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A Critical Role for C5L2 in the Pathogenesis of Experimental Allergic Asthma

Xun Zhang,§,1 Inken Schmudde,§,1 Yves Laumannier,§,1 Manoj. K. Pandey,* Jennifer R. Clark,§ Peter König,§ Norma P. Gerard,§ Craig Gerard,§ Marsha Wills-Karp,§ and Jörg Köhl*,†

The Journal of Immunology

The complement fragment C5a plays dual roles in the development of experimental allergic asthma. It protects from pulmonary allergy by a regulatory effect on dendritic cells during allergen sensitization, but is proallergic during the effector phase. C5a can bind to two distinct receptors (i.e., C5a receptor and C5a receptor-like 2 [C5L2]). The functional role of C5L2 in vivo remains enigmatic. In this study, we show in two models of OVA- and house dust mite (HDM)-induced experimental allergic asthma that C5L2-deficient mice are protected from the development of airway hyperresponsiveness, Th2 cytokine production, eosinophilic airway inflammation, serum IgE, or mucus production. Surprisingly, HDM-induced experimental asthma in C5L2-deficient mice was associated with increased pulmonary IL-17A production and increased airway neutrophil numbers. To directly assess the role of C5L2 on myeloid dendritic cells (mDCs) during allergen sensitization, we performed single or repeated adoptive transfers of C5L2-deficient mDCs into wild-type mice. HDM-pulsed C5L2-deficient mDCs induced strong Th2 cytokine production, which was associated with marked IFN-γ and IL-17A production, decreased eosinophil numbers, and reduced IgE production as compared with HDM-pulsed mDCs from wild-type mice. HDM stimulation of C5L2−/− mDCs in vitro resulted in production of Th17-promoting cytokine IL-23, which was absent in wild-type mDCs. Our findings suggest that C5L2 acts at the mDC/T cell interface to control the development of Th1 and Th17 cells in response to airway HDM exposure. Furthermore, it drives Th2 immune responses independent of mDCs, suggesting a complex role for C5L2 in the development of experimental allergic asthma. The Journal of Immunology, 2010, 185: 6741–6752.

Allergic asthma is a common chronic inflammatory disease with a high prevalence in western countries. It develops as a severe lung disease in response to exposure to inhaled allergens, air pollutants, chemicals, and diverse environmental factors. The clinical manifestations of asthma include wheezing, breathlessness, chest tightness, and coughing. The pathophysiology includes bronchoconstriction, airway hyperresponsiveness (AHR), mucus hyperplasia, elevated IgE production, and pulmonary infiltration of leukocytes such as eosinophils, lymphocytes, and mast cells. Intensive investigations have been focusing on the immunologic basis of asthma. It is now generally believed that asthma arises as a result of dysregulated immune responses in which CD4+ Th2 cells play a central role in disease pathogenesis and pathology (1).

The complement system is an important arm of innate immunity. Besides its well-known function as a lytic effector system in host defense against pathogens, it has recently been shown to regulate adaptive immunity both under steady state conditions and in an inflammatory environment (2). The anaphylatoxins (ATs) C3a and C5a are generated following complement activation and are recognized as proinflammatory mediators of allergic asthma. However, recent studies suggest an unexpected immunoregulatory role for C5a during allergen sensitization (3, 4). C5a exerts its effects mainly through binding to the C5a receptor (C5aR) that is widely expressed (2). C5aR signaling in pulmonary dendritic cells (DCs) has been suggested to regulate DC function and subsequent DC-mediated activation of naïve T cells as a means to control the development of allergen-induced adaptive immune responses (5). In addition to C5aR, the orphan receptor C5aR-like 2 (C5L2) has lately been identified as a second receptor for C5a (6, 7).

C5L2 binds C5a with the same high affinity as C5aR, but has a 20-fold higher affinity for the desarginylated C5a metabolite, C5adesArg, than C5aR (6, 7). C5L2 has also been considered a receptor for other complement fragments including C3a, C3a-desArg, C4a, and C4a-desArg (8, 9), although this still remains controversial as in vitro ligand-binding assays demonstrated no interaction of C5L2 with C3a, C4a, or their metabolites in various cell lines (10).

Binding of C5a to C5aR activates cells through the recruitment of G protein subtypes, including Gαq, Gα13, or Gα16 resulting in increased intracellular calcium concentration (11), MAPK (7, 12), PI3K (13), and protein kinase B/Akt (14) activation among other pathways (reviewed in Ref. 15). In contrast, C5L2 is uncoupled from G proteins due to the lack of critical intracellular amino acid

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The online version of this article contains supplemental material.

Abbreviations used in this paper: AHR, airway hyperresponsiveness; AT, anaphylatoxin; BAL, bronchoalveolar lavage; BM, bone marrow; C5aR, C5a receptor; C5aRKO, C5aR-deficient mice; C5L2, C5a receptor-like 2; C5L2KO, C5L2-deficient mice; DC, dendritic cell; Eo, eosinophil; HDM, house dust mite; i.t., intratracheal; Ly, lymphocyte; mDC, myeloid DC; MHC-II, MHC class II; Ne, neutrophil; PAS, periodic acid-Schiff; To, total cells; WT, wild-type.
Th17 differentiation, which otherwise promote airway neutrophilic inflammation. HDM-loaded C5L2-deficient myeloid DCs (mDCs) were harvested on day 10 and washed, and 1 × 10^5 DCs were injected i.t. into the airways of naive BALB/c mice. Recipient mice were challenged once after 10 d with 100 μg/ml HDM i.t. Unpulsed DCs were injected into BALB/c recipients following the same protocol as controls. After 72 h, airway responsiveness was determined, and tissue as well as blood samples were harvested for further analysis.

