The tick salivary protein sialostatin L inhibits the Th9-derived production of the asthma-promoting cytokine interleukin-9 and is effective in the prevention of experimental asthma[1]

Helena Horka[*,a], **Valérie Staudt**[#,a], **Matthias Klein**[#,a], **Christian Taube**[§], **Sebastian Reuter**[+], **Nina Dehzad**[+], **John F. Andersen**[§], **Jan Kopecky**[*], **Hansjörg Schild**[#], **Michalis Kotsyfakis**[*], **Markus Hoffmann**[#], **Bastian Gerlitzki**[#], **Michael Stassen**[#], **Tobias Bopp**[#,b], and **Edgar Schmitt**[#,b]

[*] Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic and Faculty of Science, University of South Bohemia, 37005 České Budějovice, Czech Republic [#]Institute for Immunology University Medical Center of the Johannes Gutenberg-University Mainz, 55131 Mainz, Germany [+]III-Medical Clinic, University Medical Center of the Johannes Gutenberg-University Mainz, 55131 Mainz, Germany [§]Department of Pulmonology, C3-P, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands [§]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, USA

Abstract

Ticks developed a multitude of different immune evasion strategies in order to obtain a blood meal. Sialostatin L is an immunosuppressive cysteine protease inhibitor present in the saliva of the hard tick *Ixodes scapularis*. Herein we demonstrate that sialostatin L strongly inhibits the production of IL-9 by Th9 cells. Since 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.

Keywords

Th9 cells; Lung; Allergy; Parasites; Rodents

Correspondence to: Dr. Tobias Bopp and Prof. Edgar Schmitt Institute for Immunology, University Medical Center of the Johannes Gutenberg-University Mainz, Building 708, Langenbeckstraße 1 D-55131 Mainz www.immunologie-mainz.de phone: +49 (0) 6131 17-6175 (TB), 6195 (ES) fax: +49 (0) 6131 17-6260 boppt@uni-mainz.de; eschmitt@uni-mainz.de;.
[a]contributed equally
[b]joined senior authors

Introduction

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 immune suppressive 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 antigen processing and presentation, phagocytosis, modulate cytokine expression, and nitric oxide 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 antigen-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 downregulated and allergic airway hyperreactivity 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, by 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 since 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 recombinant IL-9 instilled into airways (15) showed that IL-9 is sufficient to produce a classical Th2-like response *in vivo*, and upregulates airway hyperresponsiveness (AHR), lung eosinophilia, and serum total IgE. Administration of IL-9-neutralizing antibody ablated an OVA-induced asthmatic response (16) and phase I clinical trials have been started using anti-IL-9 antibodies (17). Recently, we have shown that IL-9 from Th9 cells induces and maintains asthmatic symptoms (7).

Herein we describe a novel mechanism of immunomodulation in which tick cystatin (sialostatin L) 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 Jackson Lab (Bar Harbor). BALB/c and DO11.10 mice, transgenic for the OVA$_{323-339}$-specific TCR-$\alpha\beta$ (18), were bred in our animal facility (Mainz). Males and females were used at the age of 6–12 weeks. Animal procedures were performed in accordance with the national convention for the use and care of animals.

Cytokines, antibodies and reagents

Mouse rIL-4 was affinity purified using a column with IL-4 mAb 11B11. Proleukin (Chiron Cooperation) served as a source of human IL-2. Porcine TGF-beta1 was obtained from R&D Systems. Murine rIL-9 and hamster IL-9 mAb C12 were given to us by Dr. J. Van Snick (Ludwig Institute, Brussels, Belgium). Rat IL-9 mAb 229.4 was generated as described (19). In addition we used the following antibodies: 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, APC-conjugated IL-9 mAb RM9A4 (BioLegend).

SialoL preparation and LPS decontamination

SialostatinL 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 detergent extraction; endotoxin presence by the end of the procedure was estimated as $<4\times10^{-5}$ endotoxin U/μg protein (roughly, $<3\times10^{-14}$ g of endotoxin per microgram of protein) with a sensitive fluorescent-based endotoxin assay (PyroGene recombinant factor C endotoxin detection system; LonzaBiologics).

CD4+ T cell isolation

Murine naïve CD4+CD25− T cells were isolated as described previously (20, 21).

