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Tick Salivary Sialostatin L Represses the Initiation of Immune Responses by Targeting IRF4-Dependent Transcription in Murine Mast Cells

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Coevolution of ticks and the vertebrate immune system has led to the development of immunosuppressive molecules that prevent immediate response of skin-resident immune cells to quickly fend off the parasite. In this article, we demonstrate that the tick-derived immunosuppressor sialostatin L restrains IL-9 production by mast cells, whereas degranulation and IL-6 expression are both unaffected. In addition, the expression of IL-1 β and IRF4 is strongly reduced in the presence of sialostatin L. Correspondingly, IRF4- or IL-1R-deficient mast cells exhibit a strong impairment in IL-9 production, demonstrating the importance of IRF4 and IL-1 in the regulation of the *Il9* locus in mast cells. Furthermore, IRF4 binds to the promoters of *Il1b* and *Il9*, suggesting that sialostatin L suppresses mast cell-derived IL-9 preferentially by inhibiting IRF4. In an experimental asthma model, mast cell-specific deficiency in IRF4 or administration of sialostatin L results in a strong reduction in asthma symptoms, demonstrating the immunosuppressive potency of tick-derived molecules. *The Journal of Immunology*, 2015, 195: 000–000.

Development and reproduction of hard ticks (Ixodidae) depend on blood meals that require a close attachment to their host for several days. As a consequence, the penetration of the skin initially provokes innate immune reactions in addition to an adaptive immune response when a vertebrate host is repeatedly infested. Therefore, suppression of such an immune reaction is of vital importance for these parasites, and tick saliva contains a multitude of immune-modulating ingredients that ensure an adequate supply of blood (1). The cystatin sialostatin L (sialoL) was isolated from *Ixodes scapularis* saliva and was shown to inhibit the maturation of dendritic cells, as well as the Ag-specific stimulation and proliferation of CD4⁺ T cells (2, 3). Recently, we demonstrated that primary IL-9 production of Th9 cells is strongly

suppressed by sialoL in vitro, and its administration in vivo inhibited airway hyperresponsiveness (AHR) and eosinophilia in mice suffering from experimental asthma (4). These results suggested that suppression of Th9-derived IL-9 by sialoL prevents the development of asthmatic symptoms.

Initially, IL-9 was found to have mast cell growth-enhancing activity, and IL-9–induced mastocytosis was shown to be essential in protective immune responses during parasitic worm infections (5). Interestingly, mast cells also probably represent the most important innate source of IL-9, thus amplifying such reactions in an autocrine fashion. Mast cells preferentially reside beneath epithelial surfaces, which are considered interfaces between host and environment. This localization and the expression of a great va-

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Abbreviations used in this article: AHR, airway hyperresponsiveness; ChIP, chromatin immunoprecipitation; KL, Kit ligand; m, murine; RNA seq, RNA sequencing; sense/chal, sensitization and subsequent challenge; sialoL, sialostatin L; WT, wild-type.

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riety of TLRs (6, 7) predetermine mast cells to perceive invading parasites like ticks and to boost an immediate early immune response. In particular, their ability to rapidly release mediators, such as histamine and leukotrienes, and to serve as a primary source of cytokines, including TNF- α , IL-1 β , and IL-9, enable mast cells to finely shape the fate and magnitude of an antiparasitic immune response (8). With regard to IL-9, our analyses revealed that IL-1, IL-10, Kit ligand (KL), or the TLR4 ligand LPS synergistically enhance the production of this cytokine by mast cells (9–11). At the transcriptional level, GATA-1 was found to regulate the expression of the *Il9* gene upon IgE-mediated activation of mast cells (12). However, the detailed molecular mechanisms underlying *Il9* gene regulation in mast cells are far from being definitive.

In this study, we demonstrate that the tick saliva protein sialoL represses mast cell-derived IL-9 by inhibiting the expression of IL-1 β , as well as the transcription factor IRF4.

Materials and Methods

Mice

Mice of strain C57BL/6 were obtained from Charles River Laboratories (Sulzfeld, Germany) and bred in our own animal facility, the Translational Animal Research Center. *Ir4*-deficient mice (*Ir4*^{-/-}) (13) on a C57BL/6 background were a gift from Prof. Magdalena Huber. *Il1r1*^{-/-} (14) and *Il1a/b* double-deficient mice (15) on a C57BL/6 background were a gift from Prof. Esther von Stebut-Borschitz (University Medical Center of the Johannes Gutenberg-University Mainz).

