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*1100529
http://www.jimmunol.org/content/early/2012/02/10/jimmunol.1100529

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The Tick Salivary Protein Sialostatin L Inhibits the Th9-Derived Production of the Asthma-Promoting Cytokine IL-9 and Is Effective in the Prevention of Experimental Asthma

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Ticks developed a multitude of different immune evasion strategies to obtain a blood meal. Sialostatin L is an immunosuppressive cysteine protease inhibitor present in the saliva of the hard tick *Ixodes scapularis*. In this study, we demonstrate that sialostatin L strongly inhibits the production of IL-9 by Th9 cells. Because we could show recently that Th9-derived IL-9 is essentially involved in the induction of asthma symptoms, sialostatin L was used for the treatment of experimental asthma. Application of sialostatin L in a model of experimental asthma almost completely abrogated airway hyperresponsiveness and eosinophilia. Our data suggest that sialostatin L can prevent experimental asthma, most likely by inhibiting the IL-9 production of Th9 cells. Thus, alternative to IL-9 neutralization sialostatin L provides the basis for the development of innovative therapeutic strategies to treat asthma. *The Journal of Immunology*, 2012, 188: 000–000.

When feeding on their hosts, hard ticks face the problem of host inflammation and immunity. Therefore, the feeding success of hard ticks depends on an array of immunosuppressive substances in tick saliva, among others cysteine protease inhibitors (cystatins), which have been demonstrated to have strong immunomodulatory activity (1, 2). Cystatins from various ectoparasites interfere with Ag processing and presentation, phagocytosis, modulate cytokine expression, and NO production, and thereby impair the immune response (3). The salivary cystatin sialostatin L (sialoL) from the tick *Ixodes scapularis* inhibited the proliferation of the mouse T cell line CTLL-2, and reduced an inflammatory reaction (footpad swelling) induced by carrageenan (1). Furthermore, it prevented maturation of dendritic cells and inhibited proliferation of Ag-specific CD4+ T lymphocytes due to inhibition of cathepsin S that results in prevention of invariant chain degradation in dendritic cells (2). Recently, a cystatin from parasitic nematodes has been shown to reduce allergic and inflammatory responses (4). This filarial cystatin suppressed Th2-related inflammation and the Th2-mediated asthmatic disease in a murine model of OVA-induced allergic airway hyperresponsiveness (AHR). In addition, eosinophil recruitment was inhibited, the level of IgE was reduced, IL-4 production was downregulated, and allergic AHR was suppressed.

Experimental allergic asthma was shown to rely on a combination of cytokines (IL-4, IL-5, IL-9, IL-13) that were initially thought to be of Th2 cell origin. Concerning IL-9, it has meanwhile been shown that this cytokine can also be produced by mast cells, eosinophils, and, very recently, Th9 cells (5–7). Obviously, IL-9 can be produced by cell types closely associated with induction and maintenance of allergic diseases. Additionally, IL-9 was termed a candidate gene for asthma because it was found by linkage analyses to reside within a cytokine gene cluster together with IL-3, IL-4, and IL-5 on human chromosome 5 (8). In line with these findings, IL-9, as a pleiotropic cytokine, has several activities that favor the development of allergic asthma (9, 10). IL-9 enhances the IL-4–mediated production of IgE in human and murine B cells (11), promotes eosinophil maturation in synergy with IL-5 (12), and stimulates mucin transcription in respiratory epithelial cells (13). Studies on IL-9 transgenic mice (14) and on rIL-9 instilled into airways (15) showed that IL-9 is sufficient to produce a classical Th2-like response in vivo, and upregulates AHR, lung eosinophilia, and serum total IgE. Administration of IL-9–neutralizing Ab ablated an OVA-induced asthmatic response (16), and phase I clinical trials have been
started using anti–IL-9 Abs (17). Recently, we have shown that IL-9 from Th9 cells induces and maintains asthmatic symptoms (7).

