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A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma

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Complement component 5 (C5) has been described as either promoting or protecting against airway hyperresponsiveness (AHR) in experimental allergic asthma, suggesting pleomorphic effects of C5. Here we report that local pharmacological targeting of the C5a receptor (C5aR) prior to initial allergen sensitization in murine models of inhalation tolerance or allergic asthma resulted in either induction or marked enhancement of Th2-polarized immune responses, airway inflammation, and AHR. Importantly, C5aR-deficient mice exhibited a similar, increased allergic phenotype. Pulmonary allergen exposure in C5aR-targeted mice resulted in increased sensitization and accumulation of CD4⁺CD69⁺ T cells associated with a marked increase in pulmonary myeloid, but not plasmacytoid, DC numbers. Pulmonary DCs from C5aR-targeted mice produced large amounts of CC chemokine ligand 17 (CCL17) and CCL22 ex vivo, suggesting a negative impact of C5aR signaling on pulmonary homing of Th2 cells. In contrast, C5aR targeting in sensitized mice led to suppressed airway inflammation and AHR but was still associated with enhanced production of Th2 effector cytokines. These data suggest a dual role for C5a in allergic asthma, i.e., protection from the development of maladaptive type 2 immune responses during allergen sensitization at the DC/T cell interface but enhancement of airway inflammation and AHR in an established inflammatory environment.

Introduction
Asthma is a chronic inflammatory disorder of the lung that manifests as recurrent episodes of wheezing, breathlessness, chest tightness, and coughing in response to exposure to environmental stimuli. Airflow obstruction, airway hyperresponsiveness (AHR), and airway inflammation are pathophysiological characteristics of the disease that are associated with the presence of lymphocytes, eosinophils, and mast cells along with epithelial desquamation, goblet cell hyperplasia, and thickening of the submucosa. Although asthma is multifactorial in origin, it is generally accepted that it arises as a result of inappropriate immunological responses to common environmental antigens in genetically susceptible individuals (1, 2). Specifically, a multitude of evidence suggests that CD4⁺ Th2 lymphocytes play a critical role in disease pathogenesis.

The complement system comprises a network of more than 30 proteins that are crucial to host defense. Serine proteases generated in response to activation of the complement system, as well as allergen- (3) and tissue-derived (4) proteases, can cleave the low molecular weight anaphylatoxins complement component 3a (C3a) and C5a from C3 and C5, respectively. The release of these anaphylatoxins contributes significantly to the benefit and burden of inflammation (5–7). Several studies have demonstrated that both C3a and C5a are specifically released into the challenged asthmatic lungs and that the inflammatory infiltrate of eosinophils and neutrophils correlates highly with the amount of anaphylatoxins present (8, 9). Importantly, many pathophysiologic features of allergic asthma, such as smooth muscle contraction, increased vascular permeability, mucus secretion, and recruitment of inflammatory cells, have long been known to be consonant with well-defined effects of the anaphylatoxins. Along these lines, deficiencies in C3 (10, 11) and the receptor for C3a (8, 12, 13) protect from development of acute bronchoconstriction, AHR, airway inflammation, and Th2 cytokine production, although the effects on inflammation and Th2 cytokine production are controversial and appear to be strain- and antigen-dependent (14). These data suggest a critical role for C3a during the effector phase of the allergic response. In addition to C3 and C3a, pharmacological targeting of C5 and the C5a receptor (C5aR) in an established allergic environment has been shown to reduce early and late phase airway constriction, AHR, and airway inflammation in rodent models of pulmonary allergy (15–17), indicating that both C3a and C5a positively regulate the allergic phenotype at the level of receptor function on airway epithelial, smooth muscle, and/or infiltrating cells. In contrast, we previously identified a strong association between C5 deficiency and allergen-induced AHR in a murine model of asthma (18). Surprisingly, C5 deficiency was linked to enhanced airway responsiveness, challenging the paradigm that downstream cleavage products C5a and C5b (nucleating the membrane attack complex) are mere proinflammatory mediators. Further, we found defective IL-12 production by C5a-deprived human...
monocytes and C5-deficient macrophages, providing a plausible mechanism for the regulation of susceptibility to asthma by C5a, as IL-12 drives type 1 adaptive immune responses, preventing or reversing experimental allergic asthma (19, 20).

Dissecting the pro- and antiallergic roles of C5 and C5a in the pathogenesis of allergic asthma is of critical importance in light of the potential role of C5a as a therapeutic target (17, 21). Previous studies have focused entirely on the role of C5 or C5a during the effector phase of the allergic response (16–18, 22), neglecting the possible impact of C5a on allergen sensitization that may account for the controversial findings. The current study was designed to fill this gap and dissect the role of C5a in each phase of the allergic response independently. For this purpose, we focused on 2 models of pulmonary exposure, using either OVA leading to inhalation tolerance (23) or HDM inducing Th2 sensitization, airway inflammation, and AHR (24). We found that in vivo C5aR targeting during initial pulmonary allergen exposure is associated with either the induction or the enhancement of Th2 adaptive immunity, eosinophilic airway inflammation, and AHR. In contrast, C5aR targeting in an established inflammatory environment reduced airway inflammation and AHR, suggesting a second, proallergic role of C5a through its proinflammatory properties on resident and/or infiltrating cells.