Stimulation and differentiation of murine T cells

Murine naïve CD4+ T cells were cultured in IMDM (Sigma), supplemented with 5% FCS, 1% Penicillin/Streptomycin, 1% L-Glutamin and Na-Pyruvat and 50μM β-Mercaptoethanol. For Th9 differentiation cells were stimulated with plate bound CD3 mAb (3μg/ml) and CD28 mAb (5μg/ml) in the presence of 300 U/ml IL-4, 20 μg/ml IFN-γ mAb, and 4 ng/ml porcine TGF-ß1.

Supernatants were used on day three to determine primary IL-9 secretion by IL-9-specific ELISA and activated T cells were used on day two or three for intracellular FACS staining and qRT-PCR analyses. For secondary stimulation Th9 cells were harvested on day five and restimulated with CD3 mAb (5μg/ml) for two days to detect IL-9 by ELISA, intracellular FACS and qRT-PCR analyses.

Lymphokine assays (ELISA)

Mouse IL-2 was detected using mAb JES6-1A12 (1μg/ml) and biotinylated mAb JES6-5H4 (1μg/ml). IL-9 was detected by mAb 229.4 (1μg/ml) and biotinylated mAb C12 (1μg/ml). ELISAs were evaluated according to reference standard curves by using known amounts of the specific cytokine.

mRNA detection

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 HGPRT forward: 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′

Murine HGPRT reverse: 5′GAG GGT AGG CTG GCC TAT AGG CT-3′

Murine IL-9 forward: 5′-CTG ATG ATT GTA CCA CAC CGT GC-3′

Murine IL-9 reverse: 5′-GCC TTT GCA TCT CTG TCT TCT GG-3′

Murine IL-13 forward: 5'-GGA GCT GAG CAA CAT CAC ACA-3'

Murine IL-13 reverse: 5'-GGT CCT GTA GAT GGC ATT GCA-3'

Oligonucleotides were chosen to span at least one intron at the level of genomic DNA. qRT-PCR analyses were performed in triplicates on an iCycler (Bio-Rad,) using the SYBR GreenER qPCR Supermix (Invitrogen). After normalization of the data according to the expression of HGPRT mRNA, the relative expression level of IL-9 mRNA was calculated.

Intracellular staining (FACS analyses)

For intracellular staining of IL-9 murine naïve CD4^{+} T cells were stimulated for 48–72 h as outlined in *Stimulation and differentiation of murine T cells* chapter of this section. BrefeldinA (Sigma) was added to the cells 4 h before harvest and washed with PBS. Fixation and permeabilization was performed with buffers from a FoxP3 staining kit (eBiosciences). Cells were stained for IL-9 (RM9A4-APC and rat IgG isotype control).

Asthma experimental protocols

OVA-induced asthma model—10- to 12-wk-old BALB/c mice were sensitized by i.p. injection of 20 µg OVA (Grade V; Sigma) 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 days (days 28, 29, and 30), using ultrasonic nebulization (NEU17, 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 2×10^6 Th9 cells derived from naïve 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. 10µg/challenge). On day 7 airway reactivity was assessed and bronchoalveolar lavage (BAL) fluid was collected on the same day (see Fig. 7A).

Measurement of airway reactivity

Measurements of the airway resistance (R_L) were performed on anesthetized, intubated and mechanically ventilated mice (FlexiVent, Scireq, Montreal, QC) in response to increasing doses of inhaled methacholine (MCh: 3.125, 6.25, 12.5, 25, and 50 mg/ml). Measurements of R_L were performed every 15 seconds 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 cytocentrifuged preparations fixed and stained with the Microscopy Hemacolor ®-Set (Merck).

Histology

Lungs were fixed by inflation (1ml) and immersion in 4% formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (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 280 units/ml collagenase type IA (Sigma) in PBS in a 37°C water bath. After 1h of incubation, a single-cell suspension was achieved by pushing the digested lung tissue

fragments through a 0.9×40-mm canula (BD Microlance) and a 70-µm nylon cell strainer (BD Falcon). Red blood cells were removed using EDTA containing Gey's lysis buffer.

Isolated lung cells (4×10^6/ml in a 24 well plate) were stimulated with 2.5µg/ml OVA peptide 323–339 for 48h (mRNA expression) and for 72h (protein production), respectively.