In this study, we describe a novel mechanism of immunomodulation in which tick cystatin (sialoL) inhibits host hypersensitivity by suppression of Th9 cell-derived IL-9 production. Accordingly, sialoL inhibits asthmatic symptoms in a model of experimental asthma.

Materials and Methods

Mice

BALB/c RAG1−/− mice were obtained from The Jackson Laboratory. BALB/c and DO11.10 mice, transgenic for the OVA232-239–specific TCR-αβ (18), were bred in our animal facility (Mainz). Males and females were used at the age of 6–12 wk. Animal procedures were performed in accordance with the national convention for the use and care of animals.

Cytokines, Abs, and reagents

Mouse rIL-4 was affinity purified using a column with IL-4 mAb 11B11. Proteukin (Chiron) served as a source of human IL-2. Porcine TGF-β1 was obtained from R&D Systems. Murine IL-9 and hamster IL-9 mAb C12 were given to us by J. Van Snick (Ludwig Institute, Brussels, Belgium). The following Abs: CD28 mAb 37.51, IFN-γ mAb XMG 1.2, IL-4 mAb 11B11, IL-2 mAb S4B6.1, JES6-1A12, biotinylated JES6-5H4, CD3 mAb 145-2C11, CD4 mAb GK1.5, and allophycocyanin-conjugated IL-9 mAb RM9A4 (BioLegend).

SialoL preparation and LPS decontamination

SialoL, was expressed in Escherichia coli, and the corresponding active protein was purified, as previously described (1). Any potential LPS contamination in the stock solution was removed by Arvys Proteins using protein was purified, as previously described (1). Any potential LPS contamination in the stock solution was removed by Arvys Proteins using

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Differences in responsiveness to methacholine were assessed by repeated measures ANOVA. Numbers of eosinophils were initially analyzed by trypan blue dye exclusion. Differential cell counts were made from cytospin-prepared preparations fixed and stained with the Microscopy Hemacolor Set (Merck).

Statistical evaluations of cytokine production and qRT-PCR analyses were performed every 15 s following each nebulization step until a plateau phase was reached (22).

Bronchoalveolar lavage

After assessment of airway function, cells were isolated by lavage of the lungs via a tracheal tube with PBS (1 ml). Numbers of living cells were counted by using trypan blue dye exclusion. Differential cell counts were made from cytospin-prepared preparations fixed and stained with the Microscopy Hemacolor Set (Merck).

Lungs were fixed by inflation (1 ml) and immersion in 4% formalin and postfixed in 70% ethanol. They were sliced and mounted on slides. Subsequently, sections were stained with hematoxylin and eosin for light microscopy. Airway hyper-reactivity was determined using the FlexiVent apparatus (Scireq, Montreal, QC, Canada).

Measurement of airway reactivity

Measurements of the airway resistance were performed on anesthetized, intubated, and mechanically ventilated mice (FlexiVent; Scireq, Montreal, QC, Canada) in response to increasing doses of inhaled methacholine (3.125, 6.25, 12.5, 25, and 50 mg/ml). Measurements of airway resistance were performed every 15 s following each nebulization step until a plateau phase was reached (22).

Assay experimental protocols

OVA-induced asthma model. Ten- to 12-wk-old BALB/c mice were sensitized by i.p. injection of 20 μg OVA (grade V; Sigma-Aldrich) in a total volume of 100 μl on days 1 and 14. Mice were challenged (20 min) via the airways with OVA (1% in saline) alone or together with sialoL (i.v. 10 μg/challenge) for 3 d (days 28, 29, and 30), using ultrasonic nebulization (NE-117; Omron). Control mice groups received OVA challenge without prior sensitization. Assays were performed 24 h after the last challenge on day 31 (see Fig. 4A). Th9-induced asthma model. BALB/c RAG1−/− mice received 2 × 106 Th9 cells derived from naive CD4+ T cells from DO11.10 BALB/c mice on day 1 by i.v. injection. Mice were then left unchallenged or challenged via the airways using nebulized OVA from day 1 to 6. Thirty minutes before each challenge, mice received sialoL (i.v. 10 μg/challenge). On day 7, airway reactivity was assessed and bronchoalveolar lavage (BAL) fluid was collected on the same day (see Fig. 7A).

