



Use of monoclonal antibodies to assess expression of anaphylatoxin receptors in rat and murine models of lung inflammation

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Abstract

The anaphylatoxins C3a and C5a are involved in the pathophysiology of microbial as well as allergic inflammation in the lungs. Besides their expression in leukocytes, receptors for C3a and C5a (C3aR and C5aR) have been noted in alveolar and bronchial epithelial cells, bronchial smooth muscle cells as well as in vascular endothelial and smooth muscle cells of normal and inflamed human and murine lungs. Recently, however, expression of anaphylatoxin receptors in parenchymal cells of the lung (and kidney) has been challenged. Using well-characterized monoclonal antibodies (mabs) against murine and rat anaphylatoxin receptors, we reexamined the pulmonary distribution of C3aR and C5aR. Immunohistochemistry was performed on frozen sections of lung tissues from normal mice and rats as well as from animals subjected to lipopolysaccharide (LPS)-induced inflammation or from MRL/lpr mice suffering from autoimmune disease. Furthermore, ovalbumin (OVA)-induced models of allergic asthma in the rat and mouse were investigated. Prominent expression of both anaphylatoxin receptors was detectable in resident as well as infiltrating leukocytes. No C3aR protein was observed in alveolar macrophages. Upon LPS- and OVA-challenge as well as in autoimmune inflammation, numbers of infiltrating leukocytes expressing prominent amounts of anaphylatoxin receptors increased. Even under these highly inflammatory conditions, however, expression of C3aR and C5aR was not inducible in parenchymal cells. Thus, our findings identify infiltrating leukocytes as a prominent source of anaphylatoxin receptors in inflamed lungs. A direct involvement of parenchymal cells in anaphylatoxin-mediated pulmonary inflammation is unlikely.

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Introduction

Complement can be activated through the classical, alternative and lectin pathways. The third component of the complement system, C3, is cleaved upon complement activation with generation of C3a and C3b,

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followed by the generation of C5a and C5b (Walport, 2001). The anaphylatoxins C3a and C5a are responsible for recruiting and activating leukocytes, particularly phagocytic cells such as granulocytes and monocytes/macrophages. Anaphylatoxins perform their functions by engaging specific receptors on target cells. Anaphylatoxin receptors are closely related members of the rhodopsin family of seven transmembrane-spanning G protein-linked receptors (Ames et al., 1996).

Anaphylatoxins are involved in the pathophysiology of allergic asthma. C3a and C5a were increased in bronchoalveolar lavage fluid or plasma in subjects with asthma (Krug et al., 2001; Nakano et al., 2003; Humbles et al., 2000). Genetic C3aR defects resulted in decreased bronchoconstriction in experimental asthma models (Drouin et al., 2002; Humbles et al., 2000; Bautsch et al., 2000). Similarly, pharmacological targeting of C5a or C5aR suppressed airway hyperresponsiveness and inflammation (Abe et al., 2001; Baelder et al., 2005). On the other hand, the role of C5a in allergic disease may be diverse since it was noted to exert anti-inflammatory effects as well (Drouin et al., 2006; Karp et al., 2000).

C3aR and C5aR expression could be detected in bronchial epithelial and smooth muscle cells, alveolar epithelial cells, and vascular smooth muscle and endothelial cells in humans and mice (Drouin et al., 2001; Haviland et al., 1995; Melendi et al., 2007). Upregulation of parenchymal anaphylatoxin receptor expression was found in animal models of sepsis and asthma (Drouin et al., 2001; Melendi et al., 2007) and in fatal asthma in humans (Fregonese et al., 2005). However, a direct involvement of parenchymal anaphylatoxin receptors in C3a- or C5a-induced pulmonary inflammation has not been demonstrated. Recently, the view of a widespread expression of anaphylatoxin receptors in parenchymal cells of the lung (and other organs such as the kidney) has been challenged (Fayyazi et al., 2000; Thangam et al., 2005; Kiafard et al., 2007). In order to improve our understanding of the pathophysiological role of anaphylatoxins in pulmonary inflammation, a reexamination of anaphylatoxin receptor expression in the lung appeared warranted.