Results

Ablation of C5aR signaling during initial pulmonary OVA exposure induces airway inflammation, Th2 skewing, mucus production, and AHR. Intranasal administration of OVA has been shown to induce antigen-specific inhalation tolerance (25, 26). Similarly, in our model of intratracheal (i.t.) OVA administration (detailed in Figure 1), we found no increase in the production of either Th2 (IL-4, IL-5, IL-10, or IL-13) or Th1 cytokines (IFN-γ; data not shown) from pulmonary cells isolated 24 hours after the final exposure to OVA when compared with PBS controls (Figure 2A). Further, serum IgE concentrations were indistinguishable from PBS controls (Figure 2B). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic inflammation (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C). In pulmonary tissue, we found minor peribronchiolar infiltrates and mucus production (Figure 2D). Total cell numbers in bronchoalveolar lavage fluid (BAL) were slightly elevated, which was mainly due to increased neutrophilic numbers (Figure 2C).
in BAL) when C5aR-mediated signaling was ablated by the utilization of either C5aR-deficient mice (Supplemental Figure 2) or by pharmacological targeting of C5aR prior to initial pulmonary allergen exposure, using a neutralizing anti-C5aR–specific mAb (27) (Figure 2, A–C). The anti-C5aR mAb has been demonstrated to neutralize C5aR signaling in several in vivo models of allergic asthma (16) and immune complex disease (28). Of note, the magnitude of IL-5, IL-13, and IL-10 production was 10-fold higher in response to pharmacological targeting as compared with C5aR ablation due to C5aR deficiency (Figure 2A versus Supplemental Figure 2A). Similarly, the magnitude of airway inflammation was more pronounced in response to pharmacological C5aR blockade (Figure 2C versus Supplemental Figure 2B). Histological examination of the lung 24 hours after the last OVA challenge showed dense perivascular and peribronchiolar infiltrates, comprising mainly eosinophils and lymphocytes, and abundant mucin in epithelial cells when C5aR signaling was ablated in response to pharmacological targeting (Figure 2D and Supplemental Figure 1B). Again, the effect was less pronounced in C5aR-deficient mice (data not shown). In addition, we found a strong increase in allergen-induced AHR in response to anti-C5aR treatment (Figure 2E). C5aR deficiency

Figure 2
C5aR targeting induces Th2 adaptive immune responses and eosinophilic airway inflammation in response to pulmonary OVA exposure. (A) Cytokine profile of pulmonary cells harvested from BALB/c mice 24 hours after final OVA exposure. Supernatants were collected after 72 hours in vitro culture. (B) Serum concentration of total IgE. (C) Total and differential cell counts in BAL. (D) Histological examination of airway inflammation. Sections were stained for mucus production with PAS (left panels) and with H&E (right panels). Original magnification, ×200. (E) Airway responsiveness to i.v. acetylcholine (Ach). Airway responsiveness is expressed as the time-integrated change in airway pressure over baseline pressure (APTI). In all figures, values shown are the mean ± SEM; n = 8–10 per group. **P < 0.001; *P < 0.05.

had no impact on AHR development (Supplemental Figure 2C).
Importantly, pharmacological targeting of the C5aR in the absence of OVA exposure induced minor airway inflammation, production of Th2 cytokines, increased serum IgE, and AHR (Figure 2). These data suggest that C5aR activation during allergen sampling provides a critical signal preventing the development of Th2 adaptive immune responses.

C5aR blockade during pulmonary HDM exposure increases airway inflammation, Th2 skewing, mucus production, and AHR. To determine whether this protective effect of C5a applies to natural antigens that are associated with airway sensitization in human asthma, we assessed the impact of C5aR blockade in a murine model of HDM-induced allergic asthma (Figures 1B and 3). In contrast to the inhalation with OVA, respiratory exposure to HDM did not induce tolerance but led to Th2 skewing associated with airway inflammation, mucus production, and AHR (24). As expected, we found production of Th2 cytokines (IL-5, IL-13, and IL-10) from pulmonary cells isolated 72 hours after the last allergen challenge (Figure 3A), elevated serum IgE and IgG1 levels (Figure 3B), and airway inflammation (Figure 3C) as well as a dramatic increase in allergen-induced AHR (Figure 3D). Administration of the anti-C5aR mAb during initial antigen encounter was associated with markedly increased Th2 cytokine production from pulmonary cells and increased IgE and IgG1 serum concentrations as well as enhanced airway inflammation (Figure 3, A–C). Importantly, pulmonary cells did not produce IFN-γ in response to HDM immunization either.

Figure 3
C5aR targeting enhances Th2 adaptive immune responses and eosinophilic airway inflammation in response to pulmonary HDM exposure. (A) Cytokine profile of pulmonary cells harvested from BALB/c mice 72 hours after the final in vivo HDM exposure. (B) Serum concentrations of total IgE and allergen-specific IgG1. (C) Histological examination of airway inflammation. Sections were stained for mucus production with PAS (left panels) and with H&E (right panels). (D) Airway responsiveness to i.v. Ach. n = 8–10 per group. **P < 0.001; *P < 0.05.
Figure 4

Generation and characterization of C5aRA Tg mice. (A) Generation of C5aR Tg mice (see Methods). rCCSP, rat Clara cell–specific promoter; hGHpA, human growth hormone polyadenylation signal; bGHpA, bovine growth hormone polyadenylation signal. (B, upper left panel) Ccsp-tTA sTg mice (545 bp, lane 1) were bred to (tetO)-CMV-C5aRA sTg mice (228 bp, lane 2) to generate C5aRA-Ccsp dTg progeny (C5aRA-Ccsp dTg, lane 3). M, marker (200–900 bp). (Upper right panel) Kinetic of induction of pulmonary C5aRA mRNA in C5aRA-Ccsp dTg mice. RT-PCR was performed from whole-lung samples of C5aRA-Ccsp dTg mice that received drinking water without dox (day 0) or dox-supplemented water (0.5 mg/ml) for 1 day, 3 days, or 7 days. Control reactions with GAPDH primers are shown in the lower panel. (Lower panel) Organ specificity of Tg activation in C5aRA-Ccsp dTg mice treated with dox for 7 days. Lane 1, lung from C5aRA sTg mouse; lanes 2–7, liver, spleen, kidney, heart, brain, and lung from C5aRA-Ccsp dTg mouse; lane 8, genomic DNA from C5aRA-Ccsp dTg (positive control). (C) C5aRA protein expression in lung epithelial cells (indicated by arrows) from C5aRA-Ccsp dTg mice treated with dox (data not shown). (D, left panel) Total and differential cell counts in BAL. (Right panel) Cytokine profile of pulmonary cells harvested from BALB/c mice 72 hours after final in vivo HDM exposure. Cytokine profiles and BAL cell counts obtained from HDM-exposed non-Tg littermates were indistinguishable from those of HDM-exposed C5aRA sTg mice (data not shown). *P < 0.05; **P < 0.001.
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The presence or absence of C5aR blockade (data not shown). In addition, C5aR blockade further enhanced the already pronounced allergen-induced AHR in HDM-exposed mice (Figure 3D).