Allergen-induced AHR

AHR was determined by measuring dynamic airway pressure (21) or by forced oscillation technique. Briefly, dynamic airway pressure was determined in anesthetized mice at the indicated time points after the final i.t. injection. Mice were intubated and ventilated at a rate of 120 breaths per minute with a constant tidal volume of air (0.2 ml), and paralyzed with 25 mg/kg weight of decamethromon bromide. After establishment of a stable airway pressure, 25 μg/kg weight of acetylcholine was injected i.v., and dynamic airway pressure measured as airway pressure time index in cm H2O × s^{-1} was followed for 5 min. Alternatively, alternative airway resistance was measured in anesthetized mice that were mechanically ventilated using a FlexiVent (SciRq, Saint-Urbain, Quebec, Canada). Aerosolized metacholine (50 mg/ml; Sigma-Aldrich) was generated by ultrasonic nebulizer and delivered in-line through the inhalation port for 10 s and airway resistance was measured 2 min later.

Collection of blood and bronchoalveolar lavage cell differentials

Blood was drawn from abdominal aorta and centrifuged. Serum was stored at −80°C for IgE measurements. Bronchoalveolar lavage (BAL) samples were obtained by cannulating the trachea, injecting 1.0 ml ice-cold PBS, and subsequently aspirating the BAL fluid. BAL cells were washed once in PBS and counted using a hemocytometer (Paul Marienfeld, Lauda-Koenigshofen, Germany). Differential cell counts were obtained from BAL cells spun down onto slides and treated with Diff-Quick stain (Dade Behring, Deerfield, IL). A total of 500 cells was morphologically differentiated by light microscopy.

Isolation of pulmonary cells and cytokine measurements

Liberase/DNase I digest of the lungs were prepared to obtain single lung cell suspensions. Single-cell suspensions (2.5 × 10^6) were restimulated ex vivo with 1 μM OVA for the OVA model, 30 μg/ml HDM for the HDM model, or with medium alone, and incubated at 37°C for 72 h in RPMI 1640 culture medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate (Cellgro Technologies Invitrogen, Carlsbad, CA), and 50 μM 2-ME (Sigma-Aldrich). Production of IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN-γ in culture supernatants was determined and tissue samples were harvested for further analysis. The HDM-induced allergic asthma model was performed, as described previously (20).

mDC preparation and induction of the allergic phenotype in vivo

Bone marrow (BM) cells were isolated from naive BALB/c and C5L2-deficient mice by flushing femurs and tibias with ice-cold PBS. RBCs were lysed using Tris-ammonium chloride. BM cells were washed and then cultured at 2–3 × 10^6 cells/ml in complete RPMI 1640 culture medium and 20 ng/ml murine rGM-CSF (PeproTech, Rocky Hill, NJ). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 10 d. On day 9, cells were pulsed overnight with 30 μg/ml HDM (crude extract; Greer Laboratories, Lenoir, NC) in vitro. To induce pulmonary allergic phenotype in vivo, BM-derived HDM-pulsed DCs were harvested on day 10 and washed, and 1 × 10^5 DCs were injected i.t. into the airways of naive BALB/c mice.Recipient mice were challenged once after 10 d with 100 μg/ml HDM i.t. Unpulsed DCs were injected into BALB/c recipients following the same protocol as controls. After 72 h, airway responsiveness was determined, and tissue as well as blood samples were harvested for further analysis.

Materials and Methods

Mice

BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) and C5L2-deficient mice on BALB/c background were bred and maintained in the Cincinnati Children’s Hospital Medical Center-specific pathogen-free facility or Gemeinsame Tierhaltung at the University of Lübeck and used at 8–12 wk of age. Animal care was provided in accordance with National Institutes of Health guidelines. These studies were reviewed and approved by the Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committee or the Ministerium für Landwirtschaft, Umwelt und ländliche Räume (Kiel, Germany) (V312-72241.122-39 [5-1/09]).

Protocols for mouse models of experimental allergic asthma

OVA-induced asthma model was performed, as described previously, with minor modifications (14). Briefly, mice were sensitized i.p. with 20 μg OVA (grade II; Sigma-Aldrich, St. Louis, MO) and 2 mg Inject Alum (Pierce, Rockford, IL) on days 0 and 7. On days 14, 16, 18, and 20, mice were challenged with 50 μl 1.5% OVA solution intratracheally (i.t.) to generate allergic airway disease. On day 21, airway responsiveness was determined and tissue samples were harvested for further analysis. The HDM-induced allergic asthma model was performed, as described previously (20).
of OVA-specific IgE were assessed. Plates were coated with 50 μl OVA at a concentration of 10 μg/ml. Two-fold dilutions of mouse sera were then incubated in wells. OVA-specific IgE binding was detected in the same way, as described above.

**Lung histology**

Lung histological staining, detection, and quantification of mucus cell content were done, as described (15). Briefly, lungs were excised and fixed in 10% formalin. Fixed tissues were then washed with 70% ethanol, dehydrated, embedded in paraffin, and cut into 5-μm sections. Slides were stained with H&E and periodic acid-Schiff (PAS). For quantification of mucus production in the airway epithelium, PAS-positive and PAS-negative airways were counted by light microscopy for a total of four lung sections per animal. The percentages of PAS-positive airways were calculated.