To achieve this goal, established mabs against rat and murine anaphylatoxin receptors were applied in the immunohistochemical examination of normal as well as diseased lung tissues. Beside an abundant expression of C5aR and C3aR in resident and infiltrating leukocytes, no anaphylatoxin receptors were noted in parenchymal pulmonary cells in situ under normal or inflammatory conditions. We conclude that the abundance of anaphylatoxin receptors in pulmonary leukocytes may account for the significant contribution of anaphylatoxins in lung pathology.

Materials and methods

Monoclonal antibodies

Monoclonal ab R63 (mouse IgG1) against rat C5aR, mab 10/92 (rat IgG2a) against mouse C5aR, mab 74 (mouse IgG1) against rat C3aR as well as mabs 1G4 (rat IgG1) and 14D4 (rat IgG2a) against mouse C3aR have been demonstrated to be applicable in immunohistochemistry (Kiafard et al., 2007).

Tissue specimens

All animal work was conducted in accordance with guidelines for animal welfare and was approved by the government of Lower Saxony, Germany.

Normal Balb/c mice as well as Lewis and Brown-Norway (BN) rats were obtained from our local animal facilities. MRL/lpr mice (4–6-month old) suffered from lupus-type nephritis (as judged by massive proteinuria).

To induce systemic inflammation, Balb/c mice and Lewis rats were injected i.p. with 5 mg/kg bodyweight LPS (serotype 0128:B12, Sigma, Munich, Germany) and killed 24 h later by asphyxiation.

OVA-dependent allergic hyperresponsiveness in rats (Schmiedl et al., 2005) and mice (Weigt et al., 2005) was induced as described. Lungs were harvested 24 h after the challenge.

Immunohistochemistry

Five micron thick sections were prepared from frozen lung tissues and placed on glass slides that were coated with poly-lysine. Immunohistochemistry was performed as described previously for the rat (Lehmann et al., 2001) and mouse (Bernier et al., 2001). In short, tissues were fixed in acetone at -20°C for 10 min, incubated with PBS/5% normal mouse serum and subsequently with primary antibody (5 $\mu\text{g}/\text{ml}$ in PBS/1% BSA) followed by rabbit-anti-rat (staining of mouse tissue) or rabbit-anti-mouse (staining of rat tissue) immunoglobulins and rat/mouse alkaline phosphatase-anti-alkaline phosphatase (APAAP; 1:100 in PBS; Dako, Hamburg, Germany) for 30 min each. The secondary antibody steps were repeated with incubation times of 15 min each. The color was developed with Fast blue (Sigma) as a substrate for the APAAP. No counterstaining was performed to lower the threshold for antigen detection. Isotype control mabs consistently yielded negative results.

Results

Murine lungs

To study anaphylatoxin receptor expression in murine lungs, mab 10/92 reactive against murine C5aR and two

mabs, 1G4 and 14D4, reactive against independent epitopes of the murine C3aR were applied. It is important to note that mabs 1G4 and 14D4 always yielded undistinguishable staining results.

In normal Balb/c mice, we found interstitial macrophages to express C5aR as well as C3aR protein, whereas no other cell type was stained. Alveolar macrophages exclusively expressed C5aR but not C3aR (data not shown).

Next, anaphylatoxin receptors were investigated in LPS-induced pneumonitis which was characterized by a distinct influx of leukocytes. In this model of inflammatory lung disease, anaphylatoxin receptors were detectable in infiltrating leukocytes but not in parenchymal cells such as epithelial, endothelial and smooth muscle cells. Similar to normal mice, alveolar macrophages exclusively expressed C5aR but not C3aR (Fig. 1a–f).

In MRL/lpr mice which suffered from spontaneous autoimmune disease, the above mentioned findings were confirmed indicating that parenchymal anaphylatoxin

receptor expression is not inducible in vivo under conditions of generalized inflammation (Fig. 2a, b and data not shown).

Similarly, in OVA-induced allergic asthma in Balb/c mice (Fig. 2c, d and data not shown), no anaphylatoxin receptor proteins were detectable in parenchymal pulmonary cells, in contrast to infiltrating leukocytes which were strongly stained. Again, alveolar macrophages remained C3aR-negative.