In a complementary approach, C5aR signaling was blocked by lung-specific expression of a C5aR antagonist (C5aRA) (29) in C5aRA Tg mice. Specifically, we have generated mice expressing the soluble C5aRA A8371-73 under conditional control of the Clara cell secretory protein (Ccsp) promoter and the reverse tetracycline transactivator (rtTa) (Figure 4A) (30). A8371-73 is a C5a mutant that was selected after random mutagenesis from a phage display library and that efficiently blocks C5a-mediated tissue injury (29, 31, 32). Induction of C5aRA was initiated 1 week prior to the initial antigen sensitization by administration of doxycycline (dox), resulting in lung-specific expression in the proximal airway epithelial cells (Figure 4B). Again, we found increased production of Th2 cytokines and eosinophilic airway inflammation when the C5aR was blocked, even in the absence of allergen exposure (Figure 4D).

Together, these data confirm the results obtained in the OVA model and suggest that the presence of C5a in the airways at the time of initial antigen encounter serves to prevent (OVA) or downregulate (HDM) the development of Th2-mediated immune responses. Of note, we found evidence of complement activation in

Figure 5
Flow cytometric and functional characterization of pulmonary DC populations. (A, middle left panel) Dot plot of pulmonary cells isolated from naive BALB/c mice and double stained for the expression of CD11c (x axis) and 7-amino-actinomycin D (7AAD; y axis). (Upper middle panel) Dot plot of 7AAD− and CD11c+ cells that were double stained for Gr-1 (x axis) and CD11b surface expression (y axis). (Lower left panel) Dot plot of CD11c−CD11b+ gated cells that were stained for mPDCA-1/B220 expression; percentages in boxed regions indicate the frequency of CD11c− cells that were mPDCA-1−B220− and mPDCA-1−B220+, respectively. (Lower middle panel) Dot plot of CD11c−CD11b+ gated cells that were stained for mPDCA-1−Gr-1 expression. Percentages in boxed regions indicate the frequency of CD11c+ cells that are mPDCA-1−Gr-1− and mPDCA-1−Gr-1+ respectively. (Upper left panel) C5aR expression on mDCs (CD11c+, CD11b+, and Gr-1− cells). (Upper right panel) C5aR expression on mDCs (CD11c+, CD11b+, and Gr-1− cells). (Lower right panel) C5aR expression on pDCs (CD11c+, CD11b−, and Gr-1+ cells). Black histogram profiles, specific staining with anti-C5aR mAb 20/70: Alexa488. White histogram profiles, ISO-Ctrl.). (B) IL-10 and IL-13 productions from cocultures of FACS-sorted CD11c+ DCs and CD4+ lymphocytes. (C, left panels) Ability of FACS-sorted mDCs and pDCs to induce production of Th2 cytokines from sorted CD4+ lymphocytes. (Right panels) Ability of pDCs to suppress mDC-induced production of Th2 cytokines from CD4+ lymphocytes. n = 8–10 per group. **P < 0.001; *P < 0.05.
BAL of OVA- or HDM-exposed mice comparable to that obtained in BAL of human asthmatics 48 hours after segmental allergen challenge (9) (Supplemental Figure 3).

Distinct pulmonary DC subsets express C5aR. Our data indicating that ablation of C5aR signaling prior to allergen sensitization enhances Th2 adaptive immune responses let us hypothesize that C5a has an impact on the interaction of pulmonary DCs and naive CD4+ lymphocytes. DCs are not only considered to be crucial for initial Th2 differentiation of naive CD4+ lymphocytes in response to aeroallergens, but are also essential for maintaining airway inflammation through controlling the recruitment and activation of primed Th2 effector cells in pulmonary tissue. Thus, to begin to determine the impact of C5a on the interaction between pulmonary DCs and CD4+ lymphocytes, we phenotyped distinct lung DC subsets in naive BALB/c mice and determined their C5aR expression. First, we defined pulmonary DCs as CD11c+ cells (Figure 5A). We then distinguished distinct DC subsets by the presence of Gr-1 (a myeloid differentiation antigen) and CD11b markers (Figure 5A). We identified 3 different populations of DCs as follows: (a) CD11c+CD11b−Gr-1−; (b) CD11c+CD11b−Gr-1+; and (c) CD11c−CD11b+Gr-1− cells. In addition, we identified a population of CD11c+CD11b−Gr-1− cells, most of which were F4/80+ macrophages (data not shown). We focused mainly on CD11c+CD11b−Gr-1− cells that we considered myeloid DCs (mDCs) and CD11c+CD11b+Gr-1− cells that we considered plasmacytoid DCs (pDCs). The pDCs were further characterized through the expression of mouse pDC antigen-1 (mPDCA-1), a surface marker that is selectively expressed on murine pDCs (33), B220 (Figure 5A, lower left panel), and Gr-1 (Figure 5A, lower middle panel). Importantly, all DC subsets expressed the C5aR, albeit to a different extent (Figure 5A).