**In vitro stimulation of BM mDCs**

BM-derived mDCs (1 x 10^5/plate) were stimulated with HDM (30 μg/ml) in RPMI 1640 culture medium supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies Invitrogen) at 37°C for 72 h. Secretion of IL-1β, IL-6, IL-12p40, IL-12p70, IL-23, and TGF-β in culture supernatants was determined using DuoSet ELISA kits (R&D Systems) following the manufacturer’s protocol. The sensitivities were 16 pg/ml for IL-1, 32 pg/ml for IL-6, IL-12p40, IL-23, and TGF-β, and 32 pg/ml for IL-23. Expression of surface co-stimulatory molecules was assessed by flow cytometry using fluorochrome-labeled Abs with MHC-II, CD86, and CD80. The sensitivities were 16 pg/ml for IL-12, IL-12p40, and TGF-β. Expression of surface co-stimulatory molecules was assessed by flow cytometry using fluorochrome-labeled Abs with MHC-II, CD86, and CD80. The sensitivities were 16 pg/ml for IL-12, IL-12p40, and TGF-β.

**Statistical analysis**

Statistical analysis was performed using the SigmaStat version 3.5 statistical package (Systat Software, Evanston, IL). Statistical difference of data was assessed by either ANOVA or unpaired t test. The p values <0.05 were considered statistically significant.

**Results**

**CSL2 deficiency results in a reduced asthmatic phenotype and Th2 adaptive immune responses in a mouse model of OVA-induced experimental allergic asthma**

C5a contributes to asthma pathogenesis through mechanisms involving C5aR signaling on APCs during allergen sensitization and myeloid effector cells during the allergic effector phase (3, 20). The role of the second receptor for C5a, C5L2, in asthma pathogenesis is less clear. To examine the role of CSL2 in the induction of pulmonary allergy as well as maladaptive Th2-biased immune responses, we focused on the frequently used model of OVA-induced allergic asthma (Fig. 1A). As expected, i.t. OVA challenge significantly enhanced AHR in WT mice as compared with PBS-treated controls. CSL2KO mice on a BALB/c background also exhibited a marked increase in AHR upon OVA exposure; however, its magnitude was substantially lower as compared with WT littermates (Fig. 2A). Histological staining and cell differential analysis of BAL fluid showed marked reduction in OVA-induced pulmonary accumulation of inflammatory cells and mucus secretion in CSL2KO as compared with WT mice (Fig. 2B, 2C, Table 1). Furthermore, we observed reduced total BAL cell numbers and significantly reduced eosinophil and lymphocyte numbers in OVA-treated CSL2KO as compared with WT controls. Neutrophil (Fig. 2B) and macrophage numbers (data not shown) were virtually indistinguishable between the two strains.

In addition to airway reactivity and inflammation, we also determined the impact of CSL2 deficiency on the development of maladaptive T and B cell responses in the OVA-induced asthma model. The i.t. OVA challenge enhanced the production of Th2 cytokines (IL-5, IL-13, IL-4, and IL-10) from pulmonary cells of both WT and CSL2KO, which was associated with increased levels of serum total IgE and OVA-specific IgE. Importantly, serum IgE levels and pulmonary Th2 cytokine production were markedly attenuated in CSL2KO mice as compared with WT mice (Fig. 2D, 2E). We did not detect any IFN-γ or IL-17 secretion from pulmonary cells of either strain (data not shown). Taken together, we demonstrated that mice with a defect in CSL2 show a reduced allergic phenotype as evidenced by decreased AHR, airway responsiveness, and mucus production, as well as decreased serum IgE responses following repeated pulmonary OVA exposure.

**CSL2 contributes to the expression of AHR and dysregulated Th2-polarized adaptive immunity in a mouse model of HDM-induced experimental allergic asthma**

In previous studies, controversial data have been generated regarding the role of ATs in different animal models of allergic asthma, which may result from differences in mouse strains and/or different allergic stimuli.
the nature of allergen used for immunization (22, 23). To exclude the possibility of an OVA-specific effect of C5L2 and to extend our studies to an allergen that frequently drives allergic asthma in humans (24), we next examined the impact of C5L2 deficiency on the development of HDM-induced allergic inflammation and pulmonary Th2 adaptive immunity (Fig. 1B). Similar to what we found in the OVA model, airway responsiveness was substantially increased in both WT and C5L2KOs upon i.t. HDM exposure. Importantly, C5L2KOs experienced significantly attenuated AHR when compared with WT littermates in response to either PBS or
HDM treatment, suggesting that C5L2 contributes to enhancement of airway reactivity under steady state conditions as well as during pulmonary inflammation (Fig. 3A). Furthermore, HDM-induced secretion of serum total IgE and mucus secretion were attenuated in C5L2KOs (Fig. 3C, 3D, right panels, Table II), whereas levels of HDM-specific IgE were very low and indistinguishable between the two strains (data not shown). In addition, we observed substantial leukocyte infiltration into the airways in HDM-treated mice as compared with PBS controls. The number of total cells, eosinophils, and lymphocytes in the BAL fluid was comparable between WT and C5L2-deficient mice (Fig. 3B, 3D). Interestingly, HDM-induced airway accumulation of neutrophils was markedly elevated in C5L2KOs, suggesting that C5L2 is not required for typical HDM-induced airway eosinophilia, but is important for the regulation of pulmonary neutrophil accumulation. This increase in neutrophil numbers was associated with a 4-fold increase in IL-17A production from lung cells of C5L2-deficient mice as compared with WT mice (Fig. 3E). As previous studies have shown that IL-17 contributes to neutrophil recruitment in experimental asthma (25, 26), it is tempting to speculate that the increased IL-17 in C5L2-deficient mice drives airway neutrophil trafficking in response to inhaled HDM allergen, although differences in chemokine levels may also account for this effect. Importantly, the development of adaptive Th2 responses was impaired in the absence of C5L2 as evidenced by substantially reduced production of Th2 cytokines in C5L2KOs compared with WT controls (Fig. 3E). As in the OVA model, we found no IFN-γ production (data not shown). These data suggest that C5L2 ligation contributes to the development of maladaptive immunity by promoting Th2 cytokine production.

