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Th17 Cytokines Are Critical for Respiratory Syncytial Virus-Associated Airway Hyperresponsiveness through Regulation by Complement C3a and Tachykinins

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Respiratory syncytial virus (RSV) infection is associated with serious lung disease in infants and immunocompromised individuals and is linked to development of asthma. In mice, acute RSV infection causes airway hyperresponsiveness (AHR), inflammation, and mucus hypersecretion. Infected cells induce complement activation, producing the anaphylatoxin C3a. In this paper, we show RSV-infected wild-type mice produce Th17 cytokines, a response not previously associated with viral infections. Mice deficient in the C3aR fail to develop AHR following acute RSV infection, and production of Th17 cytokines was significantly attenuated. Tachykinin production also has been implicated in RSV pathophysiology, and tachykinin receptor-null mice were similarly protected from developing AHR. These animals were also deficient in production of Th17 cytokines. Tachykinin release was absent in mice deficient in C3aR, whereas C3a levels were unchanged in tachykinin receptor-null animals. Thus, our data reveal a crucial sequence following acute RSV infection where initial C3a production causes tachykinin release, followed by activation of the IL-17A pathway. Deficiency of either receptor affords protection from AHR, identifying two potential therapeutic targets. *The Journal of Immunology*, 2011, 187: 000–000.

Infections with respiratory syncytial virus (RSV) in young children and immunocompromised individuals are a leading cause of hospitalization for respiratory illness in the United States and worldwide (1). It is estimated that >50% of infants contract RSV during their first year of life, and >90% are infected by the end of their second season of exposure. Most primary infections are symptomatic, presenting in the form of bronchiolitis and/or pneumonia. RSV is also associated with asthma exacerbations and AHR in nonasthmatic individuals (2). Although anti-RSV Abs are evident following infection, they do not confer complete protection against re-exposure. Efforts toward vaccine development during the 1960s used formalin-fixed virus and were met with dire results. Administration of formalin-fixed virus to infants resulted in severe respiratory disease and significant mortality following subsequent infection (1), and studies have implicated a role for acute immune complex injury as a component of the mechanism (3).

In humans, the pathophysiology associated with severe RSV lung disease includes airway hyperresponsiveness (AHR), mucus

hypersecretion, and a cellular inflammatory response consisting of lymphocytes, neutrophils, macrophages, and eosinophils (4, 5). Mechanistically, this results from activation of a number of interacting proinflammatory pathways, including complement, tachykinins (substance P), TLRs, and chemokines, including CXCL2/3, CCL5, and CX3CL1 (6–10). In addition, alterations in the neurogenic control of respiration have been associated with RSV infection, resulting at least in part from actions of substance P (11).

Mouse models of acute RSV disease are reasonably faithful to the spectrum of changes seen in humans (5). Thus, through studies of knockout strains, they offer the possibility of examining the role of individual components of inflammatory pathways to better understand the pathophysiologic mechanisms involved. Virally infected airway epithelial cells have been shown to induce activation of complement (12), with production of the anaphylatoxins C3a and C5a. These cells are also induced to secrete chemokines, which selectively recruit leukocytes to the infected airways (13). In studies of the role of individual chemokines in RSV pathophysiology, we demonstrated a dependence of AHR, mucus cell hyperplasia, and IFN- γ production on actions of RANTES (9). Mice deficient in CXCR2 were similarly protected (10), as were animals treated with a neutralizing Ab against CX3CR1 (14).

A number of studies have focused on the involvement of substance P and demonstrated protection from RSV-mediated inflammation in animals treated with neutralizing Ab against the tachykinin (15). Pretreatment with the NK1 receptor antagonist, Sendide, also blocked RSV-associated AHR (16). More recent work has identified a substance P homolog, hemokinin-1 (HK-1), which shares full agonist properties at the NK1 receptor (17) but derives primarily from hematopoietic cells, whereas substance P originates predominantly from neuronal sources (18). Because the sequence similarity between the two peptides is so great (SP: RPKPQQFFGLM-NH₂; HK-1: RSRTRQFYGLM-NH₂ [bold-face amino acids indicate sequence identity]), distinguishing them immunologically has not been possible. As a result, studies that concluded a mechanism, based on neurogenic inflammation for

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Abbreviations used in this article: AHR, airway hyperresponsiveness; C3aRI, complement anaphylatoxin C3a; HDM, house dust mite; HK-1, hemokinin-1; MCh, methacholine; P.I., postinfection; RSV, respiratory syncytial virus; *TACR1*^{-/-}, tachykinin receptor-null.

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RSV-mediated pathophysiology, may be subject to reinterpretation, based on understanding the contributions of HK-1 derived from hematopoietic cells. The two tachykinin peptides derive from discrete genes, however, with *TAC1* generating substance P and *TAC4* producing HK-1, enabling the possibility of determining their relative roles by quantitative or RT-PCR (18).

To our knowledge, no studies have been reported relating to the contribution of complement activation products to acute RSV-associated pathophysiology. Our previous studies of mice with targeted deletion of the receptor for the complement anaphylatoxin C3a (*C3aRI*) revealed protection from AHR associated with OVA hypersensitivity (19, 20). C3aR activation in allergen-induced AHR is also implicated in asthma in humans (19, 21) and guinea pigs (22). Furthermore, AHR induced by inhalation of particulate matter was absent in mice deficient in complement C3, the C3a precursor (23). These data support a common mechanism for AHR involving C3aR activation. We thus anticipated that *C3aRI* deficiency would also protect animals from AHR associated with acute RSV infection. In mice sensitized and challenged with house dust mite (HDM) Ag, C3a–C3aR signaling resulted in generation of IL-17A, and pretreatment with a blocking Ab against the cytokine abrogated AHR (24). We further expected that analysis of the resultant constellation of cytokines and tachykinins involved would provide insights about their relative roles in RSV lung disease. By comparing responses of tachykinin receptor-null (*TAC1*^{−/−}) mice with those of *C3aRI*^{−/−} animals, we determined a relationship between RSV-mediated activation of complement and production of tachykinins. Work presented in this paper reveals protection from viral-induced AHR by deletion of the *C3aRI* or *TAC1*, and both strains exhibited significant reduction in the levels of Th17 cytokines, including IL-17A, consistent with a role for activation of this pathway in generation of AHR.

Materials and Methods

Mice

The generation and phenotypic characterization of *C3aRI*^{−/−} and *TAC1*^{−/−} mice have been described previously (19, 25). Animals backcrossed through at least 12 generations to the BALB/c background and wild-type BALB/c mice 6–8 wk of age were used for the studies described in this paper. All studies were conducted in accordance with the Institutional Animal Care and Use Committee of Children's Hospital (Boston, MA).

Virus

Human RSV strain A2 (American Type Culture Collection) was propagated in Hep-2 cells maintained in Eagle's MEM containing 10% heat-inactivated FBS and antibiotics. Cells were harvested at maximum cytopathic effect, and virus was purified under endotoxin-free conditions as described previously (7). Titers were determined by standard plaque assay on Hep-2 cells (26).

RSV inoculation

Mice were inoculated under light anesthesia (isoflurane) by intranasal instillation of ~10⁶ PFU of purified virus in 75 µl endotoxin-free PBS. Sham-infected animals were inoculated with UV-inactivated virus under identical conditions.