C5aR targeting sensitizes CD4+ lymphocytes to produce Th2 cytokines and alters the relative proportion of mDC and pDC numbers in lung tissue. The enhanced Th2 cytokine production from whole lung cells in response to C5aR ablation in vivo may result from sensitized CD4+ lymphocytes, activated resident cells, or infiltrating inflammatory cells (1, 14). To directly assess the source of the Th2 cytokines, we examined cytokine production in ex vivo cocultures of FACS-sorted CD11c+ DCs and CD4+ lymphocytes isolated from anti-C5aR–treated and untreated mice. We found no Th2 cytokine production in cocultures from PBS controls and minor production in cocultures from HDM-treated mice but large amounts of Th2 cytokines in cocultures of HDM-treated mice in the presence of C5aR blockade (Figure 5B). Importantly, CD11c+ DCs, CD4+ lymphocytes, and CD11c−CD4− cells of any treatment group did not produce Th2 cytokines (data not shown). These data indicate the following: (a) CD4+ lymphocytes are likely to be the major source of the Th2 cytokines; (b) pulmonary DCs are the driving force of this cytokine production; and (c) C5aR signaling regulates the DC-induced activation of CD4+ effector T cells.

Next, we determined which of the distinct DC subsets accounts for the Th2 cytokine production. Previous data have demonstrated that mDC and pDC subsets not only express different phenotypic markers but have different functions. The mDCs are potent antigen-presenting cells and induce Th2 responses to inhaled antigens (34). In contrast, pDCs are considered a subset of immature DCs that possess only a modest capacity to activate naive T cells (35). To assess the ability of mDC and pDC to promote Th2 cytokine production independently ex vivo, we cocultured pulmonary mDCs, pDCs, and CD4+ lymphocytes purified 72 hours after the final HDM exposure from the different treatment groups (PBS, HDM, and HDM + anti-C5aR). CD4+ lymphocytes from PBS controls and HDM-exposed mice did not produce any Th1 or Th2 cytokines when they were cocultured with pulmonary DC subsets (data not shown). In contrast, CD4+ lymphocytes from mice that had been exposed to HDM in vivo in the presence of anti-C5aR mAb produced high levels of IL-10, IL-13 (Figure 5C, left panels), and IL-5 (data not shown) in cocultures with mDCs from PBS controls. The pDCs, however, failed to stimulate Th2 cytokine production from CD4+ lymphocytes. Importantly, in the same setting, pDCs suppressed the mDC-induced production of Th2 cytokines (Figure 5C, right panels). This effect was most pronounced at an
mDC/pDC ratio of 10:1 and decreased with lower numbers of pDCs. In fact, at an mDC/pDC ratio of 100:1, the inhibitory effect was almost gone. These data demonstrate the following: (a) ablation of C5aR signaling during initial allergen exposure sensitizes CD4+ lymphocytes to produce large amounts of Th2 cytokines in response to interaction with pulmonary mDCs but not with pDCs; and (b) pDCs have the ability to suppress the mDC-induced production of Th2 cytokines.

To assess whether these in vitro findings are related to our in vivo findings that C5aR ablation prior to sensitization is associated with increased Th2 production, we determined the number of mDCs and pDCs in the lung 72 hours after the final HDM exposure. Pulmonary exposure to HDM led to a much higher increase in pulmonary mDC numbers than in pDC numbers (Figure 6A), resulting in an increased mDC/pDC ratio as compared with PBS controls (Figure 6A). Importantly, when the C5aR was targeted, the mDC/pDC ratio increased to almost 17.5 in anti-C5aR–treated mice as compared with a ratio of 5 in untreated mice (Figure 6B). Further, the mDC/pDC ratio increased 2-fold when the C5aR was blocked in the absence of allergen exposure (Figure 6B). These data suggest a direct effect of C5a on pulmonary DCs that regulates the balance between mDCs and pDCs in the pulmonary environment under steady state conditions as well as in response to allergen exposure.

C5a suppresses the accumulation of Th2 effector cells through negative regulation of CCL17 and CCL22. In addition to their ability to activate CD4+ lymphocytes, pulmonary DCs play an important role in recruiting Th2 effector cells. Human blood–derived mDCs but not pDCs have been found to secrete CC chemokine ligand 17 (CCL17), also known as thymus and activation-regulated chemokine (TARC), and CCL22, also known as macrophage-derived chemokine (MDC), both of which are ligands for CC chemokine receptor 4 (CCR4), preferentially expressed on Th2 polarized effector cells (36). We hypothesized that the high concentrations of Th2 cytokines in the supernatants of CD11c+ DC/CD4+ T cells from PBS- or anti-C5aR–treated mice (Figure 5B) may result from high numbers of Th2 effector cells in naive mice 16 hours after primary HDM exposure in 4 different groups: (a) PBS–isotype control (PBS ISO-Ctrl); (b) PBS–anti-C5aR; (c) HDM ISO-Ctrl; and (d) HDM–anti-C5aR. Importantly, mDC numbers from HDM-exposed mice were much higher in the presence of C5aR targeting than in the absence of C5aR blockade (Figure 6B), resulting in an mDC/pDC ratio of 17.5 in anti-C5aR–treated mice as compared with a ratio of 5 in untreated mice (Figure 6B). Further, the mDC/pDC ratio increased 2-fold when the C5aR was blocked in the absence of allergen exposure (Figure 6B). These data suggest a direct effect of C5a on pulmonary DCs that regulates the balance between mDCs and pDCs in the pulmonary environment under steady state conditions as well as in response to allergen exposure.

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Opposing proallergic and antiallergic effects of C5a during the effector phase of pulmonary allergy. C5aR was blocked 1 day prior to the final HDM exposure by i.t. administration of a neutralizing anti-C5aR mAb. All experiments were performed 72 hours after the final in vivo HDM exposure. (A) Total and differential cell counts in BAL. (B) Cytokine profiles of pulmonary cells. (C) Airway responsiveness to i.v. Ach. n = 8–10 per group. **P < 0.001; *P < 0.05.