Bronchoalveolar lavage

After assessment of airway function, cells were isolated by lavage of the lungs via a tracheal tube with PBS (1 ml). Numbers of living cells were counted by using trypan blue dye exclusion. Differential cell counts were made from cytospin-prepared preparations fixed and stained with the Microscopy Hemacolor Set (Merck).

Histology

Lungs were fixed by inflation (1 ml) and immersion in 4% formalin and embedded in paraffin. Tissue sections were stained with H&E and periodic acid-Schiff (PAS).

Lung single-cell preparation and OVA peptide-specific activation

Isolated lungs (day 31) from BALB/c mice were minced and enzymatically digested with 200 U/ml collagenase type IA (Sigma-Aldrich) in PBS in a 37°C water bath. After 1 h of incubation, a single-cell suspension was achieved by pushing the digested lung tissue fragments through a 0.9 × 40-mm cannula (BD Microance) and a 70-μm nylon cell strainer (BD Falcon). RBCs were removed using EDTA containing Gey’s lysis buffer. Isolated lung cells (4 × 106/ml in a 24-well plate) were stimulated with 2.5 μg/ml OVA peptide 323–339 for 48 h (mRNA expression) and for 72 h (protein production), respectively.

RNA was isolated using TRIzol (Invitrogen), and cDNA was synthesized with RevertAid M-MuLV reverse transcriptase following the recommendations of the supplier (MBI Fermentas). Quantitative qRT-PCRs were performed using the following oligonucleotides: murine Hprt forward, 5′-GGT GGA TAC AGG CCA GCC TTT GGT G-3′; murine Hgprt reverse, 5′-GAG GGT AAG CTG GCC TAT AGG CT-3′; murine IL-9 forward, 5′-CTG ATG ATT GTA CCA CAC CCT GC-3′; murine IL-9 reverse, 5′-GGC TTT GCA TCT CTG TCT TCT GG-3′; murine IL-13 forward, 5′-GGA GCT GAG CAA CAT CAC ACA-3′; and murine IL-13 reverse, 5′-GTT CCT GAT GAC ATT GCA-3′.

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Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Differences in responsiveness to methacholine were assessed by repeated measures ANOVA. Numbers of eosinophils were initially analyzed by nonparametric ANOVA (Kruskal–Wallis test) for overall differences. In case of significant results, Mann–Whitney U test was used to elucidate which specific differences were statistically significant. The p values for significance were set at 0.05. Values for all measurements are expressed as the mean ± SEM.

Statistical evaluations of cytokine production and qRT-PCR were performed by GraphPad Prism software (Version 5.0) using the Unpaired Student t test. The p values < 0.05 were considered statistically significant.
Results

IL-9 production of Th9 cells is impaired in the presence of recombinant sialoL

We have previously shown that polyclonal activation of naive CD4+ T cells in the presence of IL-4 combined with TGF-β induces within 3 d a strong production of IL-9 and leads to the development of Th9 cells (19). The addition of recombinant sialoL strongly reduced primary IL-9 production in a concentration-dependent manner (Fig. 1A). This was confirmed by intracellular staining of IL-9 as well as by quantifying mRNA expression via qRT-PCR (Fig. 1B, 1C). Admittedly, it is possible that sialoL inhibits the activation of CD4+ T cells in general. However, this was ruled out because neither the expression of CD25 and CD122 nor the proliferation of CD4+ T cells was reduced by sialoL (Fig. 1D, 1E).

