

Pharmacological Targeting of Anaphylatoxin Receptors during the Effector Phase of Allergic Asthma Suppresses Airway Hyperresponsiveness and Airway Inflammation¹

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Airway hyperresponsiveness and airway inflammation are hallmarks of allergic asthma, the etiology of which is crucially linked to the presence of Th2 cytokines. A role for the complement anaphylatoxins C3a and C5a in allergic asthma was suggested, as deficiencies of the C3a receptor (C3aR) and of complement factor C5 modulate airway hyperresponsiveness, airway inflammation, and Th2 cytokine levels. However, such models do not allow differentiation of effects on the sensitization phase and the effector phase of the allergic response, respectively. In this study, we determined the role of the anaphylatoxins on the effector phase of asthma by pharmacological targeting of the anaphylatoxin receptors. C3aR and C5a receptor (C5aR) signaling was blocked using the nonpeptidic C3aR antagonist SB290157 and the neutralizing C5aR mAb 20/70 in a murine model of *Aspergillus fumigatus* extract induced pulmonary allergy. Airway hyperresponsiveness was substantially improved after C5aR blockade but not after C3aR blockade. Airway inflammation was significantly reduced in mice treated with the C3aR antagonist or the anti-C5aR mAb, as demonstrated by reduced numbers of neutrophils and eosinophils in bronchoalveolar lavage fluid. Of note, C5aR but not C3aR inhibition reduced lymphocyte numbers in bronchoalveolar lavage fluid. Cytokine levels of IL-5 and IL-13 in bronchoalveolar lavage fluid were not altered by C3aR or C5aR blockade. However, blockade of both anaphylatoxin receptors markedly reduced IL-4 levels. These data suggest an important and exclusive role for C5aR signaling on the development of airway hyperresponsiveness during pulmonary allergen challenge, whereas both anaphylatoxins contribute to airway inflammation and IL-4 production. *The Journal of Immunology*, 2005, 174: 783–789.

Allergic asthma is a chronic inflammatory disease of the bronchial airways caused by an inappropriate immune response to common aerosol Ags. The disease is characterized by reversible airway obstruction and airway hyperresponsiveness (AHR)³ associated with pulmonary inflammation, and the elevation of total and allergen-specific IgE. Data obtained from animal models of pulmonary allergy suggest that Th2 cells and their secreted cytokines IL-4, IL-5, and IL-13 are critical to pulmonary inflammation and AHR. However, the inflammatory status in asthmatic patients is heterogeneous and the benefit of strategies aimed at inhibiting the adaptive immune responses at the

level of IL-4, IL-13, or their corresponding receptors for the allergic phenotype in human asthmatics needs to be verified (1, 2).

The complement system has long been appreciated for its proinflammatory properties, including degranulation of mast cells, cytokine and chemokine release from eosinophils and mast cells, as well as for its ability to induce smooth muscle contraction. Despite the fact that all of these properties are relevant to the allergic phenotype, complement has not been considered to contribute to the pathogenesis of allergic asthma. During the past few years, however, data from animal models of allergic asthma and from human asthmatics have accumulated that have fueled new interest in the role of complement in the allergic response (3–5). In particular, these data suggest a critical role for the anaphylatoxins (AT) C3a and C5a, cleavage products of C3 and C5, in the development of the allergic phenotype. A natural C3a receptor deficit in guinea pigs protects against bronchoconstriction seen after allergen challenge in a model of OVA-induced asthma (6). Mice with an engineered deficiency of the C3a receptor (C3aR) gene exhibited diminished AHR. Of note, C3aR deficiency on a C57BL/6 background is associated with a decreased Th2 response in a model of OVA and *Aspergillus fumigatus* (AF) induced pulmonary allergy (7, 8). Similar results were found by blocking complement activation at the level of C3 and C4 using a soluble form of mouse membrane complement inhibitor complement receptor-related gene y (Crry) fused to the IgG1 hinge, CH2 and CH3 domains (Crry-Ig) (9). In contrast, no change in the Th2 response was found in C3aR-deficient BALB/c mice sensitized and challenged with OVA, reflecting the complex trait of the asthmatic response. In fact, several susceptibility loci have been described for experimental allergic asthma, the allelic variation of which impacts the allergic phenotype. A role for C5 was suggested by Karp et al. (10)

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Received for publication February 20, 2004. Accepted for publication November 10, 2004.

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¹ This work was supported by Deutsche Forschungsgemeinschaft grant (Graduiertenkolleg 705; to R.B. and A.B.) and the Fraunhofer Institute of Toxicology and Experimental Medicine.