Discussion

In an attempt to reconcile the conflicting data on the role of C5/C5a in experimental allergic asthma (15–18), we hypothesized that abrogation of C5aR signaling during allergen sensitization or during the effector phase of pulmonary allergy has opposing effects. More specifically, we proposed that C5aR signaling during allergen sensitization protects from the development of pulmonary allergy but enhances the allergic phenotype in an inflamed pulmonary environment during the effector phase. To test this hypothesis, we first targeted the C5aR prior to initial antigen encounter. We focused on models in which the allergen is administered solely

CD4+CD69+ lymphocytes was significantly higher in mice exposed to HDM in the presence than in the absence of anti-C5aR mAb treatment (Figure 7A). This increase in Th2 effector cells was associated with high concentrations of CCL17 and CCL22 in CD11c+ DC/CD4+ lymphocyte cocultures from mice in which C5aR signaling was ablated (Figure 7B). CCL17 and CCL22 concentrations in cocultures from the PBS group and from mice exposed to HDM were 3- to 4-fold lower, suggesting that C5a negatively regulates CCL17 and CCL22 production in C5aR-deficient mice that had been exposed to OVA as compared with C5aR-sufficient mice (Supplemental Figure 2D). Alternatively, however, the increased concentration of CCL chemokines in C5aR-targeted mice may result from the increased inflammation and the known positive regulatory effects of Th2 cytokines IL-4 and IL-13. To minimize such inflammatory effects, we determined CCL17 and CCL22 production from CD11c+ DC/CD4+ lymphocyte cocultures of naive mice 16 hours after primary exposure to HDM. Again, CCL17 and CCL22 concentrations were significantly higher in the presence than in the absence of anti-C5aR treatment (Figure 7C). Importantly, we found no production of IL-4 or IL-13 in such cocultures (data not shown).

C5aR signaling promotes airway inflammation and bronchoconstriction during the effector phase of the allergic response. Our findings that C5aR signaling prevents excessive airway inflammation and type 2 adaptive immunity is in contrast to data suggesting that C5a contributes to the allergic phenotype (15–17). To assess the role of C5aR signaling during the effector phase, we targeted C5aR signaling solely prior to the final HDM challenge (Figure 8). In agreement with previous results (15–17), allergic inflammation was reduced as evidenced by decreased numbers of eosinophils and lymphocytes in BAL (Figure 8A). Further, airway responsiveness was significantly reduced (Figure 8C). In contrast to C5aR blockade during sensitization (Figures 2 and 3), we found no impact of C5aR signaling on the production of IL-4 and IL-10 from pulmonary cells (Figure 8B); however, the production of IL-5 and IL-13 was enhanced, albeit to a lesser extent as compared with the effect of C5aR blockade prior to initial allergen exposure (Figure 8B versus Figure 3A). Of note, C5aR blockade had no impact on the production of CCL17 and CCL22 chemokines (data not shown). Thus C5a exerts proallergic properties in an established allergic environment.

C5aR signaling promotes airway inflammation and airway contraction through infiltrating inflammatory cells but has no direct impact on airway smooth muscle contraction. To determine the mechanism that may account for the suppressed allergic phenotype in response to C5aR signaling during the effector phase, we determined the impact of C5a and its degradation product, C5adesArg, in which C-terminal Arg has been cleaved off, on airway smooth muscle (ASM) contraction ex vivo and in vivo. C5a and C5adesArg have been described as inducing ASM contraction in naive guinea pigs, both ex vivo, using tracheal strips (37), and in vivo (38). However, no data exist on the effect of C5a/C5adesArg on murine ASM contraction. In contrast to data obtained with guinea pigs, C5a/C5adesArg administration neither induced contraction of isolated tracheal strips (Figure 9A) nor ASM contraction in vivo after i.t. instillation into naive BALB/c mice (Figure 9B) although ASM cells express the C5aR (39). In contrast, we observed strong ASM contraction after i.t. administration of C5a/C5adesArg into mice that had been repeatedly exposed to HDM (Figure 9B). Further, airway inflammation was enhanced after local C5adesArg, but not after C5a challenge, as evidenced by increased eosinophil numbers (Figure 9C). In addition, administration of both C5a and C5adesArg increased lymphocyte numbers, suggesting that C5a/C5adesArg serve as chemoattractants for activated lymphocytes.
through the pulmonary route to model the events leading to the Th2-biased immune response in allergic asthma. As predicted, pulmonary OVA exposure in the absence of C5aR signaling did not result in inhalation tolerance (25, 40) but induced a strong activation of the CD4+ T cell and B cell compartments, as evidenced by marked Th2 cytokine and IgE production, associated with marked airway inflammation, mucus production, and increase of allergen-induced AHR. Of note, the absolute increase in Th2 cytokine production, in particular that of IL-13 (41), was lower in C5aR−/− mice than in C5aR-targeted mice, which is likely to account for the lack of increased allergen-induced AHR in C5aR−/− mice. Although suggestive, our data provide no direct evidence that tolerance is broken in the absence of C5aR signaling.

To explore the impact of C5aR signaling on allergen sensitization in a setting that is of relevance to human asthma and atopy, we focused on the HDM species Dermatophagoides pteronyssinus, which accounts for atopic symptoms in 10% of individuals with asthma (42). Importantly, proteases from Dermatophagoides species can cleave C3 and C5 into their active fragments in vitro (3). As therefore expected, we found high concentrations of C3a in BAL after repeated exposure to HDM. As in the OVA model, local C5aR blockade with the neutralizing anti-C5aR mAb or the C5aRA prior to pulmonary HDM sensitization resulted in excessive production of Th2 cytokines, associated with a Th2-skewed B cell response, strong airway inflammation, and increased AHR.

Thus, we have demonstrated by 3 independent approaches in 2 independent animal models that C5aR signaling during pulmonary allergen sensitization protects from the development of vigorous Th2 adaptive immune responses. Importantly, ablation of C5aR signaling in the absence of allergen exposure induced a modest Th2-skewed immune response, suggesting that some signaling through constitutively expressed C5aR on antigen-sampling, pulmonary DCs is necessary to prevent the induction of Th2-biased effector lymphocytes.