Measurement of RSV-induced AHR

For analysis of AHR, at 7 d following inoculation, mice were anesthetized (125 mg/kg pentobarbital) and intubated. Animals were ventilated at 150 breaths/min with a tidal volume of 0.25 ml at 3 cm H₂O-positive end-expiratory pressure. After a stable baseline was established, animals were administered graded doses of MCh by aerosol from 0 to 50 mg/ml, and changes in airway resistance was recorded as cm H₂O × seconds per milliliter (27). Animals were subsequently sacrificed, and lung tissues were processed to evaluate additional parameters of injury.

Assessment of lung cytokines

Lung samples were prepared by homogenization in PBS-containing protease inhibitors (Complete; Roche Diagnostics) and used in ELISAs to measure IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-1β (BD Pharmingen), IL-21 (eBioscience), and IL-17A (BioLegend) following protocols described by the manufacturer. Mouse C3a was determined by ELISA (BD Pharmingen).

Isolation of lung cells

Lungs were excised and minced in serum-free RPMI 1640 medium under aseptic conditions and incubated with 0.22 U/ml Liberase (Roche) and 1 U/ml DNase I for 1 h at 37°C. The total cellularity was determined using an automated cell counter. Cell differentials were determined from Wright–Giemsa-stained cytocentrifuge preparations, counting a minimum of 300 cells from each sample. The remaining cells were stained for flow cytometric analyses.

Flow cytometry

Cells were stained at 4°C in PBS containing 4% goat serum with anti-CD16/CD32 (BD Biosciences) to block FcRs for 30 min, followed by Abs to the surface markers CD45 (allophycocyanin-Cy7 conjugated, clone 30-F11; BD Biosciences), CD4 (Pacific Blue conjugated, clone RM4-5; BD Biosciences), CD8 (PE-Cy7 conjugated, clone 53-6.7; BD Biosciences), CD11b (PerCP/Cy5.5 conjugated, clone M1/70; BD Biosciences), and Ly6G (PE conjugated, clone 1A8; BD Biosciences). For intracellular cytokine staining, cells were permeabilized with saponin (Cytofix/CytoPerm; BD Pharmingen) and stained with anti-IL-6 (allophycocyanin conjugated, clone MP5-20F3; BD Biosciences), IL-17A (Alexa 647 conjugated, clone TC11-18H10; BD Biosciences), or substance P (rabbit polyclonal; Abbiotec), followed by Alexa 647-conjugated anti-rabbit IgG (BD Biosciences). Fixed cells were analyzed using a MoFlo cytometer (Dako-Cytomation/Beckman Coulter) with Summit software.

Measurement of gene expression by quantitative real-time PCR

RNA was purified from lung tissue (RNeasy, Qiagen), reverse transcribed (Superscript III First Strand; Invitrogen), and used as the template for real-time PCR as previously described (Bio-Rad) (27), with primers specific for the RSV matrix protein gene (28), the gob5 gene *mclca3* (9), *C3aRI* (24), *RORγt* (29), IL-23p19 (30), *TAC1*, *TAC4* (31), and GAPDH (16). Results were normalized to GAPDH.

Statistical evaluation

Data are expressed as mean ± SEM and evaluated for significance by Student *t* test (Prism software; GraphPad). Differences are considered significant for *p* ≤ 0.05.

Results

C3aRI^{−/−} mice do not develop AHR subsequent to RSV infection

To determine the impact of the complement anaphylatoxin C3a on RSV-mediated AHR, we examined the reactions of mice with targeted deletion of the C3a receptor gene (*C3aRI*^{−/−}) in comparison with wild-type BALB/c mice following RSV infection. Seven days after intranasal infection with ~10⁶ PFU of human RSV-A2 we tested lung function by evaluating the increases in pulmonary resistance following aerosol administration of graded concentrations of methacholine (MCh) in anesthetized and mechanically ventilated animals (Fig. 1A). Sham-infected mice were administered UV-killed virus under identical conditions. As we and others have previously demonstrated, RSV-infected wild-type mice exhibited significant increases in airway resistance compared with sham-infected animals, with values reaching >5-fold over baseline at 50 mg/ml MCh (9, 10). Contrasting this, the airway resistance for RSV-infected C3aR-null animals was not significantly different from sham-infected animals of either strain.

C3aR deficiency reduces RSV-associated lung inflammation and mucus production

Previous studies have also shown that RSV infection in wild-type mice results in an influx of inflammatory cells into the lungs (32).