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Differences in responsiveness to MCh were assessed by repeated measures ANOVA. Numbers of eosinophils, were initially analyzed by non-parametric 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. *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 Graph Pad Prism software (Version 5.0) using the Unpaired Student's *t*-test. Values of $p < 0.05$ 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 naïve CD4^{+} T cells in the presence of IL-4 combined with TGF-β induces within 3 days 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 by quantifying mRNA-expression *via* qRT-PCR (Fig. 1B/C). 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/E).

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 three days of culture in the presence of conditions that favor Th9-development and secondary production upon restimulation after five days. 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 to 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 airway hyperresponsiveness (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 while 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 comparatively analyzed in the different groups of mice. OVA challenge in the absence of sensitization caused no symptoms while OVA challenge of sensitized mice resulted in profound AHR and severe eosinophilia (Fig. 4B/C). 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/C). These data were corroborated by staining of lung tissue sections with hematoxylin and eosin (H&E) and periodic acid-Schiff (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) were strongly reduced after additional treatment (i.v.) with sialoL (Fig. 5, sens/chal + SialoL).

IL-9 could not be detected in the bronchoalveolar lavage (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 & Methods with OVA peptide 323–339 resulted in a significant production of IL-9 on the protein and mRNA level (Fig. 6A/B). This IL-9 production was strongly reduced when lung cells were analyzed that had been prepared from mice treated additionally with sialoL (Fig. 6A/B, 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 which 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). Since 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 *Ixodes scapularis* were found to be slow feeders that need up to 10 days 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 in order 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 immunmodulatory 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, IL12p70) 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 antigen-specific T cell proliferation while polyclonally (ConA-) induced proliferation was not affected (1). We concluded that sialoL preferentially suppressed the accessory function of DC rather than inhibited 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). Herein, we can show that sialoL inhibits the production of IL-9 by Th9 cells that had been activated polyclonally by platebound 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 ConA-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 while 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 non-physiological 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 reevaluation of findings which 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 from IRF4 (7). IRF4-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 asthmatogenic 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 co-stimulator IL-1 prompted us to apply this cystatin 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 (Fig. 4–6).

The finding that AHR caused by transfer of Th9 cells is strongly reduced by i.v. treatment with sialoL while 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, antigen 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 BMDC as accessory cells showed a comparatively strong inhibition of secondary 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 will be completed in 2012 aims to evaluate the efficacy and safety of a humanized IL-9 mAb (MEDI-528) for the treatment of adults with uncontrolled asthma (42) (ClinicalTrials.gov Identifier: NCT00968669).

In conclusion, these data indicate that the inhibition of IL-9 is an auspicious approach for the treatment of asthma. Hence, the exploitation of the immunosuppressive potency of sialoL could be important for the development of new strategies -alternatively to humanized IL-9 mAb- for the treatment of allergic asthma.

Acknowledgments

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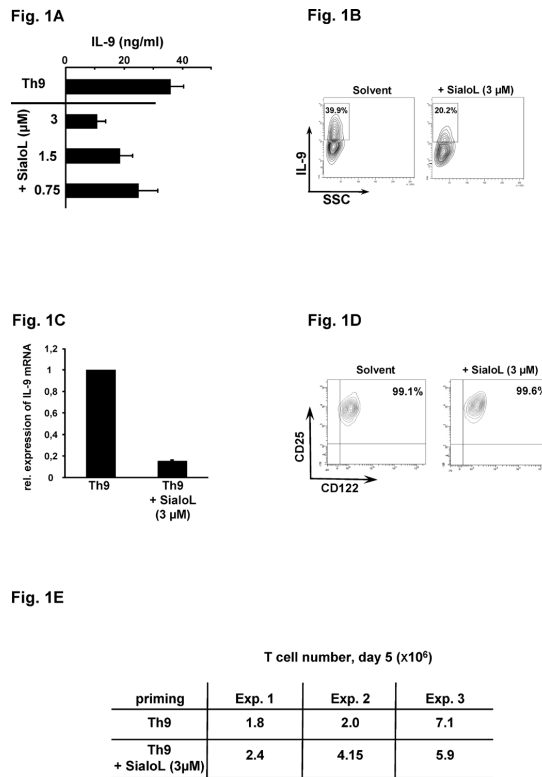
Fig. 1A

IL-9 (ng/ml)

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 1E

priming	Exp. 1	Exp. 2	Exp. 3
Th9	1.8	2.0	7.1
Th9 + SialoL (3µM)	2.4	4.15	5.9

T cell number, day 5 (x10⁶)