Rat lungs

Mab R63 reactive against rat C5aR and mab 74 reactive against rat C3aR were used for the immunohistochemical examination of frozen sections from rat lung tissues. In normal animals, interstitial macrophages expressed C5aR as well as C3aR protein, whereas no parenchymal cell type was stained. Similar to mice, alveolar macrophages exclusively expressed C5aR but not C3aR (data not shown).

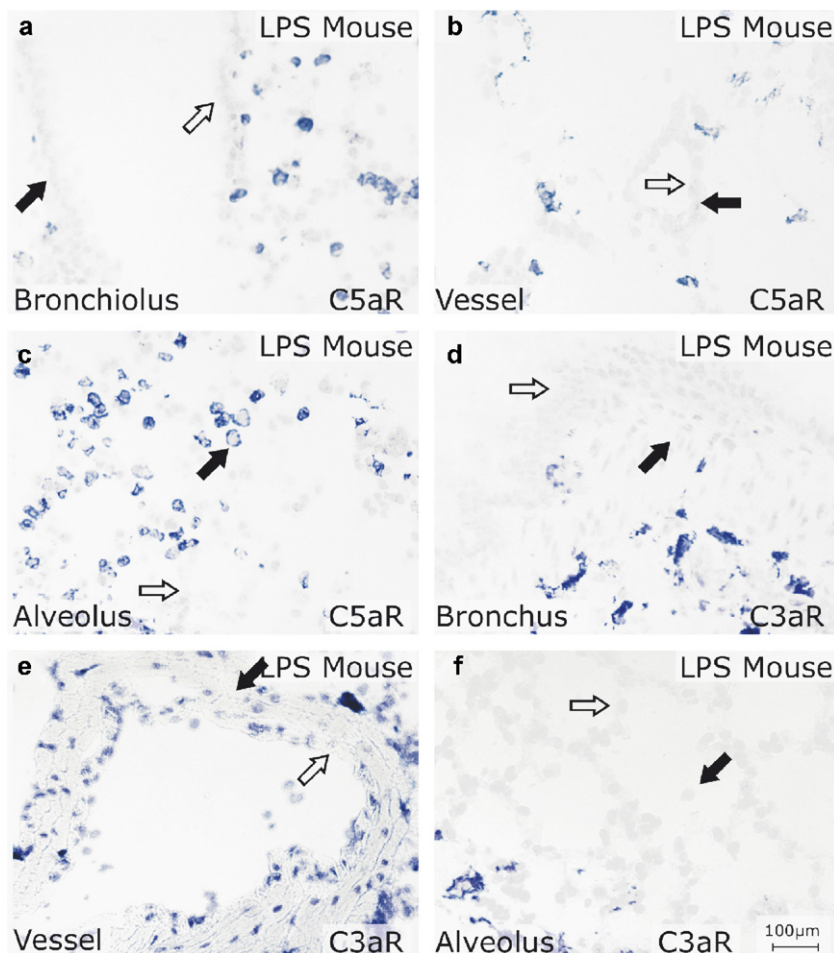


Fig. 1. C3aR and C5aR protein detection in lungs from LPS-treated mice. Frozen sections from murine lung tissues after systemic LPS-treatment were stained with anti-mouse C5aR mAb 10/92 (a–c) and anti-mouse C3aR mAb 14D4 (d–f). Binding of mAbs was visualized by indirect immunohistochemistry. Empty arrows indicate airway epithelial cells (a, d), vascular endothelial cells (b, e), and alveolar epithelial cells (c, f); filled arrows indicate airway (a, d) and vascular (b, e) smooth muscle cells and alveolar macrophages (c, f).