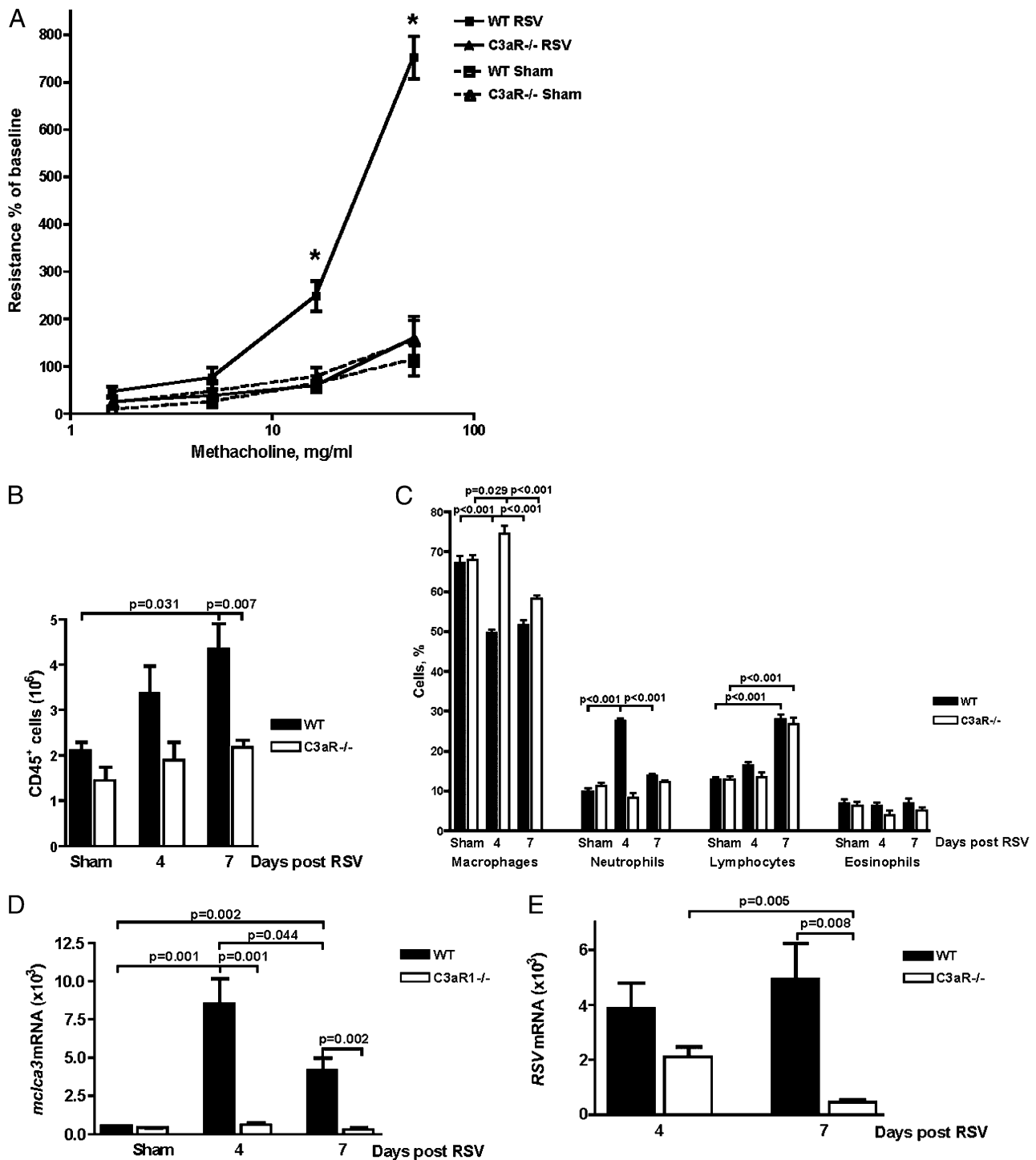


FIGURE 1. *C3aR1*-deficient mice are protected from RSV associated airway pathophysiology. **A**, AHR in RSV-infected *C3aR1*^{-/-} and wild-type BALB/c mice assessed as changes in pulmonary resistance as a function of MCh concentration relative to PBS control. Sham-infected mice were administered UV-killed RSV under identical conditions. ■, RSV-infected wild-type mice; ▲, RSV-infected *C3aR1*^{-/-} mice; □, sham-infected wild-type mice; and △, sham-infected *C3aR1*^{-/-} mice. **p* < 0.001 relative to RSV infected *C3aR1*^{-/-} mice. **B**, Total CD45⁺ cells isolated from lungs of RSV- or sham-infected wild-type and *C3aR1*^{-/-} mice. **C**, Differential analysis of inflammatory cells isolated from lungs of RSV- or sham-infected wild-type and *C3aR1*^{-/-} mice. **D**, Expression analysis of the *mlca3* gene as a function of time postinfection with 10⁶ PFU of human RSV-A2 or UV-killed (sham) virus. Data normalized to GAPDH; significance of differences is indicated. **E**, Assessment of the lung burden of RSV as determined by quantitative real-time PCR of RSV matrix protein RNA at 4 and 7 d following infection normalized to GAPDH. Results are representative of three independent experiments (mean ± SEM for four to five animals per group).

Our findings were similar, with increases in the total cells isolated from the lungs of wild-type mice as a function of time post-infection (Fig. 1B). The increase for *C3aR1*^{-/-} animals was markedly less (NS relative to sham), and at day 7 postinfection (P.I.), the inflammatory cell population was ~50% that of wild-

type mice. Differential analysis revealed an elevation in the neutrophil and lymphocyte populations in wild-type mice at day 4 P.I. with a concomitant decrease in macrophages (Fig. 1C). Contrasting this, *C3aR1*^{-/-} mice exhibited a decrease in the neutrophil population at day 4 P.I., with an increase in macrophages and

lymphocytes. Changes in the eosinophil populations were not significant over the course of the infection for either strain. At day 7, the lymphocyte populations in the lungs were further elevated >2-fold relative to sham treated mice with no significant difference between the two strains.

RSV infection is additionally associated with significant increases in production of airway mucus in BALB/c mice (9). We therefore determined alterations in gene expression of the mucus-associated protein gob5 (*mclca3*) in these animals. This protein has been shown to play a role in the regulation of mucus production and/or secretion and is characteristically upregulated during lung inflammatory reactions (33, 34). Consistent with previous reports, quantitative real-time PCR analysis revealed a dramatic increase in *mclca3* mRNA relative to GAPDH for wild-type mice at day 4 post-RSV infection relative to sham-infected animals, which returned toward baseline by day 7 (Fig. 1D). In marked contrast, *C3aRI*^{-/-} mice exhibited no increase in *mclca3* following RSV infection. Indeed, sham-infected *C3aRI*^{-/-} mice revealed significantly reduced *mclca3* mRNA relative to sham-infected wild-type mice, suggesting a possible role for C3a–C3aR interactions in the maintenance of basal levels of mucus production in the absence of airway inflammation.

RSV is cleared more rapidly in *C3aRI*^{-/-} mice

We used quantitative real-time PCR with primers specific for RSV matrix protein mRNA to assess the lung burden of virus (28). At day 4 P.I., the viral content of *C3aRI*^{-/-} mice was not significantly different from that of wild type mice (Fig. 1E). At day 7 P.I., the viral content of wild-type mice revealed a trend toward further increase, whereas the value for *C3aRI*^{-/-} mice dropped dramatically to ~10% of the wild-type level, ~20% as much as *C3aRI*^{-/-} mice at day 4. Thus, expression of the C3aR appears to retard the clearance of RSV.

C3aRI^{-/-} mice transplanted with wild-type bone marrow exhibit wild-type AHR following RSV infection

Expression of the C3aR has been reported on airway epithelium and smooth muscle as well as numerous inflammatory cell types (35–37). Thus, in efforts to dissect the mechanism of AHR associated with acute RSV infection, we performed reciprocal bone marrow transplantation to lethally irradiated mice. Following 10-wk engraftment, animals were infected with ~10⁶ PFU human RSV-A2 as before, and airway responsiveness to MCh was evaluated 7 d later (Fig. 2). RSV-infected *C3aRI*^{-/-} mice that received cells from wild-type animals exhibited increases in pulmonary resistance to MCh to levels comparable to those of infected wild-type animals (compare with Fig. 1A). In contrast, wild-type mice transplanted with *C3aRI*^{-/-} cells and then infected with RSV exhibited increases in airway resistance comparable to those of *C3aRI*^{-/-} animals. These data therefore suggest that a bone marrow-derived cell type(s) is the predominant driver for virus-associated AHR.

C3a stimulates tachykinin production in RSV-infected mice

As a number of reports indicate a functional role for tachykinins in RSV-associated lung disease (15, 16, 38, 39), we then asked whether mice with targeted deletion of the gene for the NK1 receptor (*TACR1*), equally selective for both substance P and HK-1 (17), were also protected from RSV-associated pathophysiology. As anticipated, *TACR1*^{-/-} mice failed to develop AHR following RSV infection (Fig. 3A). Furthermore, they exhibited no increase in expression of the mucus-associated gene *mclca3* relative to sham-infected mice (Fig. 3B), their inflammatory response was depressed much the same as the *C3aRI*^{-/-} mice (Fig. 3C, 3D),

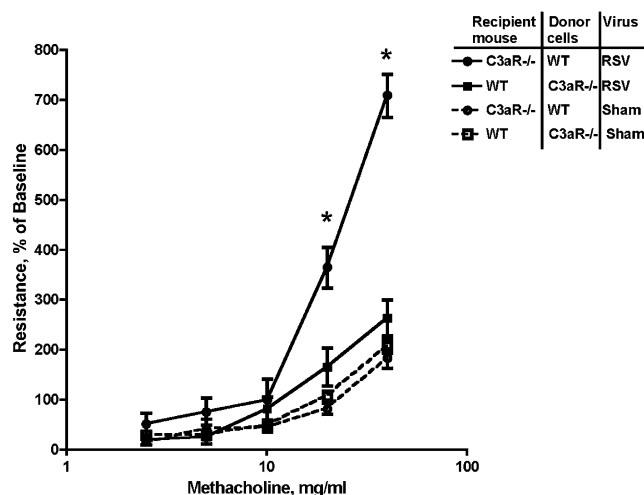


FIGURE 2. Adoptive transfer of wild-type bone marrow cells to *C3aRI*^{-/-} mice restores wild-type AHR associated with RSV infection. Airway responsiveness as a function of MCh concentration in RSV-infected *C3aRI*^{-/-} and wild-type mice after bone marrow transplantation as indicated. Sham-infected mice were administered UV-killed RSV under identical conditions. Results are expressed as the pulmonary resistance normalized to PBS control. ●, *C3aRI*^{-/-} mice transplanted with wild-type bone marrow and infected with RSV; ■, wild-type mice transplanted with *C3aRI*^{-/-} bone marrow and infected with RSV; ○, *C3aRI*^{-/-} mice transplanted with wild-type bone marrow and sham infected; and □, wild-type mice transplanted with *C3aRI*^{-/-} bone marrow and sham infected. **p* < 0.001 for RSV-infected *C3aRI*^{-/-} mice transplanted with wild-type bone marrow relative to RSV-infected wild-type mice transplanted with cells from *C3aRI*^{-/-} animals. Results are the mean ± SEM for six to eight animals per group.

and also like C3aR-null animals, viral clearance was more rapid than wild-type mice (Fig. 3E).