**C5L2-deficient mDCs have no impact on AHR, but show an impaired ability to drive B cell immunity and promote a complex Th1-, Th2-, and Th17-biased cytokine profile**

Previous studies suggest a critical role of C5a in pulmonary DC-mediated T cell activation (3, 20, 27), with a prominent role for C5aR on plasmacytoid DCs, but not on mDCs (20). To investigate potential mechanisms underlying the distinct allergic phenotype observed in C5L2KOs as compared with WT mice, we focused on the potential role of C5L2 on mDCs regarding the induction of Th2-biased adaptive immunity in response to allergen exposure. For this purpose, BM-derived mDCs from WT mice or C5L2KOs were cultured in vitro, pulsed with HDM overnight, and adoptively transferred once into the airways of naive WT BALB/c mice (Fig. 1C). Adoptive transfer of HDM-pulsed WT and C5L2−/− mDCs resulted in strong and indistinguishable AHR following pulmonary HDM challenge. In contrast, airway responsiveness was significantly lower when unpulsed mDCs from either WT mice or C5L2-deficient mice had been transferred (Fig. 4A). To evaluate pulmonary inflammation, we assessed the cellular composition in the BAL fluid. As shown in Fig. 4B, the numbers of total cells, neutrophils, eosinophils, and lymphocytes in WT recipient mice significantly increased as compared with unpulsed mDCs. Importantly, the pulmonary inflammation was dominated by neutrophils (~45–50% of cells) independent of whether WT mice had been transferred with mDCs from WT or C5L2−/− mice (Fig. 4B). In contrast, we found slightly decreased eosinophil numbers and markedly decreased lymphocyte numbers in mice that had received C5L2-deficient mDCs (Fig. 4B). Histological analysis of lung tissues revealed comparable peribronchiorial and perivascular cellular infiltrates following adoptive transfer of either C5L2-deficient or WT mDCs. Furthermore, we found substantial mucus hypersecretion after transfer of HDM-pulsed mDCs, which was significantly higher than in mice challenged with unpulsed mDCs. C5L2 deficiency in mDCs did not affect mucus production (Fig. 4D, Table III, Supplemental Table 1).

In addition to airway inflammation, we examined the development of adaptive immune responses. Th2 cytokine production (IL-5, IL-13, IL-4, and IL-10) from lung cells was markedly increased after adoptive transfer of HDM-pulsed as compared with unpulsed mDCs similar to what we had observed after i.d. HDM administration (Fig. 4E versus Fig. 3E). Except for slightly reduced IL-4 levels in the C5L2 group, we found no significant differences in Th2 cytokine levels between the WT and the C5L2 groups. Surprisingly, we found some production of the prototypical cytokine of the Th1 lineage, i.e., IFN-γ in mice that had been exposed to HDM-loaded mDCs from WT mice, which was 4-fold higher after transfer of mDCs from C5L2-deficient mice (Fig. 4E). These findings suggest that C5L2 ligation in mDCs controls the differentiation and/or effector functions of Th1 cells in response to allergen exposure, at least in part. In contrast to Th2 and Th1 cytokine levels, which were higher after transfer of HDM-pulsed mDCs as compared with unpulsed mDCs, we found no differences in the magnitude of IL-17A production in response to transfer of HDM-pulsed or unpulsed mDCs (Fig. 4E), arguing against a specific role for allergen-induced IL-17 production after single mDC transfer. In contrast, we found a strong increase in IL-17A after repeated exposure to pulsed mDCs from C5L2−/−, but not from WT mice (Supplemental Figs. 1, 2A). This increase in IL-17A was associated with higher neutrophil numbers in BAL fluid (Supplemental Fig. 2B, 2C) similar to what we had observed after i.d. HDM administration into C5L2-deficient mice (Fig. 3B, 3D). Unfortunately, repeated adoptive transfer of DCs drives induction of T cell activation in the absence of HDM due to xenogeneic proteins present in PBS used in BM cell culture, as has been reported previously (28), which somewhat limits the significance of this approach.

When we evaluated the IgE response, we found a significant increase in IgE secretion in response to transfer of HDM-pulsed mDCs. Importantly, IgE levels in the C5L2 group were significantly lower than in the WT group (Fig. 4C), which is in agreement with the reduced IL-4 levels in this treatment group (Fig. 4E).

Together, these findings suggest that allergen-loaded BM-derived mDCs are capable of inducing T cell activation and Th2-polarized adaptive immunity in the airways. Furthermore, our data indicate that C5L2 ligation on mDCs does not affect Th2 cytokine production, but modulates the differentiation and/or activation of Th1 and Th17 cells (the latter only after multiple allergen exposures). This immunoregulatory effect may result directly from C5L2-mediated regulation of CD4+ T cell polarization or indirectly from a cross talk between C5L2 and other AT receptors such as the C5aR.

