen sensitization or during an established inflammatory response (28). By describing a role for C5 in C3aR expression in the lungs, our data add a new layer of complexity to the role of complement components in pulmonary disease and offer a new mechanistic explanation, which may complement previous observations on the effects of C5 on airway reactivity. Consistent with prior observations of experimental asthma (26), C5 also appears to affect the Th bias of the immune response during ERD, as evidenced by a rise in the number of IL-5 producing lung T cells and in the serum IgG1/IgG2a ratio of antiviral responses in C5-deficient rodents. Interestingly, AHR in our ERD model was unaffected by IL-13 receptor targeting, and similar IL-13 levels were observed in the presence or absence of C5. These findings suggest that the effect of C3aR on airway reactivity in $C5^{-/-}$ mice with ERD is independent of IL-13. AHR may, in fact, be alternatively mediated by other allergic cytokines (33) or inflammatory mediators that are frequently involved in virus-induced episodes of wheezing (15). In fact, acute wheezing during viral infections has been associated with increased production of IL-6, IL-8, and eosinophilic cationic protein (15).

However, and adding to the plasticity of effects observed with the coupling of distinct G proteins (a family of receptors that includes C3aR) (1, 2, 5, 17, 26, 32, 46), the modulatory role of C5 in C3aR expression also appears to be dependent on stimulus and cell type, as evidenced by the decreased expression of the receptor in $C5^{-/-}$ animals inoculated with LPS. Although we did not elucidate the cell origin of the C3aR in the lungs of mice exposed to LPS, an excess of C3aR derived

from inflammatory cells (whether due to differences in C5-induced inflammation [32] or C5 induction of enhanced C3aR expression in this cell population [36]) may contribute to these results. Contrary to the suppressive effect of C5 on C3aR expression in bronchial epithelial and smooth muscle cells described in this paper (where we cannot exclude a role for C5b), costimulation of granulocytes with C5a has been reported to augment C3aR levels by decreasing receptor internalization (36). In the lungs of naïve mice, C3aR is expressed in bronchial epithelial and smooth muscle cells, but expression changes in a cell- and disease-specific manner (12). On a similar note, the significant differences in C3aR expression during ERD observed by RT-PCR, which are less impressive than those observed by immunohistochemistry, may be explained by the ability of immunostains to delineate the cellular distribution of C3aR expression, while RT-PCR and Western blot analyses reflect its combined expression in pulmonary epithelial, smooth muscle, and inflammatory cells.

The C3a anaphylatoxin was reported to play a critical role in AHR in several rodent models of asthma (3, 19) and can elicit AHR by direct contractile effects on smooth muscle cells (43) and/or by promoting a Th2 polarization of the immune response in the lungs (11). C3a instillation caused AHR and aggregation of leukocytes in pulmonary vessels (41), and its relevance during AHR is further supported by detection of elevated levels of C3a peptide in the lungs of patients with asthma (29). Yet, a recent report contradicted these findings, by using a C3aR antagonist in another murine model of asthma and suggesting that C3a may not have an important role in AHR (22). In our model of ERD, C3a appears to play a critical role in airway reactivity, although the mechanisms of AHR and inflammation in ERD differ from those in asthma. Differences between ERD and asthma include the timing of symptoms after challenge (later during ERD [6, 13, 25, 27]), the potential for recurrence (no recurrence has ever been observed in ERD), and a role for immune complexes (34), among other variables.

Complement, pneumonia, and lung eosinophilia in ERD. Contrary to AHR, the severity of the peribronchiolar and perivascular inflammatory infiltration is not affected by complement components. These findings confirm previous observations using $C3^{-/-}$ mice and models of ERD in mice receiving adoptive transfers of immune sera (8, 9, 34). These previous reports ascribed ERD pneumonia to IL-4 and IL-10 production by Th2-biased $CD4^+$ T cells in the lungs of infected mice (8, 9).

Importantly, our findings may affect the long-standing practice of using lung eosinophils as a surrogate marker of the severity of pneumonia during ERD (17, 21, 24, 37). Excess eosinophils detected in $C5^{-/-}$ mice were largely replaced by neutrophils in WT and reconstituted rodents while inflammation was unchanged. In fact, ERD pneumonia in certain mouse strains, cotton rats, and cattle is characterized by a neutrophilic inflammatory infiltration in the absence of eosinophils, suggesting that lung eosinophilia may not be a reproducible phenotype of ERD in different species (14, 22, 35, 39). Furthermore, a review of lung sections from children with ERD also revealed a predominance of neutrophils in the inflammatory infiltrate (35).

Interestingly, an exaggerated degree of pulmonary eosinophilic infiltration and mucus production can be seen in ERD when C3aR is overexpressed (as in B10D2 $C5^{-/-}$ mice). These findings highlight the importance of having functional C5, as its absence would lead to C3aR dysregulation and enhanced eosinophilia, mucus production, and airway reactivity. Conversely, while enhanced expression of C3aR affected these phenotypes, the absence of C3aR in BALB/c mice with ERD affected AHR but had no effect on eosinophil infiltration. With normal C3aR expression levels, the modulatory mechanisms for lung eosinophilia during ERD appear to be dependent on specific cytotoxic T-lymphocyte activity rather than complement components (21, 38) and are affected by formalin inactivation (31). Cytotoxic T lymphocytes are critical for modulating the response to RSV challenge in FIRSV-immunized mice toward Th1 and therefore for preventing eosinophilia (21, 38), while carbonyl groups in formaldehyde-treated vaccine antigens boost Th2 responses (31).

An important strength of this ERD model is its reproducibility in different murine strains, albeit with differences in the degrees of AHR (34). The variation in AHR and eosinophilia observed between BALB/c mice used for C3aR experiments and B10.D2 mice used for C5 experiments may be explained by strain-specific differences in airway reactivity and/or inflammatory responses (16, 40, 42). In addition, strain-dependent differences in RSV pulmonary replication (as shown in Table 1) (40, 42) and the variation in anti-RSV antibody levels after challenge (Table 1) might have led to enhanced or decreased formation of immune complexes promoting complement activation and C3a-mediated AHR.

In summary, we describe a novel autoregulatory mechanism in the complement cascade that modulates AHR, lung eosinophilia, and mucus production during ERD. The effect of C5 on C3aR activity plays an important role in C3a-mediated AHR. These findings provide additional evidence of the importance of complement activation in the pathogenesis of ERD and new mechanistic insight into the role of complement components in AHR. In addition, our data and those of others (5, 12, 17, 26, 28, 32, 46) suggest that interventions in pulmonary diseases aimed at modulating the complement cascade will require careful consideration of the agent eliciting disease and the specific mechanism of illness in each individual process.

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