Because the phenotype of RSV-infected *C3aRI*^{-/-} animals was virtually identical to *TACR1*^{-/-} mice, we next asked whether a relationship exists between activation of the C3aR and release of tachykinins. We performed quantitative real-time PCR analysis of *TAC1* and *TAC4* mRNAs to determine the relative expression of substance P and HK-1, respectively, and found expression of both mRNAs was increased in RSV infected wild-type mice (Fig. 4A, 4B), with *TAC1* increasing earlier than *TAC4*. Surprisingly, in *C3aRI*^{-/-} mice, the increase for both tachykinins was greatly reduced, with <50% of that observed in wild-type animals for *TAC1*, and no detectable increase for *TAC4* over sham-infected animals throughout the course of the infection.

This finding suggested a sequence of events following RSV infection in which virally infected cells induce activation of complement to generate the C3a anaphylatoxin. Ensuing C3a–C3aR interactions then elicit the production of tachykinins with subsequent development of AHR, inflammation, and mucus hypersecretion. To further test this scenario, we evaluated the lung levels of C3a in RSV-infected mice (Fig. 4C). Indeed, we found a significant elevation in C3a levels following RSV infection compared with sham-infected mice, and no difference between wild-type and either *C3aRI*^{-/-} or *TACR1*^{-/-} animals, supporting initial activation of complement by viral infection independent of the presence of receptors for downstream signaling. In addition, we found increased expression of C3aR mRNA in wild-type mice (Fig. 4D). In *TACR1*^{-/-} mice, this increase was somewhat suppressed at day 4 P.I. but not significantly different from wild-type animals at day 7. These data support a relatively intact feedback mechanism for C3aR expression despite the absence of inflammation.

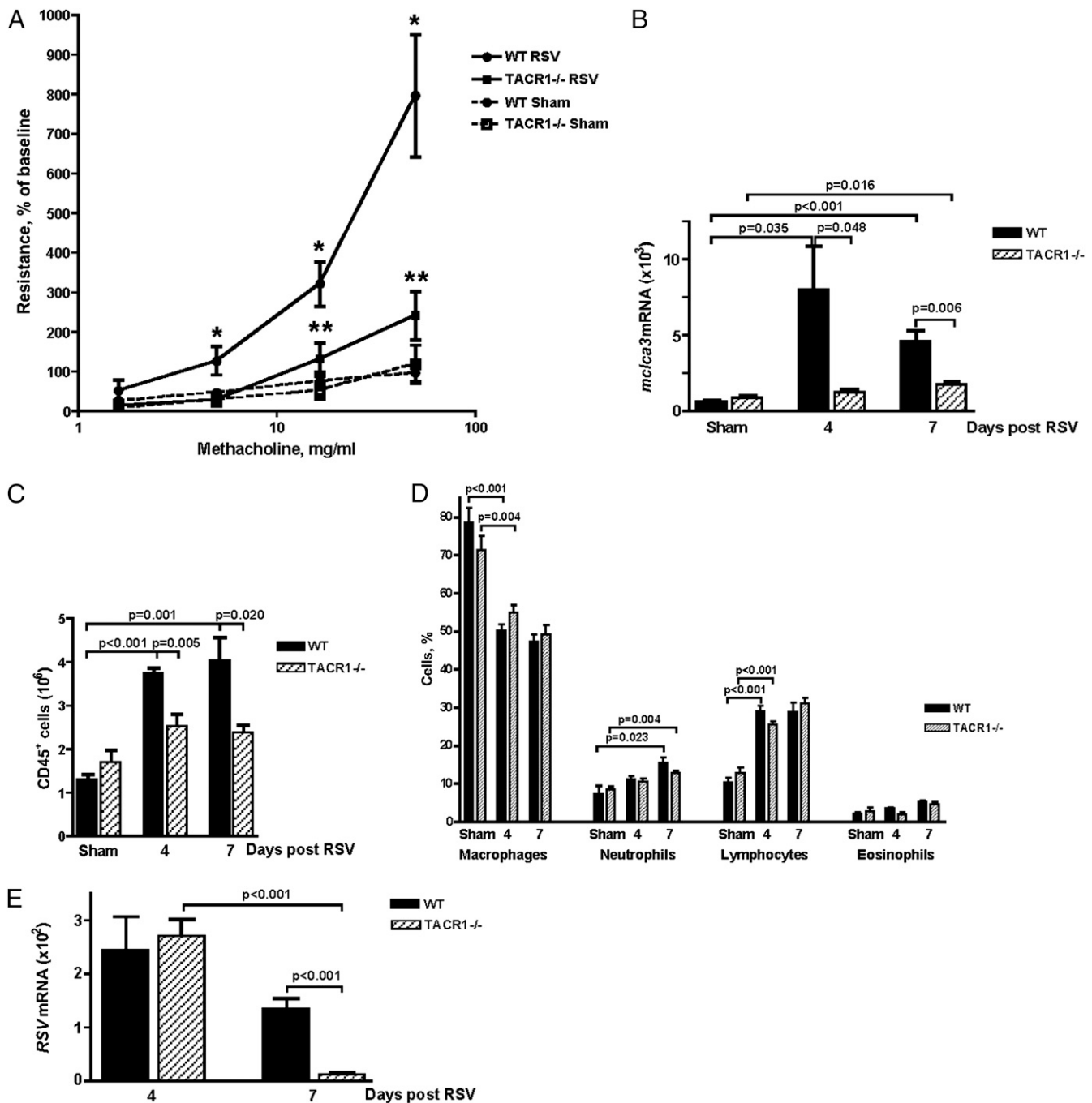


FIGURE 3. *TACR1*^{-/-} mice are protected from RSV-associated airway pathophysiology. **A**, AHR in RSV infected *TACR1*^{-/-} and wild-type BALB/c mice assessed as changes in pulmonary resistance as a function of MCh concentration relative to PBS control. Sham-infected mice were administered UV-killed RSV under identical conditions. ●, RSV-infected wild-type mice; ■, RSV-infected *TACR1*^{-/-} mice; ○, sham-infected wild-type mice; □, sham-infected *TACR1*^{-/-} mice. **p* < 0.001 for RSV-infected wild-type mice relative to RSV infected *TACR1*^{-/-} animals, ***p* < 0.01 for RSV-infected *TACR1*^{-/-} mice relative to sham-infected wild-type or *NK1R*^{-/-} animals. **B**, Expression analysis of the gob5 gene *mclca3* as a function of time postinfection with 10⁶ PFU of human RSV-A2 or UV-killed (sham) virus. Data normalized to GAPDH; significance of differences is indicated. **C**, Total CD45⁺ cells isolated from RSV- or sham-infected *TACR1*^{-/-} or wild-type mice assessed by flow cytometry. **D**, Differential analysis of inflammatory cells isolated from lungs of RSV or sham-infected wild-type and *TACR1*^{-/-} mice. Results are expressed as percentage of total cells. **E**, Assessment of the lung burden of RSV as determined by quantitative real-time PCR of RSV matrix protein RNA at 4 and 7 d following infection. Results are the mean ± SEM for five animals per group. Significance of differences is indicated.