In apparent contrast to our findings, Peng et al. reported that C5-deficient B10.D2/oSnJ mice do not suffer from increased allergen-induced AHR as compared with C5-sufficient BALB/c mice (17). This result is not surprising, taking into account that the correlation between phenotype and genotype at the C5 locus is not absolute, as we had already noted in our previous study (18). Since the genetic backgrounds of the C5−/− mice and the C5+/− mice used in the study by Peng et al. were different, the overall contribution of C5a to AHR is difficult to assess. To more definitely test the role of C5a, we compared the B10.D2/oSnJ strain and the congenic B10.D2/nSnJ strain, which carries the C5 wild-type allele. In accordance with our previous results and the data in the present study, we found that the inflammatory response and allergen-induced AHR are significantly higher in the C5−/− B10.D2/oSnJ mice as compared with their C5+/+ littermates (our unpublished data).

Figure 9
Effects of C5a and C5adesArg on ASM contraction and airway inflammation. (A) ASM contraction of tracheal rings stimulated with Ach, C5a, or C5adesArg. Shown is the maximal absolute isometric force evoked by the indicated agents. (B) Bronchoconstriction in response to i.t. administration of C5a/C5adesArg to naive mice (left panel) and to mice repeatedly exposed to HDM (right panel), respectively. (C) Total and differential cell counts in response to i.t. instillation of C5a/C5adesArg to mice repeatedly (×4) exposed to HDM. n = 6–10 per group. * P < 0.05.
The negative impact of C5a on the development of type 2 adaptive immunity is in line with recent findings that C5a is not only a danger signal of the innate immune system but provides regulatory properties that have an impact on adaptive immunity. C5a is necessary to mount an appropriate antiviral CD8+ T cell response to control acute influenza virus infections (43), which often lead to exacerbation of allergic asthma (44). Further, C5aR signaling negatively regulates TLR4-induced synthesis of IL-12 family cytokines IL-12, IL-27, and IL-23 from murine APCs, resulting in decreased Th1 adaptive immunity (7). In line with these data, Liu et al. found that CD55-deficient (DAF-deficient) mice suffer from vigorous T cell activation characterized by hypersecretion of IFN-γ and IL-2 in response to Ag recall. Strikingly, the protective effect of CD55 is related to C5 (45). These data support the view that C5a acts as a complex network of signaling pathways that either enhance or suppress CD4+ and/or CD8+ T cell immunity (for review, see ref. 46).

Although these data provide evidence for complex novel roles of C5a in regulating adaptive immune responses, it remains unclear how C3 is activated in response to OVA exposure or, even more importantly, in healthy and asthmatic individuals. In addition to hepatocytes, mononuclear phagocytes, fibroblasts, and epithelial as well as endothelial cells can produce complement proteins of the classical and the alternative pathways, in particular in the lung (47). Both the classical and alternative pathways can be activated by LPS, which we found in nanogram amounts in the OVA preparation used in this study as well as in OVA preparations used in previous studies (data not shown; refs. 17, 18, 22). Clearly, the amount of LPS administered in the present study was insufficient to mount an adaptive immune response as evidenced by the lack of Th1 or Th2 cytokine production in the OVA tolerance model (Figure 2A) and the lack of induction of DC maturation in vitro (Supplemental Figure 4). However, it is likely to be sufficient to induce low level activation of complement (Supplemental Figure 3 and refs. 48, 49). In addition to LPS, pattern recognition of carbohydrate structures on allergens may activate the lectin pathway. Alternative pathway activation may occur on allergen-related surfaces. Proteases derived from alveolar macrophages (4) or from allergens (3) have been demonstrated to cleave C3 and C5 (see above). In the context of an ongoing allergic response, the Th2 cytokines IL-4 (50) and IL-13 (our unpublished data) have both been shown to induce C3 mRNA production in human epithelial cell lines and in primary human and murine epithelial cells. In support of the view of low level complement activation in healthy individuals, we found C3a and C5a in low nanomolar concentrations in BAL (8, 9), which is sufficient to activate anaphylatoxin receptors (51).

The mechanisms that drive the Th2-biased immune response in allergic asthma are poorly understood. Clearly, pulmonary DCs play pivotal roles in the Th2 sensitization process (52) and stimulate Th2 cells in an established inflammatory environment (53). A possible role for C5aR signaling in the DC-induced Th2 skewing is suggested by our findings that (a) all pulmonary DC subsets express the C5aR (Figure 5A); (b) only CD4+ lymphocytes from mice in which C5aR signaling was ablated produce Th2 cytokines in response to DC stimulation ex vivo (Figure 5C); (c) C5aR targeting in vivo increases the relative proportion of immunogenic mDCs to tolerogenic pDCs (Figure 6); and (d) the production of Th2 cell homing chemokines CCL17 and CCL22 is only increased in cocultures of CD11c+ DC/CD4+ lymphocytes isolated from mice in which C5aR signaling has been blocked (Figure 7, B and C).

In addition to T cell priming and activation, DCs have also been demonstrated to be of critical importance for inhalation tolerance (25). Recent studies provide evidence that allergen sensitization and tolerance can be related to distinct properties of 2 phenotypic and functional distinct pulmonary DC subsets, i.e., mDCs and pDCs (35). Adoptive transfer of mDCs confers immunogenicity (34) whereas adoptive transfer of pDCs results in inhalation tolerance (25). In agreement with these data, we found that only mDCs, but not pDCs, stimulate sensitized CD4+ lymphocytes to produce high amounts of IL-5, IL-13, and IL-10 ex vivo. In addition to these findings, we demonstrate here that pulmonary pDCs have the ability to actively suppress the mDC-induced activation of sensitized CD4+ lymphocytes ex vivo. In support of the view that pDCs play a critical role in inhalation tolerance, Oriss et al. reported increased accumulation of pDCs in lymph nodes of tolerantized mice whereas under conditions that induce airway inflammation, mDCs become the predominant cell type (54). We found a shift in the relative proportion of mDCs to pDCs in response to (a) allergen exposure; and, more importantly, (b) C5aR ablation. In fact, in the absence of C5aR signaling, the mDC/pDC ratio was 2 or 3 times as high as in the presence of C5aR signaling (Figure 6, A and B). Thus, our data not only support the concept that airway tolerance versus allergy is determined by the relative proportion of distinct DC subsets but that C5aR signaling during allergen sensitization regulates this balance. At this point, we do not know the mechanisms underlying this regulation. C5a may affect the mobilization of mDC precursors from bone marrow, increase mDC recruitment, and/or increase mDC survival. The importance of this finding is supported by recent data demonstrating that asthmatic children have lower numbers of circulating pDCs than healthy controls (55).