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; AF, *Aspergillus fumigatus* extract; AT, anaphylatoxin; BAL, bronchoalveolar lavage; C3aR, C3a receptor; C3aRA, C3aR antagonist (SB290157); C5aR, C5a receptor; C5aR-mAb, anti-C5aR mAb; desArg, desarginated; EF50, midexpiratory flow; i.n., intranasally; MCh, methacholine; MSA, murine serum albumin.

who described C5 as a susceptibility gene for allergic asthma, the absence of which increased AHR. This effect has been explained by a decreased IL-12 production in C5-deficient animals resulting in an impaired ability to mount a Th1 response finally leading to an enhanced Th2 response. In human asthmatics, the concentrations of the complement cleavage products C3a and C5a in bronchoalveolar lavage (BAL) are significantly higher than those in healthy controls after segmental allergen provocation (11), suggesting substantial local complement activation under asthmatic conditions.

Although these studies highlight the importance of complement factors and cleavage products in the pathogenesis of allergic asthma, the exact contribution of each complement factor, cleavage product, and/or complement receptor signaling pathway in the sensitization or the effector phase of the allergic response remains unclear. In this study, we have determined the impact of pharmacological targeting of the C3a receptor (C3aR) and/or the C5a receptor (C5aR) during the effector phase on AHR, airway inflammation and cytokine production in a murine model of pulmonary allergy. AF was used as an allergen, as this fungus plays an important role in human asthma (12). Our data suggest that C5aR but not C3aR signaling during the effector phase is critical to AHR. Further, signaling through either receptor contributes to airway inflammation and IL-4 production.

Materials and Methods

Animals/study design

Female BALB/c mice (6–8 wk) were obtained from Charles River Laboratories and housed in a specific pathogen-free facility. Mice were maintained on laboratory food and tap water ad libitum in a regular 12-h dark/light cycle at an ambient temperature of 22°C. Experiments were performed with a protocol approved by the animal use and care committee of Bezirksregierung Hannover. Each group comprised 10 animals in experiment A and 12 animals in experiment B.

Treatment

The allergic phenotype was induced essentially as described for the acute AF model with minor modifications (13). In brief, mice were sensitized s.c. and i.p. with an equal volume of 0.1 ml using a mixture of AF (Lot XPM3A3; Greer Laboratories) in sterile saline emulsified with IFA (Sigma-Aldrich). Sensitization was performed using an allergen dose of 5.4 µg of AF per animal. Control animals received sterile saline. Fourteen days later, animals were challenged with aerosolized AF using a Pari Master system (Pari) under defined flow conditions. Particle size was measured using impactor measurement techniques. We found a mass median aerosol diameter (MMAD) of ~2 µm for AF as well as for methacholine (MCh) aerosols (Sigma-Aldrich). Aerosol concentration was measured gravimetrically and animals were exposed to AF aerosol generated out of an AF solution with a concentration of 5.4 mg/ml for 12 min, resulting in a final lung deposited dose of ~5 µg per mouse calculated with a respiratory minute volume of 35 ml/min and a deposition factor of 0.15 (14). Unsensitized control animals received aerosolized saline. Two hours before challenge, animals were treated with the receptor inhibitors intranasally (i.n.) and i.p. at the same time as described in Fig. 1. The second challenge was performed exactly as the first challenge except that the unsensitized control animals received AF as well.

AT receptor blocking reagents

To block C3a receptor signaling, the nonpeptide antagonist N²-(2,2-Diphenylethoxy)-acetyl-L-arginine (SB290157; EMD Biosciences) was used as described (15). Briefly, mice received 200 µg of the C3aR antagonist (C3aRA) i.n. and 500 µg i.p. To block the C5aR, the neutralizing anti-C5aR mAb (C5aR-mAb) 20/70 was administered as described (16, 17) at a dose of 40 µg i.n. and 100 µg i.p. per mouse. Sensitized and unsensitized controls received 100 µg murine serum albumin (MSA; Sigma-Aldrich), i.n. as well as i.p. All reagents were diluted in saline with 0.5% DMSO (Roth) and administered 2 h before allergen challenge under Ketamine/Rompun (Merial; Bayer Vital; KG Leverkusen) anesthesia. The protocol is shown in Table I.