Cytokine profile for *C3aR1*^{-/-}, *TACR1*^{-/-}, and wild-type mice following acute RSV infection

We determined the cytokine profile for *C3aR1*^{-/-} mice in comparison with *TACR1*^{-/-} and wild-type animals following RSV infection to gain further insight into the mechanisms of the signaling reactions involved. Acute viral infections generally result in Th1 responses, with enhanced production of IFN-γ and TNF-α (9,

40, 41). Infections with RSV have further been described as eliciting a "mixed" Th1 and Th2 response, with increases in IFN-γ and IL-10 (42, 43). We thus compared the Th1 and Th2 cytokine profiles of *C3aR1*^{-/-}, *TACR1*^{-/-}, and wild-type mice resulting from RSV infection (Fig. 5). Consistent with previous reports, the IFN-γ levels in lung homogenates from wild-type mice were significantly increased at day 4 P.I., with a return toward baseline

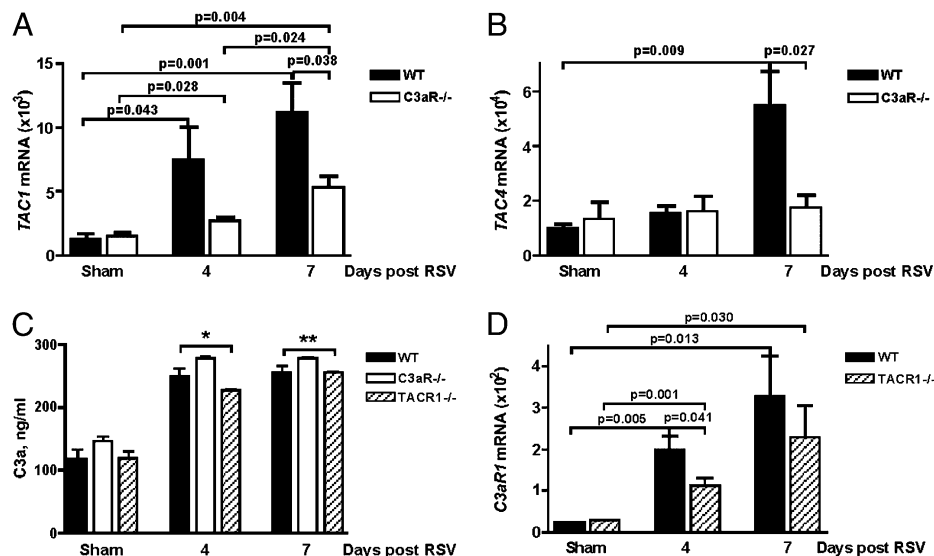


FIGURE 4. RSV-induced expression of tachykinins is reduced in *C3aR1*^{-/-} mice with no concomitant alteration in C3a generation. **A** and **B**, Expression analysis of *TAC1* (**A**, substance P) and *TAC4* (**B**, HK-1) mRNAs in the lungs of RSV and sham-infected wild-type and *C3aR1*^{-/-} mice. Results are normalized to GAPDH; mean ± SEM for four to five mice per group. Significance of differences is indicated. **C**, ELISA determination of C3a levels in the lungs of RSV- and sham-infected wild-type, *C3aR1*^{-/-} and *TACR1*^{-/-} mice. **p* < 0.05 for day 4 post-RSV relative to sham-infected mice, ***p* < 0.001 for day 7 post-RSV relative to sham-infected mice; mean ± SEM for five to nine mice per group. **D**, *C3aR1* mRNA in the lungs of RSV and sham-infected wild-type and *TACR1*^{-/-} mice. Results are normalized to GAPDH; mean ± SEM for four to five mice per group. Significance of differences is indicated.

at day 7 P.I. (32). Both *C3aR1*^{-/-} and *TACR1*^{-/-} mice mirrored this trend, and no significant differences were noted between wild-type animals and either knockout strain at the time points monitored (Fig. 5A, 5B). Similarly, TNF-α was increased in wild-type mice at day 4 P.I. with a return toward baseline by day 7 P.I. As for IFN-γ, we observed no differences between wild-type and either knockout strain at the time points studied (Fig. 5C, 5D).

Previous studies have reported either little change or decreases in Th2 cytokines following acute RSV infection in BALB/c mice (43, 44). To determine the possibility that the deficiency in C3aR signaling alters Th2 cytokine production as it does in models of allergic inflammation (24, 45), we evaluated the levels of IL-4 and IL-5 in *C3aR1*^{-/-} and wild-type mouse lungs following RSV infection. We observed no change in the IL-4 levels for either mouse strain at the time points monitored, and no difference between the two (Fig. 5E, 5F). Levels of IL-5 were increased in wild-type animals at day 4 P.I., with a return toward baseline at day 7 (Fig. 5G). A similar change was evident at day 7 in C3aR-null animals. Thus, differences in Th2 cytokines were minimal between *C3aR1*^{-/-} and wild-type mice.

The C3a-tachykinin axis promotes production of IL-17A family cytokines

AHR associated with experimental allergic reactions as well as reactions to particulate matter in mice has been linked to the production of IL-17A (24, 46). A recent investigation revealed that deficiency of the C3aR ameliorates AHR and abrogates the production of Th17 cytokines (24). Host defense against acute viral infection relies on innate immune reactions, whereas allergic airway diseases involve adaptive responses. Nonetheless, we examined the possibility that acute RSV infection also results in production of Th17 cytokines and that deficiency in the C3aR or the NK1R alters this reaction. In wild-type mice, we found the lung IL-17A content was increased ~3-fold at 4 d following RSV infection (Fig. 6A). *C3aR1*^{-/-} animals revealed a similar increase at day 4. By day 7 P.I., the level for wild-type mice was reduced by ~30% and that for *C3aR1*^{-/-} animals dropped to the level of

sham-infected mice. Contrasting this, *TACR1*^{-/-} mice revealed no detectable increase in IL-17A over sham-infected mice at any of the time points sampled (Fig. 6B).

To confirm and further understand the significance of this finding, we also assessed the levels of cytokines associated with the development and stabilization of Th17 cells (47). IL-6 induces the production of IL-21 in naive CD4⁺ T cells. IL-21 then induces the transcription factor RORγt, which drives the expression of IL-17A (48). We thus analyzed the expression of IL-6 and IL-21 by ELISA in the lungs of RSV-infected mice and found robust expression of both cytokines in wild-type animals (Fig. 6C–F). In *C3aR1*^{-/-} mice, however, the IL-6 and IL-21 levels remained unchanged relative to sham-infected animals throughout the course of the infection. *TACR1*^{-/-} mice exhibited small increases in both IL-6 (15% above sham at day 4 P.I. compared with 100% increase for wild-type animals) and IL-21 (<50% of the increases observed for wild-type mice), but levels were dramatically reduced relative to wild-type animals. We additionally analyzed the expression of RORγt by quantitative real-time PCR and again found robust expression following RSV infection in wild-type mice (Fig. 6G, 6H). C3aR-null animals revealed a small, transient increase in RORγt (<2- versus >3-fold for wild-type mice) and *TACR1*^{-/-} mice showed a slight elevation at day 7 P.I. (4- versus >100-fold relative to sham-infected animals), but these changes were minor in comparison with those observed for wild-type animals.