We have previously shown that endogenous IL-2 is essentially involved in primary IL-9 production (19). Thus, it was conceivable that the inhibitory influence of sialoL on the primary production of IL-9 was based on an impaired production of IL-2. This assumption was supported by the measurement of IL-2, which revealed that sialoL inhibited primary IL-2 production similarly to primary IL-9 production (Fig. 2A). Neutralization of endogenous murine IL-2 by mAb strongly curtailed IL-9 production (Fig. 2B). This could be completely restored by the addition of human IL-2 because it was not neutralized by murine IL-2 mAb, but perfectly active on murine T cells. However, when CD4+ T cells were activated in the presence of sialoL alone and together with human IL-2, this cytokine did not influence the inhibitory effect of sialoL, indicating that the inhibitory mechanism of sialoL is not based on the reduction of endogenous murine IL-2 (Fig. 2C).

SialoL inhibits IL-9 production promoted by IL-1

We have previously shown that the production of IL-9 by Th2 cells was strongly enhanced in the presence of IL-1 (23). In addition, it was recently shown that IL-1 can replace IL-4 in the development of Th9 cells (24). Therefore, we analyzed whether IL-1 could also enhance the primary production of IL-9 after 3 d of culture in the presence of conditions that favor Th9 development and secondary production upon restimulation after 5 d. Primary IL-9 production was strongly enhanced by IL-1β (Fig. 3A), and further analyses revealed that the same held true for IL-1α (data not shown). The addition of sialoL, however, strongly reduced this additional increase of IL-9 production promoted by IL-1. Secondary IL-9 production was also enhanced by IL-1β, but sialoL could only modestly suppress this IL-9 production (Fig. 3B). The suppressive effect of sialoL on secondary IL-9 production was less pronounced as compared with its suppressive effect on primary IL-9 production. Therefore, the expression of IL-9 was assessed with the aid of qRT-PCR to confirm the results obtained by measuring IL-9 in the supernatants. Fig. 3C clearly demonstrates that sialoL could also significantly inhibit secondary IL-9 expression even if promoted by IL-1β. These data suggest that IL-1 can enhance the production of IL-9 by Th9 cells in a similar manner to its effect on IL-9–producing T cells that develop under Th2-promoting conditions. In addition, sialoL can strongly suppress this IL-9–promoting effect of IL-1, although to a lesser extent concerning secondary production of IL-9. IL-1β from alveolar macrophages was supposed to be a mediator of asthma (25). Thus, it is tempting to speculate that a part of this activity is mediated through the upregulation of the asthma-promoting cytokine IL-9 and that
sialoL can alleviate asthma symptoms by preventing this detrimental interaction of IL-1 and IL-9. Features of experimental airway disease were strongly reduced upon the treatment with sialoL. IL-9 was found to be essentially involved in the development of airway inflammation and AHR, and we have recently shown that Th9 cells provoke AHR, eosinophilia, and increased mucus production in a model of experimental asthma (7). By neutralizing IL-9, Th9-mediated symptoms were strongly reduced, whereas Th2-mediated symptoms were only marginally affected. This indicated that the asthma-promoting activity of Th9 cells was based on the release of IL-9. In analogy to these data, the inhibitory potency of sialoL for Th9 cell-derived production of IL-9 should lead to reduced asthma symptoms in an experimental asthma model. Thus, mice were sensitized and challenged with OVA in the absence and presence of sialoL according to the experimental procedure illustrated in Fig. 4A. Subsequently, AHR and eosinophilia were comparably analyzed in the different groups of mice. OVA challenge in the absence of sensitization caused no symptoms, whereas OVA challenge of sensitized mice resulted in profound AHR and severe eosinophilia (Fig. 4B, 4C). The treatment with sialoL almost completely prevented AHR and eosinophilia, suggesting that sialoL could also inhibit Th9-derived IL-9 production under physiological conditions in vivo (Fig. 4B, 4C). These data were corroborated by staining of lung tissue sections with H&E and PAS. Cellular infiltration (Fig. 5, H&E) as well as mucus production (Fig. 5, PAS) that could be observed after challenge of sensitized mice with nebulized OVA (Fig. 5, sens/chal + sialoL).