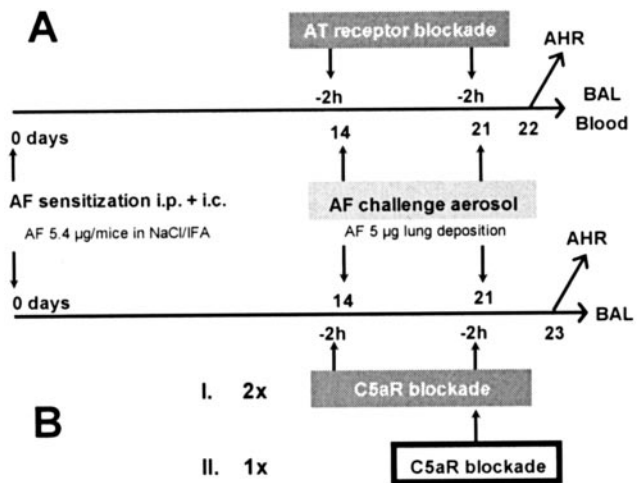


FIGURE 1. Protocol of the AF model of pulmonary allergy. Animals were sensitized at day 0 as described in *Materials and Methods*. Inhalative allergen challenge was performed at days 14 and 21. **A**, Animals were treated with the C3aRA and/or the anti-C5aR mAb (i.p. and i.n.) 2 h before each allergen challenge. Twenty-four hours after the last challenge, AHR was analyzed, and BAL as well as blood samples were taken. **B**, Mice were treated with the anti-C5aR-mAb, either before each challenge (two times) or exclusively before the last challenge (one time). The allergic phenotype was assessed 48 h after the last AF challenge.

Lung function

Lung function was determined using head-out body plethysmography, as described (18). In this setting, the respiratory signal is calculated from a pneumotachograph (PTM 378/1.2) linked to a DP45–14 differential pressure transducer (Validyne) and amplified through a Carrier Frequency Bridge Amplifier (Type 677; Hugo Sachs Electronics). Respiratory signals were analyzed using the Hem 3.4 system (Notocord Systems).

MCh aerosols were generated by a Pari Master aerosol generator. The aerosol concentration in the inhaled atmosphere was continuously determined during the provocation using a clean air mantle aerosol photometer. The photometer was calibrated gravimetrically for aerosol.

Twenty-four hours after the last allergen challenge, lung function was measured during spontaneous breathing of the animal. Flow dependent respiratory signals were transduced into lung function parameters, i.e., tidal volume, respiratory frequency, and midexpiratory flow (EF50), and registered continuously. Baseline measurements were followed by provocation with increasing doses of MCh until a predefined EF50 level was reached which was at least 50% below baseline. EF50 values and MCh doses were used to set up dose-response curves for each animal. Based on these dose-response curves, the cumulative effective inhalation dose was calculated (ED50 = micrograms of MCh needed to decrease EF50 × 50%).

Collection of blood and BAL samples

Mice were sacrificed with an i.p. overdose of pentobarbital-Na (Narcoren; Merial). Blood was drawn by puncture of the vena cava and centrifuged. Serum was shock frozen in liquid nitrogen and stored at –80°C for IgE measurements. BAL samples were obtained by cannulating the trachea, injecting 0.8 ml ice-cold saline (2×), and subsequently aspirating the BAL fluid. BAL cells were washed once in PBS. Cells were counted using a hemocytometer (Omnifab Biosystems). Differential cell counts were obtained from BAL cells spun down onto slides with a cytocentrifuge (Shandon) and treated with May-Grunwald/Giemsa stain (Sigma-Aldrich). A total of 600 cells were morphologically differentiated by light microscopy. For cytokine measurements, supernatants of BAL were shock frozen in liquid nitrogen and stored at –80°C.

Cytokine and IgE ELISA

Cytokine concentrations in BAL were measured by commercially available ELISAs (R&D Systems Duoset ELISA kits). Serum IgE concentration was measured with a Mouse IgE ELISA set (BD Biosciences). ELISAs were performed according to the manufacturer's instructions.

Table I. A study design^a

Group	Sensitization	1st Challenge	2nd Challenge	Treatment
NEG	NaCl	NaCl	AF	MSA
POS	AF	AF	AF	MSA
Expt. A				Blockade
C3aR	AF	AF	AF	C3aRA
C5aR	AF	AF	AF	C5aR-mAb
C3/5aR	AF	AF	AF	C3aRA + C5aR mAb
Expt. B				Blockade
1x	AF	AF	AF	C5aR mAb
2x	AF	AF	AF	C5aR mAb

^a BALB/c mice were sensitized and challenged with *Aspergillus* extract and saline, respectively. Control animals were treated with MSA 2 h prior to each challenge. In Expt. A, mice were treated with a C3aR inhibitor, a C5aR mAb, or both reagents in combination. The allergic phenotype was assessed 24 h after the last AF challenge. In Expt. B, mice were treated with the C5aR Ab, either before each challenge or just before the second challenge. Animals in Expt. B were analyzed 48 h after the last challenge.

Statistics

All values were first tested for normal distribution and variance differences. Statistical differences between two groups were evaluated using Student's *t* test with Welch correction. Data are mean + SEM of 10 animals in experiment A and 12 animals in experiment B. Data were considered significantly different from positive control (POS) at $p \leq 0.05$ (#); $p \leq 0.01$ (##); $p \leq 0.001$ (###); and at $p \leq 0.05$ (*) significantly different from negative control (NEG).