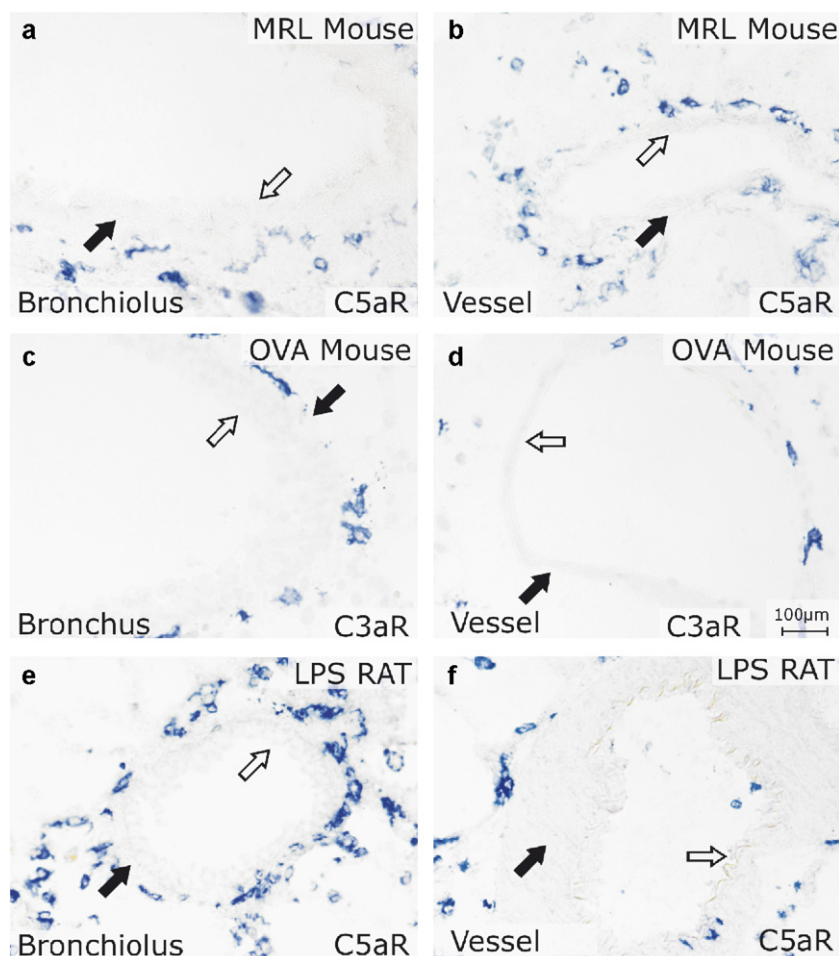


Fig. 2. Anaphylatoxin receptor detection in MRL/lpr mice, ovalbumin-challenged mice and LPS-treated rats. Staining of frozen lung sections from mice (a–d) and rats (e–f) was performed as described in Figs. 1 and 3, respectively. Empty arrows indicate airway (a, c, e) epithelial cells and vascular endothelial cells (b, d, f); filled arrows indicate smooth muscle cells from the airway (a, c, e) and vessels (b, d, f).

Lungs from rats with LPS-induced pneumonitis (Fig. 2e and f) or OVA-induced allergic asthma (Fig. 3a–f) were distinguished by prominent cellular influxes. The immunohistochemical examination further revealed abundant amounts of anaphylatoxin receptor proteins in infiltrating leukocytes, however, no other cell types including epithelial, endothelial or smooth muscle cells were stained. Again, alveolar macrophages were C3aR-negative. Our findings indicate that no upregulation of anaphylatoxin receptor expression occurs in parenchymal cells of the rat lung under conditions of local or generalized inflammation.

Discussion

In this study, we provide evidence for a prominent expression of anaphylatoxin receptors in leukocytes in normal and diseased lung tissues of mice and rats. No

other cell types indigenous to the lung expressed detectable amounts of C5aR or C3aR proteins.

Our results are in obvious contrast to the ubiquitously found expression of C5aR and C3aR in human and murine lungs. These findings were based on the use of polyclonal abs (Drouin et al., 2001; Haviland et al., 1995). However, in another study, marked immunostaining for C3aR was exclusively detected in pulmonary epithelial cells whereas smooth muscle cells were conclusively shown to be C3aR-negative (Thangam et al., 2005). In a third study, bronchial C3aR staining was low but strongly increased in C5 deficient mice suffering from enhanced respiratory syncytial virus disease (Melendi et al., 2007). These disparate findings point to a potential lack of specificity of the polyclonal abs used in these experiments.