Figure 1. SialoL impairs primary T cell-derived production of IL-9 but not T cell activation and proliferation
Naïve CD4+ T cells from BALB/c mice were stimulated (anti-CD3/CD28) in the presence and absence of different concentrations of sialoL (0.75 µM, 1.5 µM, 3 µM) under Th9-skewing conditions. Production of IL-9 was determined by (A) ELISA, (B) FACS, and (C) qRT-PCR. (D) T cell activation was determined by FACS analyses of CD25 and CD122 cell surface expression. Shown is each time one representative from three independent experiments ± SD. (E) T cell proliferation was determined by counting the cell numbers on day five after primary activation.
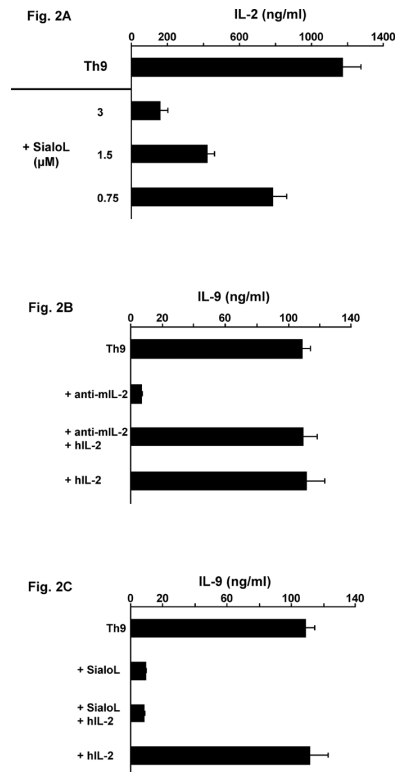
Figure 2. Exogenous human (h)IL-2 cannot compensate for the IL-9-suppressive effect of SialoL
(A) Naïve CD4^{+} T cells from BALB/c mice were stimulated (anti-CD3/CD28) in the presence or absence of different concentrations of sialoL (0.75 µM, 1.5 µM, 3 µM) under Th9-skewing conditions. Production of IL-2 was determined by ELISA. (B) Naïve CD4^{+} T cells from BALB/c mice were stimulated (anti-CD3/CD28) solely under Th9-skewing conditions or in the presence of neutralizing murine (m)IL-2 mAb (S4B6.1: 20µg/ml) or human(h)IL-2 (100ng/ml) and a combination of both. (C) Naïve CD4^{+} T cells from BALB/c mice were stimulated (anti-CD3/CD28) solely under Th9-skewing conditions or in the presence of sialoL (3 µM) or hIL-2 (100ng/ml) and a combination of both. IL-9 was determined after 72h by ELISA. Shown is one representative from two independent experiments.

Fig. 3A, 1°
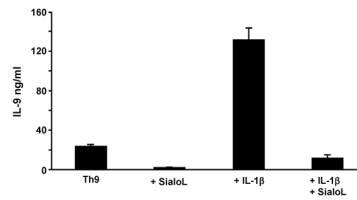
Fig. 3B, 2°
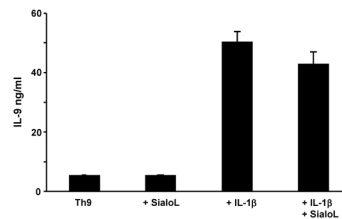
Fig. 3C, 2°
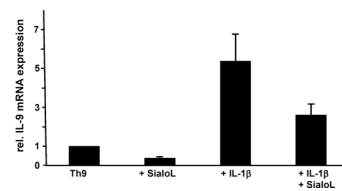
Figure 3. IL-1-mediated enhancement of secretion and expression of IL-9 is inhibited by sialoL
(A) Naïve CD4^{+} T cells from BALB/c mice were stimulated (anti-CD3/CD28) under Th9-skewing conditions in the absence or presence of sialoL (3 μM) or IL-1β (10ng/ml) and a combination of both. IL-9 was determined after 72h by ELISA. (B) Naïve CD4^{+} T cells from BALB/c mice were stimulated (anti-CD3/CD28) under Th9-skewing conditions. After five days T cells were restimulated in the absence or presence of sialoL (3 μM) or IL-1β (10ng/ml) and a combination of both. IL-9 was determined after 48h by ELISA. (C) Naïve CD4^{+} T cells from BALB/c mice were stimulated (anti-CD3/CD28) under Th9-skewing conditions. After five days T cells were restimulated in the absence or presence of sialoL (3 μM) or IL-1β (10ng/ml) and a combination of both. Expression of *Il9* was quantified after 48h by qRT-PCR. Similar results were obtained in four independent experiments.
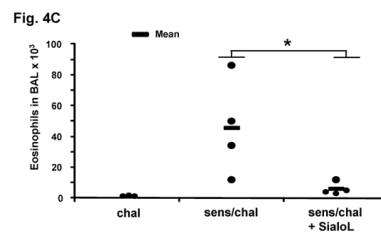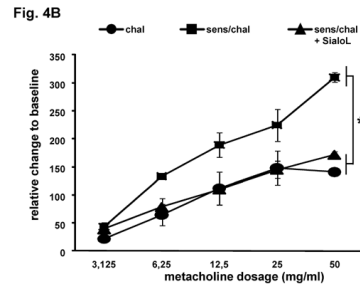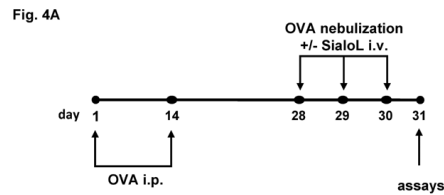
Fig. 4A