Results

Impact of AT receptor signaling on airway reactivity

An increased sensitivity of the airways toward cholinergic stimuli is a hallmark of the allergic phenotype. This AHR is strikingly attenuated in mice with an engineered deficiency of either C3 or the C3aR (7, 8, 19). In contrast, AHR is markedly increased in mice deficient in C5 (10). These data provide evidence that complement factors or signaling through complement receptors contribute substantially to the development of AHR, however, the mechanisms of this regulatory effect of complement remain unclear. Regulation may occur during allergen sensitization, allergen challenge, or both. We assessed the contribution of AT receptor signaling on the development of MCh-induced AHR during allergen challenge by pharmacological targeting of AT receptors in BALB/c mice. AHR was determined by body plethysmography 24 h after the second challenge with AF. Animals sensitized and challenged with allergen showed a much higher reactivity toward MCh as compared with the unsensitized controls as indicated by a stronger decline of the EF50 (Fig. 2, *left*). Based on individual dose-response curves, the cumulative effective inhalation dose was calculated that results in 50% decrease in EF50 (ED50). The ED50 in unsensitized controls (11.9 ± 2.4) was significantly higher ($p < 0.01$) than in allergen-treated mice (3.0 ± 0.84) (Fig. 2, *right*). Blocking the C3aR had no impact on airway reactivity toward MCh (Fig. 2). However, C5aR blockade improved lung function significantly. In fact, the dose-response curve of anti-C5aR-treated mice was indistinguishable from unsensitized controls (Fig. 2, *left*) as was the ED50 (Fig. 2, *right*). The inhibition of both AT receptors did not substantially change the reactivity of allergen-treated animals toward MCh (Fig. 2, *right*), although there was a trend toward a lower reactivity. These data strongly suggest that C5aR signaling during the effector phase of the allergic response is crucial to the development of AHR, whereas C3aR signaling is not.

We also analyzed AHR 48 h after the last AF challenge. Although AF administration induced eosinophilic inflammation, which is present up to several days after the last AF challenge, no AHR was detectable (data not shown).

Impact of AT receptor signaling on airway inflammation

In addition to increased airway reactivity, the allergic phenotype is characterized by airway inflammation. We determined airway inflammation by measuring total leukocyte numbers and differential cell counts in BAL 24 h after the second challenge with AF. Animals that were treated with saline at sensitization and first challenge, but with AF at the second challenge (Fig. 1), served as negative controls. Total leukocyte numbers were higher in AF-sensitized animals than in the appropriate controls, although the difference did not reach statistical significance. Blocking the C3aR, the C5aR, or both AT receptors significantly decreased total leukocyte numbers ($p < 0.05$, C3aR; $p < 0.01$, C5aR; or both AT receptors; Fig. 3A, *upper left panel*).

No eosinophils were found in unsensitized control animals, whereas huge numbers of eosinophils were present in BAL of allergen-treated mice (Fig. 3A, *upper right panel*). C3aR blocking as well as C5aR blocking reduced eosinophil numbers in BAL of allergen-treated mice by $>50\%$. Blocking both AT receptors did not augment the inhibitory effect, suggesting that the C3aR- and C5aR-induced attraction of eosinophils is mediated through similar redundant downstream pathways. Lymphocyte numbers in BAL of unsensitized controls were very low but increased 15-fold in AF-treated mice (Fig. 3A, *lower right panel*). Inhibition of the C3aR did not affect the lymphocyte numbers in AF-treated mice, suggesting that C3a does not contribute to allergen-induced lymphocyte accumulation in BAL. However, after blockade of the C5aR, lymphocyte numbers were significantly decreased. Blocking both AT receptors had a similar effect. These data suggest that C5a contributes substantially to lymphocyte trafficking during the effector phase of the allergic response. Neutrophil numbers in mice sensitized and challenged with AF were as high as in unsensitized controls (treated with AF at the second challenge). However, C3aR or C5aR blockade markedly reduced neutrophil transmigration into the alveolar space (Fig. 3A, *lower left panel*). This inhibitory effect was even more pronounced when both AT receptors were blocked. These data suggest that a single dose of AF is sufficient to induce a neutrophil-driven inflammatory response in the lung, which is mainly mediated by C3a and C5a. The fact that the inhibition of both AT receptors has a stronger impact on neutrophil recruitment than the inhibition of one AT receptor implies different effector mechanism downstream of C3aR and C5aR activation.