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**Table I. C5L2 deficiency results in reduced mucus production in response to pulmonary OVA exposure**

<table>
<thead>
<tr>
<th>PAS-Positive Airways (%)</th>
<th>PBS Group</th>
<th>OVA Group</th>
<th>p Value</th>
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<tbody>
<tr>
<td>WT</td>
<td>1.9 ± 1.2</td>
<td>55.4 ± 4.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C5L2KO</td>
<td>1.5 ± 1.5</td>
<td>35.5 ± 4.4</td>
<td>&lt;0.001</td>
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</table>

The quantification was assessed by determination of the percentages of PAS-positive airways from four lung histological sections of WT BALB/c mice and C5L2KOs in the OVA-induced asthma model. Values shown are the mean ± SEM; n = 8–10 per group. Values of p shown in table indicate statistical differences between PBS-treated and OVA-treated mice for each strain. p = 0.857 for comparison between PBS-treated WT and C5L2KOs; p = 0.008 for comparison between OVA-treated WT and C5L2KOs.
FIGURE 3. C5L2 deficiency results in reduced AHR and Th2 adaptive immune responses upon pulmonary HDM exposure. A, Airway responsiveness to i.v. acetylcholine. Airway responsiveness is expressed as the time-integrated change in airway pressure over baseline pressure. B, Total and differential cell counts in BAL fluid. Total cells (To), eosinophils (Eo), lymphocytes (Ly), and neutrophils (Ne). Differences in cell numbers are significant between the PBS group and the HDM group, unless otherwise indicated. C, Concentration of total IgE in serum. D, Histological examination of airway inflammation. Sections were stained with H&E (left panels) or for mucus production with PAS (right panels). Original magnification ×200; higher magnification for images at the upper left corner. E, Cytokine profile of pulmonary cells harvested from mice 72 h after the final in vivo HDM exposure. Supernatants were collected 72 h after in vitro cell culture. Values shown are the mean ± SEM; n = 7–10 per group; p values are indicated.
Table II. \( \text{CSL2} \) deficiency results in reduced mucus production in response to pulmonary HDM exposure

<table>
<thead>
<tr>
<th></th>
<th>PBS Group</th>
<th>HDM Group</th>
<th>( p ) Value</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.74 ± 0.74</td>
<td>46.8 ± 2.48</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C5L2KO</td>
<td>1.4 ± 0.87</td>
<td>39.2 ± 2.67</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The quantification was assessed by determination of the percentages of PAS-positive airways from four lung histological sections of WT BALB/c mice and C5L2KOs in the HDM-induced asthma model. Values shown are the mean ± SEM; \( n = 7–10 \) per group. Values of \( p \) shown in table indicate statistical differences between PBS-treated and HDM-treated mice for each strain. \( p = 0.81 \) for comparison between PBS-treated WT and C5L2KOs; \( p = 0.013 \) for comparison between HDM-treated WT and C5L2KOs.

\( \text{CSL2} \) regulates HDM-driven IL-23 production from BM-derived mDCs

To assess the mechanisms underlying the \( \text{CSL2} \)-mediated regulation of Th17 release in vivo, we determined the cytokine profile and the expression of MHC-II and of costimulatory molecules in WT and \( \text{CSL2} \)-deficient BM-derived mDCs stimulated in vitro with HDM. We focused on IL-1\( \beta \), IL-6, and TGF-\( \beta \), as these cytokines have been shown to drive Th17 differentiation (29), and on IL-23, which is critical for the maintenance of Th17 cells (30). IL-6 production from unpulsed \( \text{CSL2} \)-deficient mDCs was significantly enhanced as compared with WT mDCs (Fig. 5A). In the presence of HDM, IL-6 production from mDCs of either WT or \( \text{CSL2} \)-deficient mice increased substantially and reached similar levels (∼20 ng/ml). IL-1\( \beta \) production from WT or \( \text{CSL2} \)-deficient mDCs was low in the absence of HDM. HDM stimulation resulted in clear IL-1\( \beta \) production from both strains, although IL-1\( \beta \) production from \( \text{CSL2} \)-deficient mDCs was significantly lower (Fig. 5B). Total TGF-\( \beta \) production from WT and \( \text{CSL2} \)-deficient DCs was high even in the absence of HMD stimulation and did not change following HDM stimulation. Of note, the overall TGF-\( \beta \) production from \( \text{CSL2} \)-deficient DCs was lower than that of WT DCs (Fig. 5C). Importantly, \( \text{CSL2} \)-deficient DCs, but not WT DCs, produced significant amounts of IL-23, which was associated with marked production of IL-12p40 (Fig. 5D, 5E), whereas IL-12p70 was below the detection limit (data not shown). Considering the increased IL-17 production in response to repeated adoptive transfer of HDM-loaded mDCs from \( \text{CSL2} \)-deficient mice, these data suggest the regulatory impact of \( \text{CSL2} \) is not related to the induction, but rather to the maintenance of Th17 cells.

\( \text{CSL2} \) regulates steady state MHC-II expression on BM-derived mDCs

MHC-II, costimulatory molecules of the B7 family, and CD40 play important roles in mDC-mediated activation of naïve T cells in allergic asthma (31). As expected, we found only minor expression of MHC-II on WT mDCs under steady state conditions, which was not altered by HDM exposure. In contrast, MHC-II expression was 5-fold higher in \( \text{CSL2} \)-deficient mDCs (Fig. 6A). Again, HDM exposure did not change MHC-II expression. Similarly, we found a trend toward higher CD80 and CD86 expression on \( \text{CSL2} \)-deficient mDCs as compared with WT mDCs, both under steady state and under HDM-treated conditions (Fig. 6B, 6C), although the differences did not reach the level of statistical significance. CD40 expression also tended to be higher in \( \text{CSL2} \)-deficient mDCs, although this effect was observed only under steady state conditions (Fig. 6D). Taken together, our data indicate that \( \text{CSL2} \) has a strong negative regulatory impact on steady state expression of MHC-II, but no obvious effect on B7 costimulatory molecule and CD40 expression.