IL-23 produced by Th17 cells, in conjunction with IL-1β, is thought to induce positive autocrine feedback to generate additional IL-17A and stabilize Th17-committed cells (49). IL-23 consists of two subunits, p19 and p40, the latter of which is shared with IL-12. We thus determined the expression of the unique IL-23 p19 subunit using quantitative real-time PCR and again observed robust increases in RSV infected wild-type mice. A small increase was apparent at day 7 P.I. in *C3aR1*^{-/-} mice, and we found a transient elevation at day 4 P.I. for *TACR1*^{-/-} animals that dropped to the level of sham-infected mice by day 7 (Fig. 6I, 6J). ELISA analysis of IL-1β yielded similar results (Fig. 6K, 6L).

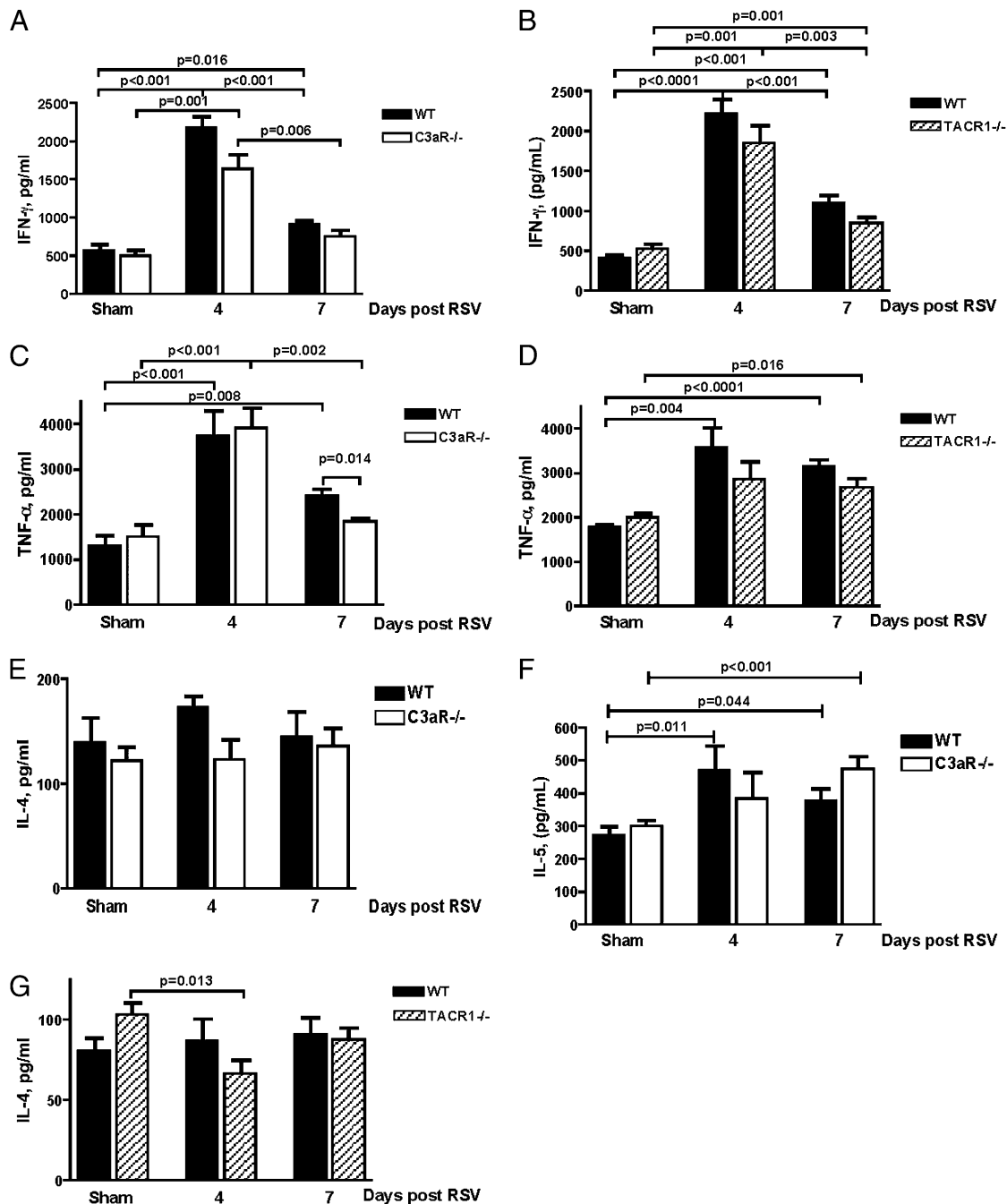


FIGURE 5. Analysis of Th1 and Th2 cytokines in the lungs of RSV- and sham-infected wild-type, *C3aR1*^{-/-}, and *TACR1*^{-/-} mice. *A* and *B*, IFN- γ levels in wild-type and *C3aR1*^{-/-} (*A*) or *TACR1*^{-/-} (*B*) mice following RSV or sham infection. *C* and *D*, TNF- α in lung homogenates of RSV- or sham-infected *C3aR1*^{-/-} (*C*) or *TACR1*^{-/-} (*D*) mice determined by ELISA. *E* and *F*, IL-4 (*E*) and IL-5 (*F*) levels in wild-type and *C3aR1*^{-/-} mice following RSV or sham infection. *G*, IL-4 levels in wild-type and *TACR1*^{-/-} mice following RSV or sham infection. Results are the mean $\pm$ SEM for five mice per group. Significance of differences is indicated.

These data support the concept that C3a and tachykinins act in concert to orchestrate the innate production of Th17 cytokines.

CD11b⁺ cells are the major source of IL-17A in RSV-infected mice

Our cytokine analyses reveal major differences between RSV-infected *C3aR1*^{-/-}, *TACR1*^{-/-}, and wild-type mice in cytokines of the IL-17 pathway, and little or no difference in Th1 and Th2 cytokines. Furthermore, our findings are consistent with the concept that C3a-mediated production of IL-17A and/or an IL-17A-related cytokine is associated with generation of AHR in acute RSV infection as was recently demonstrated in an adaptive im-

mune response (24). We sought to identify the IL-17A-producing cell type(s) in the lungs following RSV infection. Lung cells were isolated from RSV- or sham-infected mice at days 4 and 7 P.I., stained with Abs directed against cell-type specific markers and intracellular IL-17A, and analyzed by flow cytometry. We found significant elevation in IL-17A expression only in CD11b⁺ cells from wild-type mice at day 7 P.I. but not from *C3aR1*^{-/-} animals (Fig. 7*A*). Similarly, cells from *TACR1*^{-/-} mice revealed no detectable IL-17A production from the cell types assessed at any time throughout the infection (Fig. 7*B*). We also assessed IL-6-producing cells in this manner, because at least in RSV infection, IL-6 production appeared to precede IL-17A. We found the