Next, we assessed whether the strong negative impact of C5aR blockade on the development of the asthmatic phenotype reflects a true reduction or just a shift in the kinetics of the allergic response. For that purpose, we blocked the C5aR mice exclusively during the

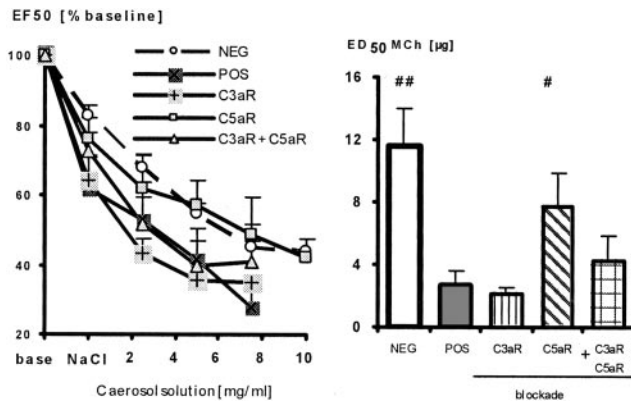


FIGURE 2. Airway reactivity of BALB/c mice to inhalative MCh challenge 24 h after the second allergen challenge. *Left panel*, Dose-response curves of the effect of increasing MCh concentrations on the EF50. *Right panel*, Airway reactivity shown as the effective dose of inhaled MCh that results in a decrease of the EF50 of 50% (ED₅₀). The ED₅₀ for MCh is significantly lower in animals sensitized and challenged with allergen than in unsensitized controls. Blockade of the C5aR during the effector phase prevented the decrease in EF50. Data are mean + SEM; #, $p \leq 0.05$ ($n = 10$ /group).

last allergen challenge. As shown in Fig. 3B, the effect of C5aR blockade on airway inflammation was more pronounced 48 h after the last challenge, when the C5aR had been blocked during each challenge as evidenced by almost abrogated eosinophil accumulation in BAL (Fig. 3B, *left panel*). Strikingly, even a single treatment before the last challenge (as a curative treatment) markedly reduced eosinophil numbers in BAL (Fig. 3B, *left panel*). Further, lymphocyte numbers were dramatically reduced in response to C5aR blockade (before both and only the last challenges (Fig. 3B, *right panel*)).

Impact of AT receptor signaling on cytokine production

The allergic lung is heavily infiltrated with CD4⁺ T cells that are polarized toward a Th2 phenotype and which produce large amounts of IL-4, IL-5, and IL-13 (20). However, the production of these cytokines is not restricted to Th2 cells. In fact, resident and infiltrating cells, such as basophils, mast cells, and dendritic cells have been demonstrated to produce these cytokines as well (21, 22). To evaluate the impact of AT receptor signaling on pulmonary cytokine production, we determined cytokine profiles in BAL. We focused on the Th2 cytokines IL-4, IL-5, and IL-13, as well the Th1 cytokine IFN- γ . As expected, we found significantly higher levels of IL-5 in BAL of AF-treated mice as compared with unsensitized control animals (Fig. 4A). Blocking the AT receptors during allergen challenge did not change the IL-5 levels. IL-4 (Fig. 4B), and IL-13 (data not shown) concentrations in BAL of allergen-treated animals did not increase as compared with unsensitized controls. However, blocking both AT receptors decreased IL-4 levels significantly (~65%, Fig. 4B) suggesting a codominant role of each of the two AT receptors. The IFN- γ concentration was significantly reduced in BAL of sensitized mice as compared with unsensitized controls (~50%, Fig. 4C). Blockade of the AT receptors had no effect on IFN- γ concentrations in BAL.

In addition to the typical Th1/Th2 cytokines, we assessed the concentrations of IL-6, an important regulator of C5aR expression in vitro and in vivo (23). We found elevated concentrations of IL-6 in BAL of sensitized mice as well as in unsensitized controls. Treatment with the C3aRa or the anti-C5aR mAb alone or in combination reduced the IL-6 concentration in BAL as compared with allergen-challenged mice. Interestingly, C5aR blockade was