There is increasing evidence that immunostaining based on the use of mabs should be also critically evaluated. Fregonese et al. (2005) demonstrated a weak

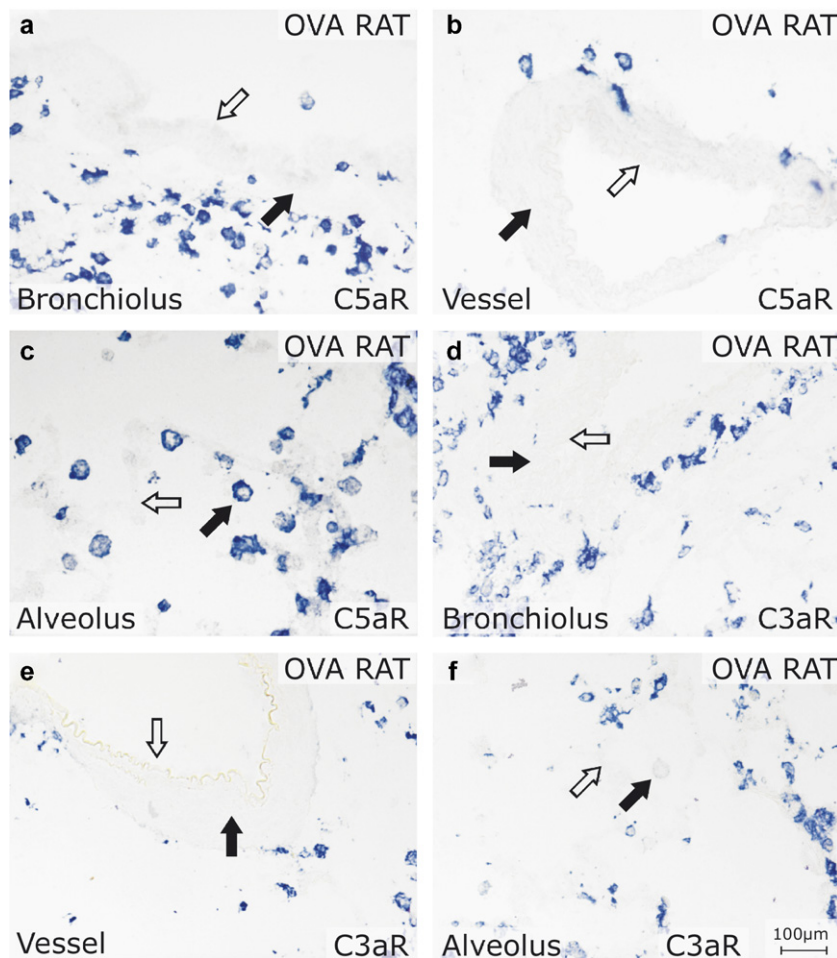


Fig. 3. Pulmonary C3aR and C5aR protein detection in OVA-induced allergic asthma. Frozen sections from lung tissues after ovalbumin-challenge were stained with anti-rat C5aR mAb R63 (a–c) and anti-rat C3aR mAb 74 (d–f). Binding was visualized by indirect immunohistochemistry. Empty arrows indicate bronchiolar epithelial cells (a, d), vascular endothelial cells (b, e) and alveolar epithelial cells (c, f); filled arrows indicate bronchiolar (a, d) and vascular (b, e) smooth muscle cells and alveolar macrophages (c, f).

in situ expression of C3aR in bronchial epithelial and smooth muscle cells as well as in blood vessels, with significant upregulation of vascular C3aR in fatal asthma. Their results were exclusively based on mab 8H1 staining. However, the prominent binding of mab 8H1 to various cell types such as placental epithelial (syncytiotrophoblasts) and vascular endothelial cells in situ was characterized in the past as unspecific since no C3aR expression was detectable using two other mabs. Only Hofbauer cells (placental macrophages) were considered truly C3aR-positive (Sohns, 2000). Furthermore, binding of mab 8H1 to renal tubular cells as shown previously (Braun et al., 2004) also proved unspecific since it was not confirmed by various mabs against human, mouse and rat C3aR (Kiafard et al., 2007).