Fig. 4B

Fig. 4C

Figure 4. OVA-induced AHR and eosinophilia are strongly alleviated by treatment with sialoL
BALB/c mice were sensitized by two injections (i.p.) of OVA (20 µg) and subsequently
challenged with nebulized OVA in the absence or presence of sialoL (10 µg, i.v.) as outlined
in (A). Airway resistance (B) and numbers of eosinophils in BAL fluid (C) were measured
in mice 24h after a third airway challenge on day 30. In (B) bars represent mean ±SEM
AHR and in (C) every dot represents the number of eosinophils from an individual mouse.
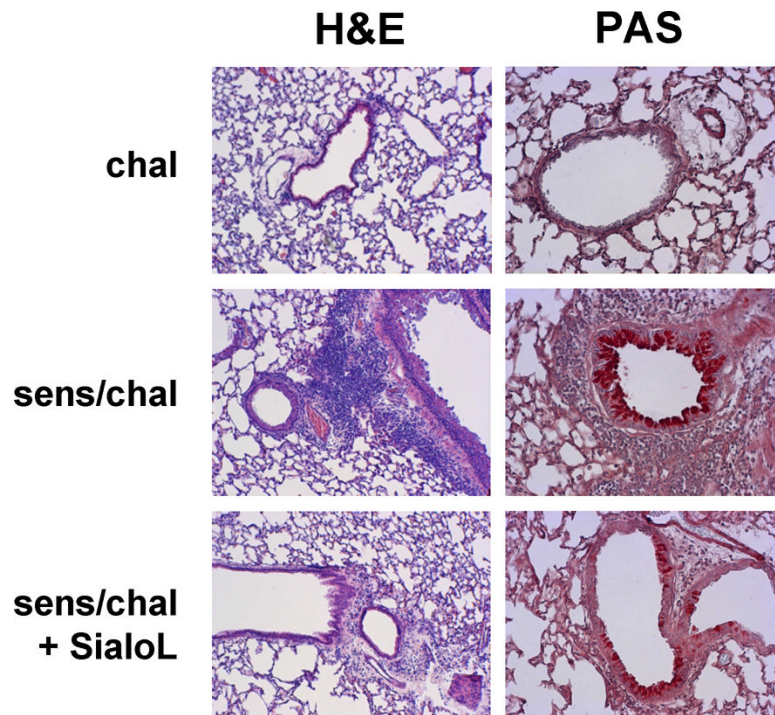In (B) and (C) * p<0.05 compared to chal and sens/chal + SialoL.