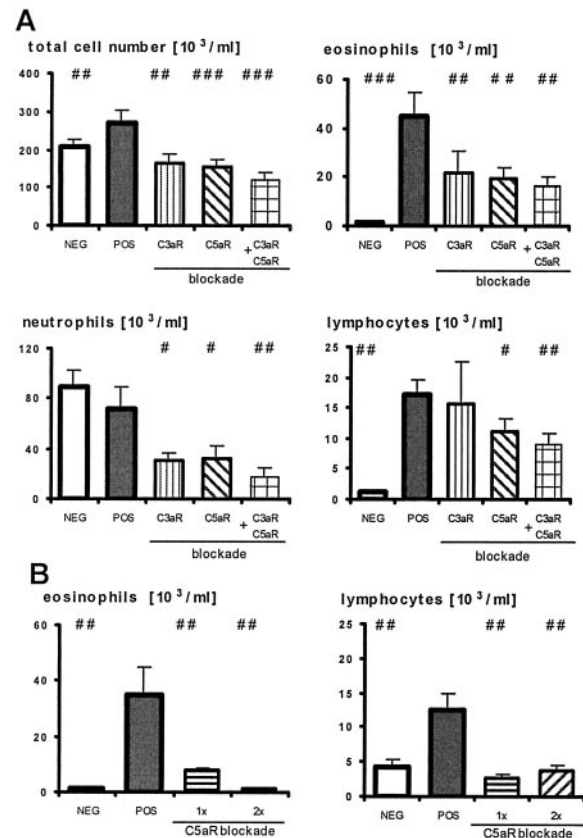


FIGURE 3. A, Leukocyte numbers in BAL of BALB/c mice 24 h after the second allergen challenge. *Upper left panel*, Total cell numbers in BAL. *Upper right panel*, Eosinophil numbers were markedly elevated in AF-sensitized and -challenged mice. Blockade of the C3aR and/or the C5aR significantly decrease eosinophil numbers. *Lower left panel*, Neutrophil numbers were high in AF-sensitized and -challenged mice as well as in unsensitized controls (NEG, exposed to AF only at the second challenge). Neutrophil numbers were significantly reduced after C3aR and/or C5aR blockade. *Lower right panel*, Lymphocyte numbers were high in mice sensitized and challenged with AF but low in unsensitized controls. C5aR blockade but not C3aR blockade decreased lymphocyte numbers. B, Leukocyte numbers in BAL of BALB/c mice 48 h after the second allergen challenge. Eosinophil numbers and lymphocyte numbers were elevated in AF-sensitized and -challenged mice similar to what was observed at 24 h (A, *upper right panel*). Blockade of the C5aR before each allergen challenge or exclusively before the last challenge markedly reduced eosinophil and lymphocyte numbers in BAL. Data are mean + SEM in experiment. #, $p \leq 0.05$; ##, $p \leq 0.01$ ($n = 10$ (A) or 12 (B)/group).

more efficient than C3aR blockade (Fig. 4D) although the difference did not reach statistical significance ($p = 0.066$).

Impact of AT receptor signaling on serum IgE levels

IL-4 is essential for the switching of B cells to IgE Ab production. As shown in Fig. 4B, blockade of both AT receptors decreased IL-4 levels in BAL substantially, suggesting that AT receptor signaling affects allergen-induced production of IgE. Thus, we determined serum IgE concentrations in mice sensitized and challenged with AF and in unsensitized controls. As expected, AF-treated mice had significantly elevated serum IgE concentrations (Fig. 5) as compared with unsensitized controls. However, neither blockade of the C3aR, the C5aR, nor the inhibition of both AT receptors changed serum IgE concentrations in mice sensitized and challenged with AF (Fig. 5).

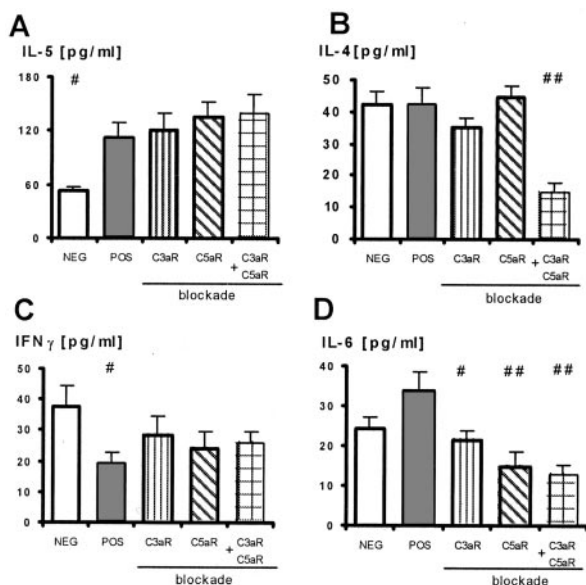


FIGURE 4. Cytokine concentrations in BAL 24 h after the second allergen challenge. *A*, IL-5 concentrations were elevated in AF-sensitized and -challenged mice as compared with unsensitized controls. AT receptor blockade did not modulate the IL-5 concentrations. *B*, IL-4 levels were unchanged after allergen sensitization and challenge. Treatment with the C3aR antagonist or the anti-C5aR mAb had no impact on the IL-4 concentration. However, blockade of the C3aR and the C5aR simultaneously strongly reduced IL-4 concentrations. *C*, IFN- γ concentrations were significantly reduced in sensitized and challenged mice compared with unsensitized controls. AT receptor blockade did not modulate IFN- γ concentrations; *D*, IL-6 was elevated in allergen-sensitized and -challenged mice compared with unsensitized controls. C3aR and/or C5aR blockade decreases IL-6 levels. Data are mean $\pm$ SEM; #, $p \leq 0.05$; ##, $p \leq 0.01$ ($n = 10$ /group).

Discussion

Genetic deletion of the C3aR markedly attenuated AHR in murine models of allergic asthma in which either OVA (8) or a combination of OVA and AF (7) had been used as allergens. Although these data convincingly demonstrated a crucial role for C3a in the development of AHR, they do not allow for differentiation of the role of C3a during the sensitization and/or the effector phase. Of note, airway inflammation, Th2 cytokines in BAL and serum IgE in C3aR-deficient BALB/c mice were indistinguishable from wild-type controls in the OVA model suggesting that C3a predominantly acts during the effector phase (8). In contrast, airway inflammation, Th2 cytokines, and serum IgE were significantly reduced in C3aR-deficient C57BL/6 mice immunized and challenged with a combination of OVA and AF. These data clearly point toward an important role for C3a during Th2 development. Our data support the latter view, as we found no change of AHR after pharmacological targeting of the C3aR during the effector phase.