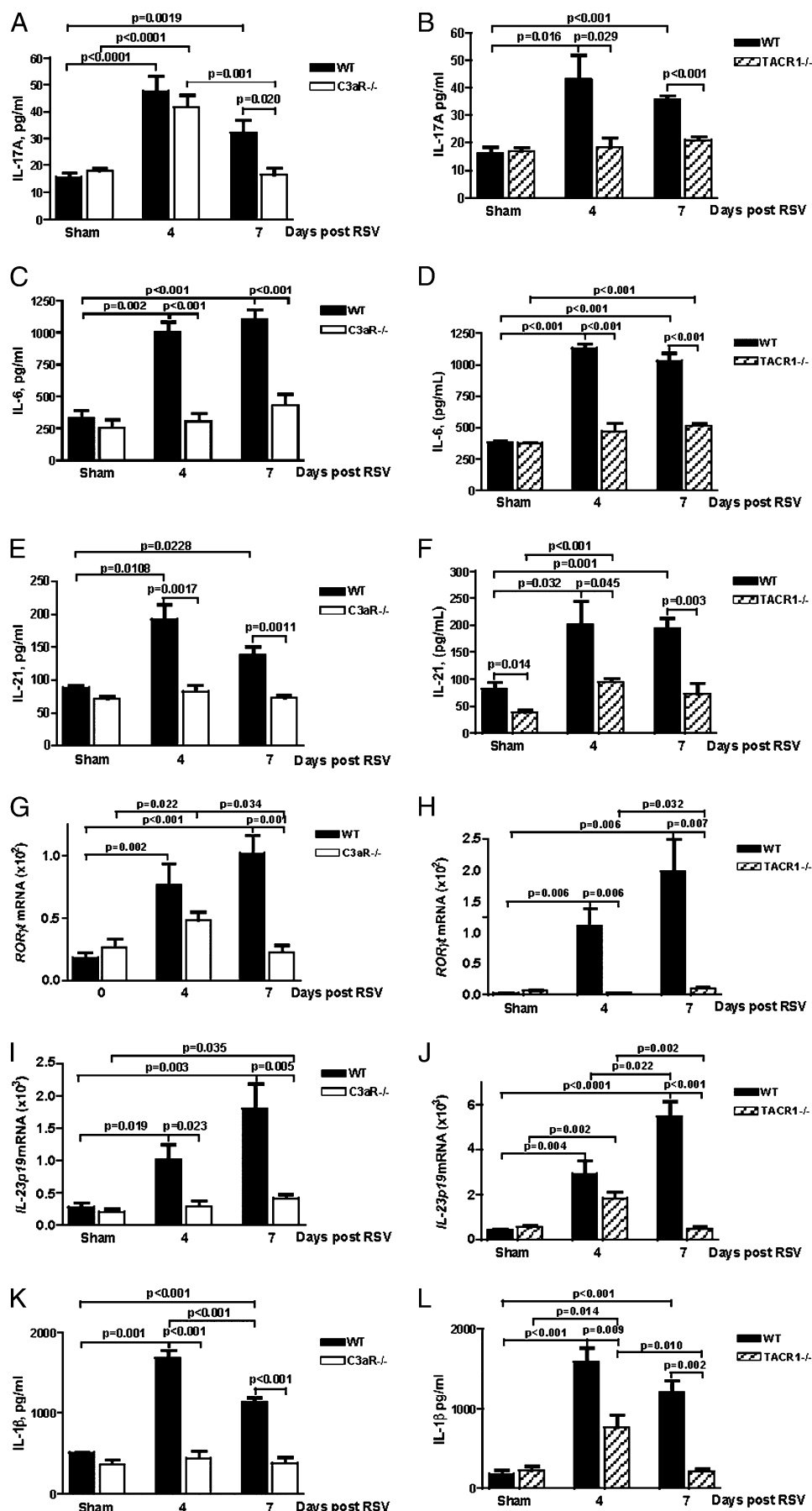


FIGURE 6. Mice deficient in the *C3aR1* or *TACR1* fail to produce Th17 cytokines following RSV infection. IL-17A (A, B), IL-6 (C, D), and IL-21 (E, F) in lung homogenates of RSV- or sham-infected *C3aR1*^{-/-} (A, C, E) or *TACR1*^{-/-} (B, D, F) mice determined by ELISA. *RORγt* (G, H) and *IL-23p19* (I, J) mRNA levels in lungs of RSV or sham infected *C3aR1*^{-/-} (G, I) or *TACR1*^{-/-} (H, J) mice determined by quantitative real-time PCR. K and L, IL-1β in lung homogenates of RSV- or sham-infected *C3aR1*^{-/-} (K) or *TACR1*^{-/-} (L) mice determined by ELISA. Results are the mean ± SEM for five mice per group. Significance of differences is indicated.

majority of this cytokine derives from neutrophils (Ly6G⁺ cells), with maximal production at day 4 following infection. As with IL-17A, and consistent with our ELISA data, neither *C3aR1*^{-/-} nor *TACR1*^{-/-} mouse cells exhibited IL-6 production relative to sham-infected animals.

In addition, in consideration of the uncertainties relating to the cellular source of the tachykinin peptides, we performed intracellular staining for substance P-like immunoreactivity on lung cells following sham or live RSV infection (Fig. 7E). We observed the majority derives from neutrophils and macrophages, peaking at day 4 following infection, with lesser quantities deriving from CD8⁺ and CD4⁺ cells. Consistent with our quantitative real-time PCR data, *C3aR1*^{-/-} mice exhibited no detectable immunoreactive material relative to sham-treated animals throughout the infection.

Discussion

A significant aspect of the pathophysiologic response to acute RSV infection in the mouse, as in humans, is generation of AHR. The work presented in this paper demonstrates, to our knowledge, for the first time, that animals with a deficiency in the receptor for the complement anaphylatoxin C3a (*C3aR1*^{-/-}), do not develop AHR following acute infection with human RSV-A2 in contrast to wild-type BALB/c mice. These animals also fail to develop the mucus hypersecretion and influx of inflammatory cells seen in virally infected wild-type mice.

Innate immune reactions associated with viral infections are characterized by a Th1 response with pronounced secretion of IFN- γ

and TNF- α (43, 50). Hypersensitivity reactions, including human allergic asthma, result from adaptive immune responses and result in production of Th2 cytokines, including IL-4, IL-5, and IL-10. Previous mechanistic studies of AHR associated with Ag sensitization and challenge demonstrated that activation of complement and generation of the C3a anaphylatoxin was causative, because mice (on both BALB/c and C57BL/6 backgrounds) and guinea pigs with deficiency in C3 or the C3a receptor did not develop airway disease (19, 20, 22). Furthermore, human asthmatics develop significant levels of C3a in bronchoalveolar lavage fluid following Ag provocation (19, 21). Studies have also demonstrated that deficiency of C3 affords protection from AHR associated with acute inhalation of particulate matter (23). Taken together, these findings raise the possibility that C3a–C3a receptor interactions are a common initiating signal for subsequent reactions that lead to the state of AHR irrespective of whether adaptive or innate immune mechanisms are invoked.