Fregonese et al. (2005) further demonstrated by the use of mab P12/1 a constitutive expression of C5aR in the human airway epithelium, which increased in fatal

asthma samples. In the past, however, mab P12/1 was found to unspecifically bind to epithelial cells of the skin (keratinocytes) (Werfel et al., 1996). Also, binding of P12/1 to another epithelial cell type, renal tubular cells, was not confirmed using three different mabs with reactivities against various epitopes of human C5aR as well as mabs against rat and mouse C5aR, respectively (Kiafard et al., 2007). Taken together, mabs 8H1 and P12/1 should not be applied any more to the immunohistochemical analysis of human anaphylatoxin receptor expression since they react with as yet undefined antigens in epithelial cells of different organs.

We conclude from our studies that, in order to minimize the risk of cross-reactivities to remain undiscovered, immunohistochemical data obtained with one mab require confirmation by at least one additional mab recognizing a different epitope of the same molecule or a homologous molecule in a different species. In the present study, this requirement was met

since two mabs directed against independent epitopes of mC3aR, 1G4 and 14D4, were found to exclusively stain murine pulmonary leukocytes similar to mab 74 in the rat lung. For the detection of murine and rat C5aR, respectively, one mab for each receptor was available. Furthermore, pulmonary C5aR expression as demonstrated herein was in full agreement with the exclusive staining of leukocytes previously observed in normal human lungs (Fayyazi et al., 2000).

In summary, our data indicate that anaphylatoxin receptor proteins are not expressed in pulmonary parenchymal cells in situ. However, functional C5aR expression has been demonstrated in cultured bronchial epithelial cells (Floreani et al., 2003) as well as, together with C3aR, in cultured endothelial cells (Schraufstatter et al., 2002). We do not argue against these findings since our results exclusively refer to the issue of protein expression in vivo. On the other hand, infiltrating leukocytes were found to be an abundant source of anaphylatoxin receptors in inflamed lungs, a finding which may account for many of the immunomodulatory effects of anaphylatoxins observed in vivo.