A role for C5a in allergic asthma has been suggested by a study in which C5 was identified as a susceptibility locus for allergic asthma. C5-deficient animals showed a more sensitive phenotype of allergen-induced AHR. In vitro functional data demonstrated defective IL-12 production by C5a-deprived monocytes and macrophages. These data suggest a mechanism in which C5a is needed to drive IL-12 production as a crucial means to mount a Th1 response, preventing or reversing allergic asthma (10). Although this is an intriguing hypothesis, no direct evidence was provided in this study for a role of C5a. We found a significant reduction of AHR in response to C5aR blockade during the effector phase, strongly

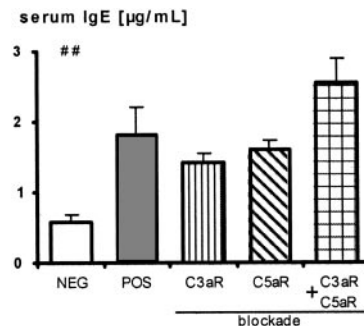


FIGURE 5. Serum concentration of total IgE 24 h after the second allergen challenge. Serum IgE concentrations were significantly elevated in allergen treated mice as compared with unsensitized controls. AT receptor blockade did not modulate the production of serum IgE. Data are mean $\pm$ SEM; ##, $p \leq 0.01$ ($n = 10$ /group).

suggesting an AHR-promoting effect of C5aR in the sensitized host. Thus, C5a may have opposing roles during the sensitization and the effector phase of the allergic response. In support of this view, Abe et al. (24) found a decreased immediate and late airway response by administering a peptidic C5aR to OVA-sensitized rats. This effect could be reversed by intratracheal instillation of rat C5a desArg. These authors did not assess AHR. Together these data suggest that targeting the C5aR may be an effective therapeutic approach for treating patients suffering from allergic asthma. Our data demonstrating that the blockade of C5aR and C3aR signaling abolish the protective effect of C5aR inhibition on AHR suggests a complex network of activating and inhibitory pathways. In support of this view, C3a binds to a specific C3aR, whereas C3a desArg (desArg) has lost its ability to bind to the C3aR. Importantly, C3a and C3a desArg can bind to another receptor, C5L2 (25). Blocking the C3aR will shift the binding of C3a from C3aR toward C5L2. Based on these data it is tempting to speculate that C5L2 activation by C3a has a proallergic effect which may also explain why the blockade of the C3aR had no impact on AHR. Further, blockade of the C3aR may abrogate the anti-inflammatory effect of C3a on mononuclear cells (26).

AHR develops in the environment of inflamed airways. Airway inflammation is driven by Th2-type cytokines including IL-4, IL-5, and IL-13. The sources for the cytokines include eosinophils, lymphocytes, mast cells, and basophils, all of which participate in the allergic responses. Further, neutrophils contribute to allergic inflammation through the release of proinflammatory cytokines (e.g., TNF- α , IL-6) and CXC chemokines. All of these cell types express AT receptors (27–31), and C3a and C5a have the potential to recruit (29, 32, 33) and to activate (27, 34) these participating cells. Our data point toward an important role for both ATs in recruiting these cells as both blockade of the C3aR as well as the C5aR reduced total cell numbers of infiltrating cells in AF-sensitized and -challenged mice. However, our data suggest a differential role for C3a and C5a in eosinophil, neutrophils, and lymphocyte recruitment. Blocking either of the AT receptors significantly reduced the numbers of infiltrating eosinophils and neutrophils, which is in agreement with findings by Abe et al. (24) who found decreased eosinophil and neutrophil numbers in response to blockade of C5aR in allergic rats. In a separate experiment in which the allergic phenotype was analyzed 48 h after the last allergen challenge, eosinophil recruitment into the lung lumen was almost abrogated by C5aR blockade. The importance of eosinophils in airway remodeling in allergic asthma has been recently demonstrated (35–38). C3a, unlike C5a, is not a chemoattractant to neutrophils (32). Consequently, the observed reduction of neutrophils in BAL in