In addition to the strong impact on pulmonary mDC accumulation and the sensitization of CD4+ lymphocytes, we found a marked increase in CD4+CD69+ T effector cells in anti-C5aR-treated mice, suggesting a regulatory role of C5a on pulmonary homing of circulating Th2 effector cells. Th2 cells express CCR8, CCR3, and CCR4, all of which have been implicated in Th2 cell trafficking and the pathophysiology of allergic asthma (56). However, CCR3 expression was found mainly after initial antigen encounter whereas CCR4 was preferentially expressed after repeated allergen exposure (57). mDCs have been described as selectively producing the 2 known ligands for CCR4, i.e., CCL17 and CCL22, as a means to recruit Th2 cells to sites of inflammation (36). In accordance with the high number of mDCs, we found high concentrations of CCL17 and CCL22 in CD11c+ DC/CD4+ lymphocyte cocultures of anti-C5aR-treated mice after initial and repeated allergen exposure and in C5aR-deficient mice. These data strongly suggest that C5a regulates homing of Th2 cells through negative regulation of CCL17/CCL22 production from pulmonary mDCs.

In contrast to the protective, antiallergic effect, we show that C5a promotes airway inflammation and AHR in an established allergic environment. Under such circumstances, C5a amplifies the allergic inflammation through its chemotactic properties on eosinophils and neutrophils and its ability to induce proinflammatory cytokines and chemokines from leukocytes and mast cells (5). Although this proallergic effect is dominant, we still found some negative regulation on Th2 cytokine production, something that argues against the C5aR as a therapeutic target in allergic asthma as suggested in a recent study (17).
In addition to previous reports, we demonstrate striking species-specific differences in the ability of C5a to induce bronchoconstriction. In contrast to data obtained in guinea pigs (37), we found no contraction of isolated tracheal strips challenged with C5a/C5adesArg despite C5ar expression on murine ASM cells. Further, we observed no ASM contraction or airway inflammation after i.t. instillation of C5a/C5adesArg to naïve mice at concentrations that resulted in respiratory distress, strong airway contraction, inflammation, and a 50% mortality in guinea pigs (38). These data suggest that C5ar has no direct effect on ASM contraction and/or that ligation of C5ar on naïve resident cells is not sufficient to release bronchoactive mediators, which induce ASM contraction in guinea pigs (58). In contrast, C5a and C5adesArg induced ASM contraction and enhanced airway inflammation in an inflamed environment, suggesting that ligation of C5ar on infiltrating cells (e.g., neutrophils, eosinophils) and/or upregulation and ligation of C5ar on pulmonary resident cells (epithelial cells, ASM) is a prerequisite for the contractile response. The higher potency of C5ar in an established allergic environment reduces airway inflammation, and AHR, suggesting a beneficial effect of C5ar blockade in human asthma. Our data support the hypothesis that C5ar signaling at the DC/T cell interface during allergen priming provides protection against inflammatory responses to harmless antigens at the mucosal surface. This contention is supported by data from genome-wide screens for asthma-susceptibility loci that have identified linkage of asthma and related traits to the chromosomal regions containing both C5 (9q34) and the C5ar (19q13.3) (60, 61). Moreover, certain C5 allelics have been found to be associated with protection from the development of both childhood and adult asthma (62). Clearly, C5ar targeting in an established allergic environment reduces airway inflammation and AHR, suggesting a beneficial effect of C5ar blockade in asthma. However, such an approach may turn out to be a pyrrhic victory, as the enhanced production of Th2 effector cytokines may promote adverse effects on the lung architecture. To this end, it is not possible to judge which of the 2 effects predominates in the long run and to decide whether the C5ar may serve as a useful therapeutic target in human asthma.

Methods

Tg mice. To generate C5ar Tg mice, C5ar cDNA was cloned into the (tetO)-CMV plasmid between the (tetO)-CMV minimal promoter and the S′ untranslated region of the bovine growth hormone (bGH) gene containing introns and a polyadenylation (pA) signal (Figure 4A). Tg mouse lines were established after microinjection of the (tetO)-CMV-C5ar construct into fertilized FVB/N oocytes (Transgenic Animal Facility, Children's Hospital, Cincinnati, Ohio, USA). Conditional temporal and spatial regulation of C5ar expression to the respiratory epithelium was achieved using the rtTa expressed under the Clara cell–specific (Ccsp) promoter (30). For conditional expression of the C5ar Tg, C5ar-rtTa activator mice (30) were bred to (tetO)-CMV-C5ar target mice (C5ar single transgenic [C5ar sTg]), producing double-Tg (dTg) progeny (C5ar-C5ar-C5ar). Genotypes were determined from tail DNA by PCR. C5ar sTg and C5ar-C5ar dTg mice used for the experiments were on a mixed FVB/N/BALB/c background.

Mice. Female BALB/c mice (Jackson Laboratory), C5ar Tg mice, and C5ar-deficient mice (63) (backcrossed to the BALB/c background; n = 7) were maintained in the Cincinnati Children's Hospital Medical Center specific pathogen–free facility and used at 6–8 weeks of age. Animal care was provided in accordance with NIH guidelines. These studies were approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee.