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References

- Abe M, Shibata K, Akatsu H, Shimizu N, Sakata N, Katsuragi T, et al. Contribution of anaphylatoxin C5a to late airway responses after repeated exposure of antigen to allergic rats. *J Immunol* 2001;167:4651–60.
- Ames RS, Li Y, Sarau HM, Nuthulaganti P, Foley JJ, Ellis C, et al. Molecular cloning and characterization of the human anaphylatoxin C3a receptor. *J Biol Chem* 1996;271:20231–4.
- Baelder R, Fuchs B, Bautsch W, Zwirner J, Kohl J, Hoymann HG, et al. Pharmacological targeting of anaphylatoxin receptors during the effector phase of allergic asthma suppresses airway hyperresponsiveness and airway inflammation. *J Immunol* 2005;174:783–9.
- Bautsch W, Hoymann HG, Zhang Q, Meier-Wiedenbach I, Raschke U, Ames RS, et al. Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. *J Immunol* 2000;165:5401–5.
- Bernier T, Tschernig T, Pabst R, Macke O, Steinmueller C, Emmendorffer A. Effects of macrophage-CSF on pulmonary macrophage repopulation after bone marrow transplantation. *J Leukoc Biol* 2001;70:39–45.
- Braun MC, Reins RY, Li TB, Hollmann TJ, Dutta R, Rick WA, et al. Renal expression of the C3a receptor and functional responses of primary human proximal tubular epithelial cells. *J Immunol* 2004;173:4190–6.
- Drouin SM, Kildsgaard J, Haviland J, Zabner J, Jia HP, McCray PB, et al. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. *J Immunol* 2001;166:2025–32.
- Drouin SM, Corry DB, Hollman TJ, Kildsgaard J, Wetsel RA. Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. *J Immunol* 2002;169:5926–33.
- Drouin SM, Sinha M, Sfyroera G, Lambris JD, Wetsel RA. A protective role for the fifth complement component (C5) in allergic airway disease. *Am J Respir Crit Care Med* 2006;173:852–7.
- Fayyazi A, Scheel O, Werfel T, Schweyer S, Oppermann M, Gotze O, et al. The C5a receptor is expressed in normal renal proximal tubular but not in normal pulmonary or hepatic epithelial cells. *Immunology* 2000;99:38–45.
- Floreani AA, Wyatt TA, Stoner J, Sanderson SD, Thompson EG, Allen-Gipson D, et al. Smoke and C5a induce airway epithelial intercellular adhesion molecule-1 and cell adhesion. *Am J Respir Cell Mol Biol* 2003;29:472–82.
- Fregonese L, Swan FJ, van Schadewijk A, Dolhnikoff M, Santos MA, Dahan MR, et al. Expression of the anaphylatoxin receptors C3aR and C5aR is increased in fatal asthma. *J Allergy Clin Immunol* 2005;115:1148–54.
- Haviland DL, McCoy RL, Whitehead WT, Akama H, Molmenti EP, Brown A, et al. Cellular expression of the C5a anaphylatoxin receptor (C5aR): demonstration of C5aR on nonmyeloid cells of the liver and lung. *J Immunol* 1995;154:1861–9.
- Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y, et al. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 2000;406:998–1001.
- Karp CL, Grupe A, Schadt E, Ewart SL, Keane-Moore M, Cuomo PJ, et al. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 2000;1:221–6.
- Kiafard Z, Tschernig T, Schweyer S, Bley A, Neumann D, Zwirner J. Use of monoclonal antibodies to assess expression of anaphylatoxin receptors in tubular epithelial cells of human, murine and rat kidneys. *Immunobiology* 2007;212:129–39.
- Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Kohl J. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med* 2001;164:1841–3.
- Lehmann C, Wilkening A, Leiber D, Markus A, Krug N, Pabst R, et al. Lymphocytes in the bronchoalveolar space reenter the lung tissue by means of the alveolar epithelium, migrate to regional lymph nodes, and subsequently rejoin the systemic immune system. *Anat Rec* 2001;264:229–36.
- Melendi GA, Hoffman SJ, Karron RA, Irusta PM, Laham FR, Humbles A, et al. C5 modulates airway hyperreactivity and pulmonary eosinophilia during enhanced respiratory

- syncytial virus disease by decreasing C3a receptor expression. *J Virol* 2007;81:991–9.
- Nakano Y, Morita S, Kawamoto A, Suda T, Chida K, Nakamura H. Elevated complement C3a in plasma from patients with severe acute asthma. *J Allergy Clin Immunol* 2003;112:525–30.
- Schmiedl A, Tschernig T, Lührmann A, Pabst R. Leukocyte infiltration of the periarterial space of the lung after allergen provocation in a rat asthma model. *Pathobiology* 2005;72:308–15.
- Schraufstatter IU, Trieu K, Sikora L, Sriramaraio P, DiScipio R. Complement C3a and C5a induce different signal transduction cascades in endothelial cells. *J Immunol* 2002;169:2102–10.
- Sohns B. Untersuchungen zur Expression des humanen C3a-Rezeptors. PhD thesis. Hannover, Germany: University of Hannover; 2000.
- Thangam EB, Venkatesha RT, Zaidi AK, Jordan-Sciutto KL, Goncharov DA, Krymskaya VP, et al. Airway smooth muscle cells enhance C3a-induced mast cell degranulation following cell–cell contact. *FASEB J* 2005;19:798–800.
- Walport MJ. Advances in immunology: complement. *N Engl J Med* 2001;344:1058–66.
- Weigt H, Nassenstein C, Tschernig T, Muhlradt PF, Krug N, Braun A. Efficacy of macrophage-activating lipopeptide-2 combined with interferon-gamma in a murine asthma model. *Am J Respir Crit Care Med* 2005;172:566–72.
- Werfel T, Zwirner J, Oppermann M, Sieber A, Begemann G, Drommer W, et al. CD88 antibodies specifically bind to C5aR on dermal CD117+ and CD14+ cells and react with a desmosomal antigen in human skin. *J Immunol* 1996;157:1729–35.