IL-9 could not be detected in the BAL of such mice that had been treated, as described in Fig. 4A. However, stimulating lung cells that had been prepared as described in Materials and Methods.
with OVA peptide 323–339 resulted in a significant production of IL-9 on the protein and mRNA level (Fig. 6). This IL-9 production was strongly reduced when lung cells were analyzed that had been prepared from mice treated additionally with sialoL (Fig. 6, sens/chal + sialoL). Expression of IL-13 that was described to be induced by IL-9 could not be detected. To further substantiate these findings, Th9 cells were transferred to T cell-deficient RAG1−/− mice that were subsequently challenged by nebulized OVA, as outlined in Fig. 7A. The challenge with OVA led to an increased AHR that was inhibited upon the treatment with sialoL (Fig. 7B). Because IL-9 was demonstrated to mediate the asthma-promoting activity of Th9 cells (7, 26), this result strongly argues in favor of sialoL-mediated inhibition of AHR via suppression of IL-9 from transferred Th9 cells.

**Discussion**

Hard ticks like *I. scapularis* were found to be slow feeders that need up to 10 d for their blood meal. Principally, this long period of time for feeding is sufficient to provoke innate and adaptive immune reactions directed against these parasites. Therefore, they acquired immunosuppressive mechanisms to successfully feed from their hosts (27). This immunosuppression is mediated by an array of different substances in the saliva of ticks, among others sialoL (1, 2, 28–33). Interestingly, several immunomodulatory substances seem to act specifically on distinct cell populations (34). For example, PGE2 from saliva of *I. scapularis* has been shown to suppress DC-derived cytokine production (TNF-α, IL-12p70) and OVA-induced IL-2 production of T cells as well (35). In addition, we have previously shown that sialoL in analogy to PGE2 can inhibit Ag-specific T cell proliferation, whereas polyclonally (Con A-) induced proliferation was not affected (1). We concluded that sialoL preferentially suppressed the accessory function of DC rather than inhibiting T cell functions directly. Data showing that sialoL impaired the expression of CD80/CD86 and the secretion of TNF-α and IL-12p70 by LPS-activated DC further supported this assumption (2). In this study, we can show that sialoL inhibits...
the production of IL-9 by Th9 cells that had been activated polyclonally by plate-bound CD3 mAb in combination with CD28 mAb, indicating a direct inhibitory effect of sialoL on T cells. A general, nonspecific impairment of T cell activation could be excluded by the observation that proliferation and CD25/CD122 expression of these T cells were not affected by sialoL, which is in agreement with our former finding that Con A-induced T cell proliferation was not inhibited by sialoL. A closely related cystatin from the saliva of *I. scapularis* is sialoL2. Notably, this cystatin showed only a marginal suppressive effect on IL-9 production of T cells (data not shown). This finding corresponds to the fact that sialoL could prevent the symptoms at the onset of EAE, whereas sialoL2 was completely ineffective (2).