Flow cytometry. To differentiate distinct DC subsets and to determine the surface phenotype of DCs and T cells, single lung cell suspensions were stained in a multicolor analysis with the following Abs: CD11c, APC (HL3); CD69, PE-Cy7 (H1.2F3); CD11b, PE-Cy7 (M1/70); Gr-1, APC-Cy7 (RB6-8C5); B220, PE (RA3-6B2) (all from BD Biosciences — Pharmingen); CD4, APC (RM4-5) (from eBioscience); mPDCA-1–APC or –PE (Miltenyi Biotec) (F4/80); FITC (C1, A1-1) (Serotec). Alternatively, they were stained with their respective, ISO-Ctrl Abs: rat IgG2b, APC-Cy7 (A95-1); rat IgG2b, PE-Cy7 (A95-1); hamster IgG1, PE (A19-3); rat IgG2a, PE (R35-95); rat IgG2b, FITC (R35-95); hamster IgG1, APC (A19-3); hamster IgG1, PE-Cy7 (G235-2356) (all from BD Biosciences — Pharmingen); hamster IgG, FITC (eBioscience). The phenotype of pulmonary DC subsets was determined using the following markers: CD11c, CD11b, Gr-1, B220, F4/80, and mPDCA-1. The expression of the C5ar on the DC subsets was determined using mAb 20/70. Alexafluor488. Surface expression of CD40, CD80, and CD86 on DCs in response to LPS (100 ng/ml) or OVA (10 µg/ml) was determined using the following mAbs: CD40, APC (IC10, eBioscience); CD80, PE (16-10A1); and CD86, PE (GL1) (both from BD Biosciences — Pharmingen). Also used were their respective ISO-Ctrl Abs (rat or hamster IgG2a). Samples were run on a FACSDiVa software (version 4.1.1; BD Biosciences). Subsequent analysis was done using FlowJo software (version 6.3.1; Tree Star Inc.).

Induction of the allergic phenotype and C5ar blockade in vivo. BALB/c, C5ar-C5ar-C5ar dTg, and C5ar sTg mice were immunized with HDM (crude extract; Greer Laboratories) (100 µg/40 µl) or PBS (as control) i.t. on days 0, 7, 14, and 21. Alternatively, BALB/c mice and C5ar-deficient mice were exposed to OVA (750 µg/mouse i.t.; LPS contamination of 2.9 ng/mg protein; Worthington Biochemical Corp.) on days 0, 20, 21, 22, and 23. C5ar was blocked in HDM-treated BALB/c mice by i.t. administration of the anti-C5ar mAb 20/70 (50 µg/40 µl) on days -1, 6, 13, and 20, and in OVA-treated mice on days -1 and 20 (Figure 1). Animals treated with an isotype-matched control mAb (50 µg/40 µl) served as controls (ISO-Ctrl). C5ar blockade in C5ar-C5ar-C5ar dTg mice was achieved by adding dox to the drinking water (0.5 mg/ml) 1 week prior to the initial HDM exposure. Dox was kept in the drinking water during the entire experiment. C5ar-C5ar-C5ar dTg mice treated in the same way served as controls. Allergen-induced AHR was determined as described (41). Briefly, mice were anesthetized 72 hours after the last i.t. HDM exposure, intubated and ventilated at a rate of 120 breaths per minute with a constant tidal volume of air (0.2 ml), and paralyzed with decamethonium bromide (25 mg/kg). After establishment of a stable airway pressure, 25 µg/kg weight of acetylcholine was injected i.v. and dynamic airway pressure (airway pressure time index [APTI] in cm-H2O x sec -1) was followed for 5 minutes. Lung leukocyte accumulation was analyzed in BAL.

Impact of C5ar blockade on Th2 cytokine production. To assess the impact of C5ar inhibition on Th2 cytokine production, Liberase/DNase I digests of the lung were prepared to obtain single lung cell suspensions. Single cell suspensions (2.5 x 10⁶) were incubated for 72 hours in culture medium (RPMI), and supernatants were used to determine cytokine expression. In some experiments, total lung cells were sorted based on CD11c-FITC (HL3) and CD4-PE (RM4-5) staining using a FACSVantage SE sorter (BD Biosciences). CD11c+ cells (10⁵), CD4+ cells (2.5 x 10⁵), CD11 C4 cells (10⁴), and CD11 C+ CD4+ cells (2.5 x 10⁴) cells were incubated for 72 hours as described above. To assess the impact of mDCs or pDCs on the production of Th2 cytokines (IL-5, IL-10, and IL-13), sorted mDCs or pDCs (each at 10⁵) and CD4+ lymphocytes (2.5 x 10⁴) from each treatment group (PBS, HDM ISO-Ctrl, and HDM anti-C5ar).
were cocultured for 72 hours. In experiments aimed at determining the ability of pDCs to suppress mDC-induced Th2 cytokine release from CD4+ lymphocytes, a constant number of sorted mDCs (10^4) were mixed with distinct numbers of sorted pDCs (10^3, 2 × 10^3, and 10^4), resulting in final mDC/pDC ratios of 10:1, 50:1, and 100:1.

Cytokine, chemokine, IgE, and C3a measurements. Serum and supernatant cytokine/chemokine release were determined by ELISA. Serial dilutions of samples were analyzed for IL-4, IL-5 (BD Biosciences—Pharmingen), IL-10, IL-13, IFN-γ, CCL7, and CCL22 (all R&D Systems) expression with commercial ELISA kits. C3a concentration in BAL was determined by ELISA.

Lung histology. Histology and determination of mucus cell content were done as described (41). Briefly, lungs were excised and fixed in 10% formalin, washed in 70% ethanol, dehydrated, embedded in glycol methacrylate, and cut into 10-μm sections. Slides were stained with H&E and PAS. Immunostaining to detect C5aRa expression in lung epithelial cells of C5aRa−/− mice was performed using anti-C5aR-specific mAb 561A Alex647.

Tracheal ring contractility. Studies of mouse tracheal contractility were performed as reported previously (64).

Statistics. Statistical analysis was performed using the SigmaStat version 2.0 statistical package (Jandel Engineering Limited). All data are given as mean ± SEM. First, we tested for a normal distribution, using the Kolmogorov-Smirnov test. Comparison of the means of more than 2 groups was performed by one-way ANOVA. When the mean values of the groups showed a significant difference, pairwise comparison was performed using the Tukey test.

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