Genetically mast cell-deficient Kit^{W-sh/W-sh} mice on a C57BL/6 background (16) were initially obtained by Prof. Marcus Maurer (Department of Dermatology, Charité, Berlin, Germany). Males and females were used at the age of 6–12 wk. Animal procedures were conducted in accordance with institutional guidelines.

Generation of mast cells and reconstitution of mast cell-deficient mice

For the generation of mast cells, mice were sacrificed by cervical dislocation. Intact femurs and tibias were removed, and bone marrow was harvested by repeated flushing with MEM supplemented with 2% FCS. Mast cell cultures were established at a density of 2×10^6 cells/ml in IMDM supplemented with 10% FCS (previously inactivated at 56°C), 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin and 100 μ g/ml streptomycin, 20 U/ml murine (m)IL-3, 50 U/ml mIL-4, and 200 ng/ml KL. Nonadherent cells were transferred to fresh culture plates every 2–3 d for ≥ 28 d to remove adherent macrophages and fibroblasts.

Kit^{W-sh/Kit} mice were systemically reconstituted with 5×10^6 mast cells derived from C57BL/6 mice, *Ir4*^{-/-} mice, or *Il1r1*^{-/-} mice by i.v. injection 8 wk before the experiments were conducted. To assess reconstitution efficiencies, lungs were fixed by inflation (1 ml), immersed in 4% formalin or Carnoy's solution, and embedded in paraffin. Tissue sections were used for avidin-based mast cell staining (17). Slides were examined in a blinded fashion with a microscope (BX-40; Olympus, Hamburg, Germany). For the assessment of mast cell numbers in each slide, mast cells were counted by a blinded investigator in five different fields, and the lung area in each field was measured using an image analysis system (Soft Imaging System; Olympus). Numbers of mast cells are expressed per cm².

Stimulation of mast cells

Stimulation of mast cells with either ionomycin or IgE/anti-IgE was performed in IMDM supplemented with 10% FCS (previously inactivated at 56°C), 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Lymphokine assays

Cytokine secretion was assessed by specific ELISA. To detect IL-9, we used mAb 229.4 (1 μ g/ml) and biotinylated mAb C12 (1 μ g/ml), which was a gift from Prof. J. van Snick (Institute de Duve, Brussels, Belgium). This ELISA detects biologically active mIL-9, as confirmed using an IL-9-specific bioassay (18). We used a Mouse IL-6 ELISA Kit (BD Pharmingen), according to the manufacturer's manual, to detect mIL-6. A Mouse IL-1 β ELISA Ready-SET-Go! kit (eBioscience) was used for the analysis

of IL-1 β content in lysates from mast cells. Lysates from 2×10^5 mast cells were prepared using 60 μ l 0.5% Triton X-100 in water; subsequently, 50 μ l was used for the detection of IL-1 β by ELISA. ELISAs were evaluated according to reference standard curves using known amounts of the specific cytokine.

Degranulation/ β -hexosaminidase release

Degranulation of mast cells was quantified by assaying the release of β -hexosaminidase. Mast cells were stimulated using 2.5 μ g/ml IgE-anti-DNP (clone SPE-7) for 72 h at 37°C. Cross-linking of receptor-bound IgE was performed using 1 μ g/ml anti-IgE for 30 min (clone EM95.3) in Tyrode's solution (10^6 cells/ml). The cells were lysed in 20 μ l 0.5% Triton X-100. Three aliquots of 20 μ l from each supernatant and the corresponding mast cell lysates were transferred to separate plates. Fifty microliters of substrate solution (1.3 mg/ml *p*-nitrophenyl-*N*-acetyl- β -D-glucosamine in 0.1 M sodium citrate [pH 4.5]) was added, and the plates were incubated for 90 min at 37°C. The reaction was stopped by adding 150 μ l 0.2 M glycine (pH 10.7). Hydrolysis of the substrate was measured at 410 nm. Activity of β -hexosaminidase in supernatant and lysate was summarized and defined as total enzyme content. The results are expressed as the percentage of β -hexosaminidase activity released into the medium.

Surface staining

IL-1R1. To determine IL-1R1 expression on mast cells, we used anti-mouse CD121a (clone JAMA-147; BioLegend).

CD117 and Fc ϵ R1. FACS analyses using anti-mouse CD117 (c-Kit) (clone ACK2; eBioscience) and anti-mouse Fc ϵ R1 (clone MAR1; eBioscience) revealed that the resulting cell populations used were >95% mast cells (data not shown).