Discussion

C5a exerts a Janus-faced function in allergic asthma, i.e., it protects from the development of maladaptive Th2 responses during allergen sensitization, but enhances the allergic phenotype during the effector phase (3). Specific targeting of the classical C5aR, CD88 (3, 32), as well as its targeted deletion (20) suggests a major role of this receptor in asthma development.

However, in the absence of C5aR, C5a and C5a-desArg can bind to \( \text{CSL2} \), which has a similar cellular distribution to CD88 (7). This is in contrast to C5aR-deficient mice, which suffer from increased airway inflammation and Th2 cytokine production (23). In contrast, when C5aR-deficient C57BL/6 mice were sensitized with a combination of OVA and Aspergillus (22) or when BALB/c mice were immunized i.t. with HDM (20), Th2 adaptive immune responses were attenuated. Thus, \( \text{CSL2} \) and C3aR appear to exert partially overlapping proallergic functions. This is in contrast to C5aR-deficient mice, which suffer from increased airway inflammation and Th2 cytokines in response to OVA (3). Recently, we have shown that the reduction of the allergic phenotype in C3aR-deficient mice results mainly from a dominant protective role of C5aR signaling in the C3aR-deficient mice (20). At this point, it is unclear whether the reduced phenotype in \( \text{CSL2} \)-deficient mice can be assigned to protective C5aR signaling or to the lack of \( \text{CSL2} \) signaling by a pathway that is independent of G protein coupling (33).

In order to investigate the role of \( \text{CSL2} \) in pulmonary allergy in a model closer to human asthma, we determined the functions of \( \text{CSL2} \) in a HDM model induced by crude extracts from \text{Derma-}

tophaeoides pteronyssinus, which accounts for atopic symptoms in 10% of individuals with asthma (24). First, we noticed that the overall response following HDM sensitization was lower as compared with OVA sensitization. This effect may be due to presence of alum, which functions as a strong adjuvant during peritoneal OVA immunization (34). Secondly, we observed substantial differences in the allergic phenotype of \( \text{CSL2} \)-deficient mice immunized with OVA or HDM. Similar to the OVA model, \( \text{CSL2} \) deficiency reduced AHR and the IgE response. In contrast to the OVA model, eosinophil and lymphocyte numbers were not affected by the absence of \( \text{CSL2} \), whereas neutrophil counts in BAL were substantially higher in \( \text{CSL2} \)-deficient mice following HDM immunization. The differential pattern of cellular responses was recapitulated by the cytokine profile. Whereas IL-4, IL-10, and IL-13 levels were markedly reduced in \( \text{CSL2} \)-/ mice, IL-5 concentrations were indistinguishable between WT and \( \text{CSL2} \)-/ mice. Importantly, the increased neutrophil count in BAL of \( \text{CSL2} \)-deficient mice was associated with elevated IL-17A production from lung cells.

Our data suggest a complex proallergic role for \( \text{CSL2} \) in HDM-mediated induction of pulmonary allergy. Extracts from HDM are known to promote allergic asthma via complex mechanisms, including TLR4 signaling on epithelial cells (35, 36) and direct ef-
Indeed, targeting of MyD88 or TLR4 decreased the magnitude of allergic airway responsiveness and inflammation (40) and TLR4 deficiency on airway structural cells reduced the development of asthma (41). Importantly, cross talk between TLRs and AT receptors has already been described (42). C5a enhances TLR4-mediated IL-6, TNF, and IL-1β secretion and

**FIGURE 4.** BM-derived C5L2−/− mDCs have an impaired ability to promote HDM-induced eosinophilic and lymphocytic airway inflammation as well as B cell responses, but drive IFN-γ production. A, Airway responsiveness to i.t. metacholine (50 mg/ml) measured as airway resistance. B, Total and differential cell counts in BAL fluid. Total cells (To), eosinophils (Eo), lymphocytes (Ly), and neutrophils (Ne). Differences in cell numbers are significant between the PBS group and the HDM group, unless otherwise indicated. C, Concentrations of total IgE measured in serum. D, Histological examination of airway inflammation. Sections were stained with H&E (left panels) or for mucus production with PAS (right panels). Original magnification ×200; higher magnification for images at the upper left corner. E, Cytokine profile of pulmonary cells harvested from mice 72 h after HDM challenge. Supernatants were collected 72 h after in vitro cell culture. Values shown are the mean ± SEM; n = 8–10 per group; p values are indicated.
suppresses IL-12p40 production by a CD88-dependent mechanism in macrophages (43, 44). More specifically, C5a was shown to inhibit LPS-induced IL-12 family cytokine production, including IL-12 and IL-23 by ERK1/2 and PI3K-mediated signaling pathways (13). The role of C5L2 in C5a-mediated modulation of TLR responses is less clear. In contrast to the data from the Song laboratory (43), Chen et al. (14) reported that C5a can reduce LPS-driven TNF-α and IL-6 production from macrophages. This effect was absent in C5L2-deficient macrophages, suggesting that C5L2 affects C5a-mediated regulation of TLR4 responses. In support of this view, C5L2 promoted high mobility group box-1 production in a model of septic peritonitis and in vitro in neutrophils and peritoneal macrophages (19). The reduced asthmatic phenotype in C5L2−/− mice in response to HDM immunization may thus result from a reduction of a synergistic cross talk between TLR4 and C5L2.