response to C3aR blockade must be due to indirect effects of C3a, e.g., through eosinophil-dependent neutrophil chemotaxis (32). In contrast, C3a and C5a are potent chemoattractants (32, 39) and activators (40) of human eosinophils suggesting a direct effect of both AT to recruit eosinophils to the airways. In fact, C3a (41) and C5a increase eosinophil adhesion to cytokine-activated and resting bronchial epithelial cells (39, 42). Of note, the reduction of eosinophils after ATs receptor blockade is not associated with decreased IL-5 levels. IL-5 and eotaxin play an important role in promoting the initial movement of eosinophils into airway tissue in asthma (43). Thus, cooperative action of several inflammatory mediators and adhesion molecules orchestrates eosinophil recruitment during allergic inflammation in the airways. The importance of eotaxin together with IL-5 is not in dispute. Our data suggest that the ATs are important, supplementary players in the network of mediators that recruit eosinophils to the airways. Investigations using primary cultured human bronchial epithelial cells clearly point toward an important role of the ATs in promoting adhesion by up-regulation of β_2 -integrin expression and shedding of L-selectin on eosinophils (14). Many other chemokines, such as eotaxin, RANTES, and MIP-1, have little or no effect on eosinophil adhesion to bronchial epithelial cells (44).

Our findings that blocking the AT receptors reduces airway inflammation are in agreement with data obtained in C3- (19) and C3aR-deficient C57BL/6 mice (7) in a model of AF- and OVA-induced pulmonary allergy. However, they are in contrast to data obtained with C3aR-deficient BALB/c mice and C3aR-deficient guinea pigs in an OVA model of allergic asthma. These data suggest that the nature of the allergen (AF vs OVA) is of major importance for the complement-mediated recruitment of inflammatory cells into the lung. Active proteases represent a critical biochemical activity underlying intrinsic AF allergenicity that is missing in OVA. Such proteases are able to cleave local C3 and C5 to generate C3a and C5a (45). Further, AF can activate the complement cascade by the lectin pathway (46).

Surprisingly, attraction of lymphocytes was affected by C5aR but not by C3aR blockade, suggesting that C5a but not C3a acts as a chemotaxin for lymphocytes in the effector phase of the allergic response. This effect was even more pronounced 48 h after the last allergen challenge. In contrast to our data, lymphocyte numbers in BAL were markedly reduced in the absence of C3 or the C3aR in the combined OVA/AF model of allergic asthma (7). Expression of C3aR has been described on activated but not on resting B (47) and T cells (48). Clearly, C3a is able to induce transient increase in Ca^{2+} in activated T cells, however, no biological function has been linked to C3aR signaling yet (48). Conflicting results have been reported for the expression and function of the C5aR on T cells. Data from several groups suggest that naive T cells do not express the C5aR (17, 48). However, Nataf et al. (29) found expression of the C5aR on a subpopulation of resting T lymphocytes, which was up-regulated after PHA stimulation. Furthermore, this population of T cells was chemotactic to C5a at nanomolar concentrations. Together, the available data suggest a model in which C5a plays an important role in T cell recruitment during the effector phase of the allergic response, whereas C3a plays an important role in T cell activation during the sensitization phase promoting Th2 skewing and proliferation.

As therefore expected, we found no effect of AT receptor blockade on IL-5 and IL-13 production. However, IL-4 BAL levels were strongly decreased after combined C3aR and C5aR inhibition. IL-4 is a typical Th2 cytokine, the major function of which is to differentiate B cells and to induce B cell isotype switch to IgE during sensitization (49). During the effector phase of the allergic response, mast cells, basophils, and T cells are considered the main

source of IL-4 (21, 22, 50). The fact that IL-4 levels in unsensitized controls and AF-sensitized and -challenged mice were indistinguishable, whereas lymphocytes were virtually absent in BAL of unsensitized animals, strongly suggests that T cells are not the primary source of IL-4. Thus, it is most likely that the inhibition of IL-4 results from AT receptor blockade on pulmonary mast cells and basophils. In agreement with this interpretation, we found no impact of AT receptor blockade on serum IgE levels.

In summary, our data provide evidence that AT receptor signaling during the effector phase of asthma has a crucial impact on the development of AHR and airway inflammation in a model of AF-induced pulmonary allergy that is relevant to human disease. Especially C5a seems to play a crucial role in the inflammatory response as even a single treatment with a blocking C5aR-mAb markedly reduced eosinophil and lymphocyte numbers in the alveolar space. In addition to data suggesting an important protective role for C5a in the development of the allergic phenotype (10), our data demonstrate that C5a promotes AHR in sensitized animals. Thus, the role of C5a in allergic asthma appears to be quite complex with opposing effects during allergen sensitization and allergen challenge. The complexity is no less for C3a. Although we found no contribution of C3aR signaling to AHR during the effector phase, airway inflammation was significantly decreased. Together with the fact that AHR is reduced in all models of pulmonary allergy with C3aR-deficient animals, our findings suggest that C3a modulates AHR during the sensitization phase, most likely through regulation of T cell skewing toward a Th2 phenotype. Although we are just at the beginning to understand the mechanism by which complement regulates the allergic phenotype, our data suggest that pharmacological targeting of AT receptors in sensitized individuals may be useful as a therapeutic strategy.

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