IL-9 was initially thought to be preferentially produced by Th2 cells, and subsequently, it has been shown that IL-9 induced asthmatic symptoms similar to those induced by IL-4 (9, 10). This asthma-promoting potency of IL-9 was found to be at least partially mediated by IL-13 that was produced from airway epithelial cells (36). However, we could not detect IL-13, presumably, because we used a comparatively mild asthma model applying OVA in the absence of alum. In addition, the studies showing that IL-13 was induced by IL-9 used IL-9–overexpressing mice, leading most likely to nonphysiological and permanent high local concentrations of IL-9 (37, 38). Nevertheless, an array of distinct studies confirmed that IL-9 is one of the key players concerning the development of asthma. Recently, the re-evaluation of findings that demonstrated that IL-9–producing T cells develop under the influence of TGF-β and IL-4 led to the definition of Th9 cells (5, 6). Furthermore, we found that Th9 development was dependent on IFN regulatory factor 4 (7). IFN regulatory factor 4-deficient mice were resistant to the induction of OVA-mediated asthma. The transfer of OVA-specific Th9 cells and consecutive challenge with nebulized OVA led to asthmatic symptoms that could be prevented when such animals were treated with neutralizing IL-9 mAb. Furthermore, it was shown that neutralizing IL-9 by IL-9 mAb i.v. not only inhibited airway inflammation and hyperreactivity, but also suppressed IL-4, IL-5, and IL-13 in the BAL, indicating that IL-9 controls the expression of Th2 cytokines and possesses higher asthmogenic potency than other asthma-promoting cytokines (39). These data clearly underscore that Th9-derived...
IL-9 is prominently involved in the development of allergic lung inflammations. Therefore, our finding that sialoL could substantially inhibit Th9 development and significantly reduce at least the expression of IL-9 by Th9 cells in the presence of the synergistic costimulator IL-1 prompted us to apply this cytokine in two preclinical asthma models. This approach clearly revealed that sialoL prevented OVA-induced AHR, cellular infiltration, mucus production, as well as the development of IL-9-producing T cells (Figs. 4–6). The finding that AHR caused by transfer of Th9 cells is strongly reduced by i.v. treatment with sialoL, whereas secondary IL-9 expression of Th9 cells in vitro is only partially affected, suggested that sialoL affects other cell types as well in vivo. Especially, Ag presentation by DC was described to be inhibited by sialoL (2). In fact, preliminary results obtained by restimulating Th9 cells with OVA in the presence of bone marrow-derived dendritic cells identified IL-9 as specifically upregulated after local allergen challenge in the lungs of asthmatic patients (40). Lymphocytes were identified as essential for the suppressive property of sialoL on IL-9 production (data not shown). Hence, sialoL represents a promising basis to develop a drug for asthma therapy.

The importance of the suppressive property of sialoL on IL-9 production is further emphasized by human studies showing that IL-9 is specifically upregulated after local allergen challenge in the lungs of asthmatic patients (40). Lymphocytes were identified as the major cellular source of IL-9. Furthermore, it was described that serum levels of IL-9 increased in rhinitis patients after allergen exposure (41). Finally, a phase 2b-randomized study that demonstrated the safety of a humanized IL-9 mAb (MEDI-528) for the treatment of adults with OVA allergy (41). Interleukin-9 is prominently involved in the development of allergic lung inflammations. Therefore, our finding that sialoL could substantially inhibit Th9 development and significantly reduce at least the expression of IL-9 by Th9 cells in the presence of the synergistic costimulator IL-1 prompted us to apply this cytokine in two preclinical asthma models. This approach clearly revealed that sialoL prevented OVA-induced AHR, cellular infiltration, mucus production, as well as the development of IL-9-producing T cells (Figs. 4–6). The finding that AHR caused by transfer of Th9 cells is strongly reduced by i.v. treatment with sialoL, whereas secondary IL-9 expression of Th9 cells in vitro is only partially affected, suggested that sialoL affects other cell types as well in vivo. Especially, Ag presentation by DC was described to be inhibited by sialoL (2). In fact, preliminary results obtained by restimulating Th9 cells with OVA in the presence of bone marrow-derived dendritic cells identified IL-9 as specifically upregulated after local allergen challenge in the lungs of asthmatic patients (40). Lymphocytes were identified as essential for the suppressive property of sialoL on IL-9 production (data not shown). Hence, sialoL represents a promising basis to develop a drug for asthma therapy.

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Acknowledgments

We cordially thank Dr. J. Van Snick for providing the IL-9 mAb and Dr. Josef Bodor for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

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