The mDC population plays a key role in the Th2 polarization process following allergen encounter (45). Thus, we assessed whether the regulatory impact of C5L2 on Th2 immunity in HDM-mediated asthma results from a direct effect on mDCs. Our data that adoptive transfer of C5L2-deficient mDCs into WT mice results in decreased eosinophilic and lymphocytic inflammation and a decreased IgE response associated with slightly lower IL-4 suggest that C5L2 controls parts of the allergic phenotype through a regulatory impact on mDCs. Importantly, C5L2 deficiency in mDCs changed the pulmonary cytokine profile as compared with WT mDCs. In the absence of C5L2, mDCs were still able to drive profound production of Th2 cytokines; however, in contrast to their WT counterparts, C5L2−/− mDCs promoted the production of prototypical Th1 (IFN-γ) and, after repeated exposure to HDM-pulsed mDCs, Th17 (IL-17A) cytokines from lung cells, which was associated with increased neutrophil recruitment. These data suggest that the decreased Th2 cytokine production in response to i.t. HDM immunization does not result from a defect of C5L2 on mDCs, whereas the increased IL-17 production can be assigned to the lack of C5L2 on mDCs, at least after multiple allergen exposures. The impact of C5L2 on Th2 cytokine production may

### Table III. Pulmonary mucus production in response to adoptive transfer of HDM-stimulated mDCs from WT or C5L2-deficient mice

<table>
<thead>
<tr>
<th>PAS-Positive Airways (%)</th>
<th>Unpulsed Group</th>
<th>HDM-Pulsed Group</th>
<th>p Value</th>
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<tr>
<td>WT</td>
<td>11.98 ± 2.18</td>
<td>44.2 ± 3.28</td>
<td>&lt;0.001</td>
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<tr>
<td>C5L2KO</td>
<td>10.4 ± 1.25</td>
<td>36.8 ± 7.07</td>
<td>=0.001</td>
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The quantification was assessed by determination of the percentages of PAS-positive airways from four lung histological sections of WT BALB/c mice and C5L2KOs in the HDM-stimulated mDC adoptive transfer asthma model. Values shown are the mean ± SEM; n = 8–10 per group. Values of p shown in table indicate statistical differences between PBS-treated and HDM-treated mice for each strain. p = 0.789 for comparison between mice injected with HDM-unpulsed mDCs from WT versus C5L2−/− animals; p = 0.220 for comparison between mice injected with HDM-pulsed mDCs from WT versus C5L2−/− animals.
result from an effect on airway epithelial cells as they are critical for TLR-driven activation of the mucosal DC network in HDM-mediated asthma (35). Furthermore, epithelial cells can express C5L2 (10) and other complement factors (46). In human asthmatics and in HDM-treated mice, high concentrations of ATs have been described in BAL fluid (3, 47).

Our data further indicate that C5L2 on mDCs is critical to suppress the development of Th17 and Th1 cells. In support of this view, we found exclusive production of IL-23 in C5L2-deficient mDCs stimulated in vitro with HDM, which was absent in WT mDCs. Stable Th17 polarization is complex and requires two complementary pathways, as follows: first, the presence of at least IL-6 and TGF-β, which drive the initial differentiation of naïve CD4+ cells into Th17 cells, and secondly, the presence of IL-23, which is critical for full and sustained differentiation of this cell type (30). The missing impact of C5L2 deficiency on the major Th17-differentiating cytokine IL-6, associated with the strong impact on IL-23 and IFN-γ, suggests that the main role of C5L2 is to suppress the maintenance and sustained differentiation of Th17 cells and the induction of Th1 cells. In line with this view, we found the impact on allergen-dependent IL-17 production only after multiple i.t. allergen exposures with HDM or HDM-pulsed mDCs. In contrast, the direct effect of C5L2 on mDCs for the HDM-mediated induction of Th2 cell is at best minor.

The contribution of IL-17 to allergic asthma is complex (48, 49). In humans, several reports show elevated IL-17 production in asthmatics in particular in patients with severe asthma (50, 51), which correlates with neutrophil numbers (52, 53). In experimental asthma, IL-17 (54) and Th17 cells (25) have been shown to drive neutrophil recruitment into the airways, which is enhanced by IL-23 (55). In contrast, exogenous IL-17 was found to markedly reduce eosinophil counts in experimental asthma (49), which is in accordance with our findings of reduced eosinophilia in mice immunized with C5L2−/− mDCs. Interestingly, Wilson et al. (26) found modest Th2, but strong Th17 polarization and neutrophilia following airway immunization with OVA in the presence of low

**FIGURE 6.** Increased MHC-II expression in C5L2-deficient mDCs. BM-derived mDCs were seeded 1 × 10⁶/plate, and stimulated with HDM, 30 μg/ml, in complete RPMI 1640 culture medium. After 72 h, cells were analyzed for the expression of MHC-II (A) and the B7 costimulatory molecules CD80 (B) and CD86 (C), as well as for expression of CD40 (D). mDCs were identified as 7-aminoactinomycin D−/−CD11c+CD11bhigh cells. Values shown are the mean ± SEM; n = 3 per group; p values are indicated.