Figure 5. OVA-induced cellular infiltration and mucus production are significantly reduced by treatment with sialoL

BALB/c mice were sensitized by two injections (i.p.) of OVA (20 µg) and subsequently challenged with nebulized OVA in the absence or presence of sialoL (10 µg, i.v.) as outlined in Fig. 4A. Tissue inflammation was evaluated 24h following the last challenge using hematoxylin and eosin staining (H&E). Periodic acid-Schiff (PAS) staining was applied to determine goblet cell metaplasia in mice which were only challenged with OVA (chal), sensitized and challenged (sens/chal) and treated in addition with sialoL (i.v.; sens/chal + SialoL).
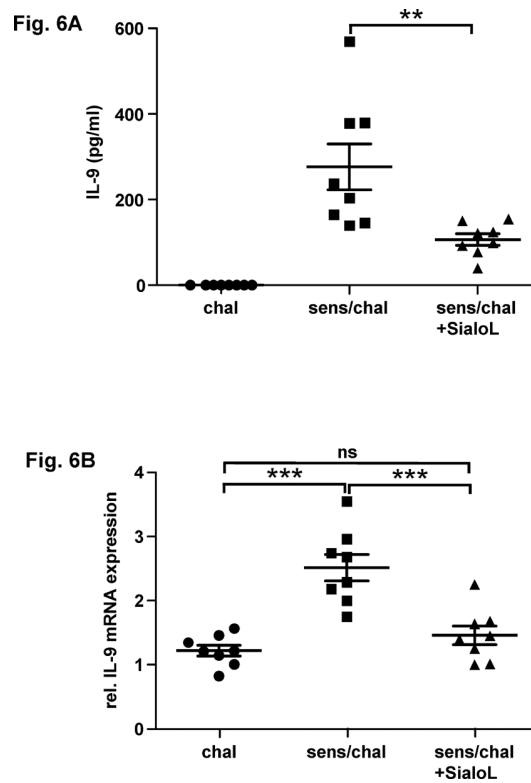
Fig. 6A

Fig. 6B

Figure 6. OVA-induced development of IL-9-producing T cells is strongly inhibited by treatment with sialoL
BALB/c mice were sensitized by two injections (i.p.) of OVA (20 µg) and subsequently challenged with nebulized OVA in the absence or presence of sialoL (10 µg, i.v.) as outlined in Fig. 4A. Lung cells were prepared from mice 24h following the last challenge (day 31) and stimulated (4×10^6/ml, 24 well plate) with 2.5 µg/ml OVA peptide 323–339 for 48h (mRNA expression) and for 72h (protein production), respectively. (A) IL-9 production was determined in the supernatants by ELISA; n= 8 mice per group. **p = 0.0084. (B) IL-9 mRNA expression levels were analyzed by quantitative real time PCR; n= 8 mice per group. *** chal vs. sense/chal p < 0.0001; ***sense/chal vs. sense/chal + SialoL p=0.0009; ns: not significant.
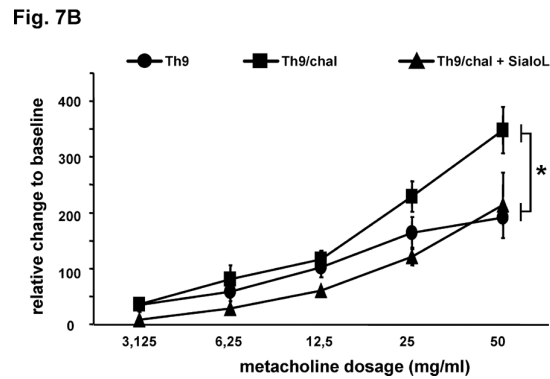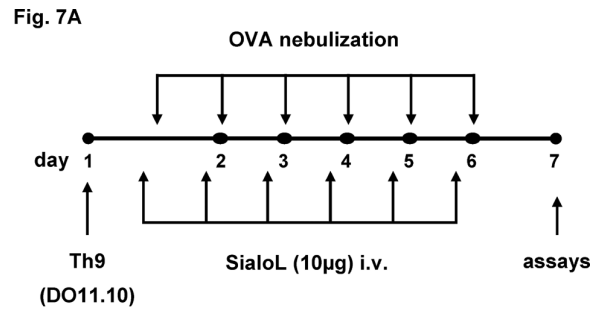
Fig. 7A

OVA nebulization

Fig. 7B

Figure 7. Th9-induced AHR is significantly reduced by treatment with sialoL

Rag1$^{-/-}$ mice were adoptively transferred with Th9 cells developed from DO11.10 mice and challenged with nebulized OVA in the absence or presence of sialoL as outlined in *Materials and Methods* and (A). Airway resistance (B) was measured in mice 24h after a final airway challenge on day 7. Bars represent mean ±SEM AHR; * p<0.05 compared to Th9 and Th9/ chall + SialoL.