Analysis of cytokines associated with severe asthma in humans has demonstrated elevated levels of IL-17A, implicating activation of the Th17 pathway (51–54). A recent study of the regulation of IL-17A cytokine production in the context of allergic AHR in mice demonstrated that both AHR and production of IL-17A were diminished in mice deficient in the C3aR in a model of HDM allergy (24). Our data showed that C3a–C3aR signaling in the context of acute RSV infection also results in production of cytokine constituents of the IL-17A pathway, a response previously considered to derive primarily from adaptive immune reactions (47). Our results further demonstrated that C3a–C3aR signaling in

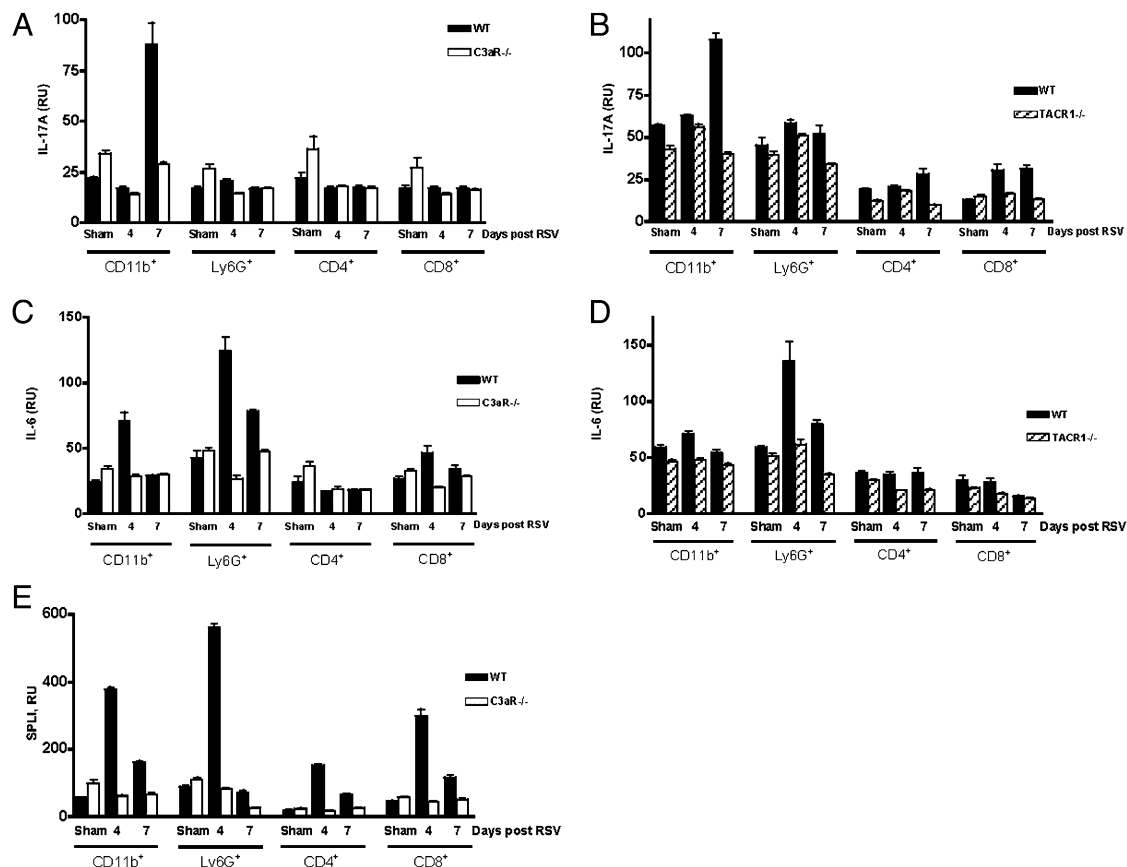


FIGURE 7. Flow cytometric identification of cells producing IL-17A, IL-6, and substance P-like immunoreactive material in the lungs following RSV infection. *A* and *B*, Intracellular cytokine staining for IL-17A in lung cells from wild-type, *C3aR1*^{-/-} (*A*), and *TACR1*^{-/-} (*B*) mice. *C* and *D*, Intracellular staining for IL-6 in lung cells from wild-type, *C3aR1*^{-/-} (*C*), and *TACR1*^{-/-} (*D*) mice. *E*, Intracellular staining for substance P-like immunoreactivity in lung cells from wild-type and *C3aR1*^{-/-} mice. Representative of two independent experiments. Results are the mean $\pm$ SEM for three to five mice per group. RU, relative units.

RSV-infected mice results in production of the tachykinins, substance P and HK-1, and that deficiency in the receptor selective for these peptides, the NK1R, resulted in a phenotype virtually identical to that of *C3aRI*^{-/-} animals. In the absence of the C3aR, tachykinin production was diminished, whereas in the absence of the NK1R, C3a production was not altered. Thus, these findings support a sequence of reactions in which RSV-infected lung cells activate complement generating the C3a anaphylatoxin. Signaling via the C3a receptor elicits production of both substance P and HK-1, and resultant downstream signaling through the NK1R then results in generation of Th17-related cytokines, including IL-17A, IL-6, IL-21, IL-1 β , and IL-23, as well as activation of the transcription factor ROR γ t to produce the pathophysiologic features observed.

C3aRI^{-/-} mice sensitized and challenged with HDM Ag revealed similar depression in production of Th17-related cytokines with the exception of IL-1 β , which remained elevated after Ag challenge. In RSV-infected mice in the absence of the C3aR or the NK1R, IL-1 β was significantly reduced relative to wild-type animals. In addition, in RSV infection, intracellular cytokine staining indicated the majority of IL-17A derives from CD11b⁺ macrophages, whereas in HDM-sensitized and -challenged animals, CD4⁺ cells appeared to be the dominant source (24). Although little information about the IL-17A biosynthetic pathway in macrophages is available, in CD4⁺ T cells, IL-1 β production is not necessarily obligate (47).

Another curious finding is that at day 4 following RSV infection in *C3aRI*^{-/-} mice, the IL-17A level was indistinguishable from that for wild-type animals, and the ROR γ t mRNA was significantly increased relative to sham infected mice. In contrast, in *TACR1*^{-/-} mice, IL-17A was not different from sham infected animals at any time following RSV infection, and ROR γ t was only slightly increased at day 7 P.I. At the same time, IL-21, IL-23, and IL-1 β were elevated at day 4 P.I. in *TACR1*^{-/-} mice, whereas in *C3aRI*^{-/-} animals, these cytokines were not different from sham. It is tempting to speculate that these differences reflect C3a–C3aR signaling in the absence of tachykinin–NK1R signaling. Alternatively, C3a may modulate tachykinin-mediated signaling to produce the overall constellation of cytokines observed for wild-type mice. Nonetheless, the readout of viral associated AHR in the absence of either receptor reveals no significant difference from sham-infected animals.

Mice with a deficiency in either the C3aR or NK1R exhibited a similar viral burden compared with wild-type animals at day 4 following infection; however, by day 7, receptor-deficient animals had virtually cleared the infection. Thus, one possible explanation for the reduction in AHR is that it reflects the reduction in viral load. Although this is formally a possibility, we note that the production of both IFN- γ and TNF- α by either mouse strain was identical to wild-type animals throughout the course of the infection. A possible mechanism for accelerated clearance in the receptor-null mice was suggested by a report of depressed NK cell cytotoxicity by addition of exogenous substance P (55). In this study, IFN- γ production and expression of NK cell activation markers were not altered by the addition of substance P, supporting an otherwise normal cellular response, but cytotoxicity and release of granzyme B were significantly attenuated. A second investigation was based on Ab-mediated blockade of lymphocyte function-associated Ag-1 and revealed a decrease in RSV-mediated pathophysiology but delayed viral clearance and prolonged expression of IFN- γ (56). Thus, although decreased viral burden may be a factor in the protection of *C3aRI*^{-/-} and *TACR1*^{-/-} mice from development of AHR, the studies cited above highlight inherent complexities in the signaling pathways

involved and in no way mitigate the importance of identification of potential therapeutic targets for this serious respiratory disease.

While this paper was under review, a publication appeared from Mukherjee, et al. (57) demonstrating IL-17 expression in primary RSV infections in BALB/C mice. Treatment with a blocking Ab against the cytokine reduced mucus production but did not alter AHR.

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Disclosures

The authors have no financial conflicts of interest.

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