<table>
<thead>
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<th>WT</th>
<th>C5L2−/−</th>
<th>p-value</th>
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<td>MFI</td>
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<td>Unpulsed</td>
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LPS. In contrast, the authors found strong Th2 polarization and eosinophilia in response to peritoneal OVA immunization with alum in C57BL/6 mice. Similarly, we observed predominant eosinophilia and strong Th2, but no Th17 immunity following peritoneal OVA immunization in the presence of alum in WT and C5L2-deficient BALB/c mice. In this context, the lack of C5L2 had no obvious impact on Th17 cell development. In contrast, airway immunization with crude HDM extract that contains substantial LPS resulted in modest Th2 cytokine production, but no IL-17. In this study, the absence of C5L2 mediated enhanced pulmonary neutrophil accumulation associated with IL-17 production. The differences with regard to the IL-17 production reported by Wilson et al. (26) and our data are likely to result from the different Ags (OVA’ LPS versus HDM + LPS) and the different mouse strains (C57BL/6 versus BALB/c) used. In fact, mite proteases play an important role in the development of asthma. For example, proteases from Dermatophagoides species can cleave C3 and C5 into their active fragments in vitro (56), and account for strong generation of C5a/C3a during allergen sensitization (3). The mechanisms underlying Th17 differentiation in asthma remain elusive. Our data suggest that complement receptor C5L2 on mDCs regulates Th17 differentiation following repeated HDM exposure in experimental allergic asthma.

In summary, our findings provide evidence for critical roles of C5L2 in the development of experimental allergic asthma through direct effects on mDCs as well as on other pulmonary cells. C5L2 exerts negative regulatory functions at the mDC/CDC4 + T cell interface, thereby suppressing the development of Th1 and Th17 differentiation and the sustained pulmonary accumulation of neutrophils by an IL-23–dependent mechanism. This function is reminiscent of the effect of C5aR in spleen-derived DCs in which the lack of C5aR signaling resulted in increased Th17 differentiation of naive T cells in response to TLR2 stimulation (57). In contrast, C5L2 on other pulmonary cells is critical for the production of Th2 cytokines leading to AHR, eosinophilic inflammation, mucus production, and increased IgE production. Thus, C5L2 plays complex and dual roles in the pathogenesis of allergic asthma, which strongly suggest functions beyond a role as a decoy receptor for C5a/C5a-des-Arg.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental data

Figure legends

Supplemental figure 1. Protocol underlying the model of experimental allergic asthma in response to repeated pulmonary exposure to HDM-pulsed mDCs. BM cells were harvested from WT BALB/c and C5L2-deficient mice. mDCs were generated by culturing BM cells in the presence of 10% FBS and 20 ng/ml recombinant murine GM-CSF for 10 days. On day 9, mDCs were pulsed overnight with HDM. One million mDCs were injected i.t. into BALB/c recipients the following day. The same treatment was repeated three times at the indicated time points. Twenty-four hours after the final injection, airway responsiveness was determined. Subsequently BAL fluid, blood samples, lung cells and tissues were collected for further analysis. BALB/c mice receiving unpulsed mDCs served as controls.

Supplemental figure 2. BM-derived C5L2<sup>-/-</sup> mDCs have an impaired ability to promote HDM-induced AHR, eosinophilic airway inflammation and B cell responses but drive IL-17, IFN-γ production associated with neutrophilic airway inflammation. (A) Cytokine profile of pulmonary cells harvested from mice 24 hours after the final adoptive cell transfer. Supernatants were collected 72 hours after in vitro cell culture. (B) Histological examination of airway inflammation. Sections were stained with H&E (left panels) or for mucus production with PAS (right panels). Original magnification, ×200; higher magnification for images at the upper left corner. (C) Total and differential cell counts in BAL fluid. To (Total cells), Eo (eosinophils), Ly (lymphocytes), Ne (neutrophils). (D) Concentrations of total IgE measured in serum. (E) Airway responsiveness to i.v. acetylcholine is expressed as the time-integrated change in airway pressure over baseline pressure. Values shown are the mean ± SEM; <i>n</i> = 10 per group; p values are indicated.
Zhang et al. Supplemental figure 1
Zhang et al. Supplemental figure 2
BM-derived WT and C5L2<sup>−/−</sup> mDCs promote significant pulmonary mucus production in WT BALB/c mice. The quantification was assessed by determination of the percentages of PAS positive airways from four lung histological sections of WT BALB/c recipient mice. Values shown are the mean ± SEM; n=10 per group. <i>p</i>-values shown in the table indicate statistical differences between mice injected with HDM-unpulsed mDCs versus HDM-pulsed mDCs from each strain, i.e. WT and C5L2<sup>−/−</sup> mice. <i>p</i> = 0.124 for comparison between mice injected with HDM-unpulsed mDCs from WT versus C5L2<sup>−/−</sup> animals. <i>p</i> = 0.267 for comparison between mice injected with HDM-pulsed mDCs from WT versus C5L2<sup>−/−</sup> animals.

<table>
<thead>
<tr>
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<th>HDM-unpulsed mDCs</th>
<th>HDM-pulsed mDCs</th>
<th>&lt;i&gt;p&lt;/i&gt;-value</th>
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<tr>
<td>WT</td>
<td>36.5 ± 1.3</td>
<td>52.5 ± 2.4</td>
<td>&lt;0.001</td>
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<tr>
<td>C5L2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>39.6 ± 1.3</td>
<td>49.3 ± 1.5</td>
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