Intracellular staining

For intracellular staining of IL-9, IL-1 β , and IL-6 murine mast cells were stimulated with ionomycin (1 μ M) for 6 h (IL-6), 24 h (IL-1 β) or 48 h (IL-9). Monensin (eBioscience) was added to the cells for the last 3–5 h of stimulation. Cells were harvested and washed with PBS. Fixation and permeabilization were performed with buffers from the Foxp3 staining kit (eBioscience). Cells were stained for IL-9 (RM9A4 and rat IgG isotype control; BioLegend), IL-1 β proform (NJTEN3 and rat IgG isotype control; eBioscience), and IL-6 (MP5-20F3 and rat IgG isotype control; eBioscience).

Western blot analyses

After stimulation with ionomycin (1 μ M) for 24 or 48 h, mast cells (10^6) were lysed in 50 μ l RIPA buffer and sonicated for 10 min (30 s on/30 s off; high power) using the Bioruptor Plus Sonication device (Diagenode). Lysates were fractionated by SDS-PAGE. After transfer to a polyvinylidene difluoride membrane, protein was analyzed by immunoblot with goat polyclonal anti-IRF4 Ab (M17) and donkey anti-goat IgG-HRP (SC2020; both from Santa Cruz Biotechnology). β -actin (clone AC-16 coupled to HRP; Sigma-Aldrich) served as control. Densitometric analysis was performed using Image Lab 4 software (Bio-Rad).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) analyses were performed using the High Cell ChIP Kit with Protein G-coated paramagnetic beads (Diagenode). After stimulation of 2×10^7 mast cells with 1 μ M ionomycin for 24 h, cells were washed twice with PBS, and protein-DNA complexes were cross-linked using formaldehyde (Thermo Scientific; 1% final concentration) for 7 min. After incubation, fixation was quenched by adding glycine to a final concentration of 125 mM. Isolation of nuclei (3×10^6 cells in 300 μ l/1.5-ml TPX tube) was performed as described in the manual using Lysis buffer 1 and Lysis buffer 2. Sonication of chromatin was conducted at 4°C in Shearing buffer S1 in the presence of a protease inhibitor mixture (Roche) using the Bioruptor Plus sonication device (Diagenode) set to high power. Four 10-min cycles (30-s pulse followed by 30 s with no pulse) were performed, leading to a distinct 100–500-bp chromatin fraction. ChIP was conducted using 6.3 μ g anti-IRF4 (M-17) or normal-goat IgG (both from Santa Cruz Biotechnology) as isotype control bound to protein G-coated magnetic beads, according to the manufacturer's instructions. After elution of DNA with DNA isolation buffer (Diagenode), conventional PCR (35 cycles) was performed with oligonucleotides flanking the desired IRF4 binding site within the murine *Il9* promoter: *Il9* prom (–285 to –265). for 5'-TTTTAAAGGGGGTTG-GGGCT-3' and *Il9* prom (–174 to –194). rev 5'-AGGCTGTCTTATGC-CAGGAA-3' or the murine *Il1b* promoter: *Il1b* prom (–1222 to –1202).

for 5'-GCTCCCTCAGCTTAAGCACA-3' and *Il1b* prom (−1034 to −1054).rev 5'-ATCGTGGTGGAATGGGCAT-3'; subsequently, PCR products were loaded onto a 2% agarose gel for visualization.

Luciferase reporter assay, plasmids, and transfection

The 5'-region of the murine *Il9* gene encompassing nt −610 to +32 (11) or the *Il1b* gene encompassing nt −758 to −1308 was cloned into the promoterless pGL3 basic luciferase reporter gene vector (Promega). Mutagenesis of two potential IRF4 binding sites (*Il1b* prom Δ−1060 to −1043 bp and *Il9* prom Δ−243 to −255 bp upstream from the translational start site) were performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Laboratories) and verified by DNA sequencing. A total of 3×10^6 mast cells in 200 μ l serum-free IMDM was transfected with 8 μ g the *Il1b* or *Il9* promoter reporter vectors or 8 μ g the mutated *Il1b* or *Il9* promoter reporter vectors in combination with 300 ng pRL-TK plasmid (Dual luciferase reporter assay system; Promega) as an internal control to exclude differences in transfection efficiency. Additionally, cells were cotransfected with 4 μ g a plasmid coding for IRF4 or empty vector pcDNA3.1

(Invitrogen). Transfections were performed by electroporation in 0.2-cm cuvettes at room temperature using a Gene Pulser II (Bio-Rad) set to 290 V and 600 μ F. Cells were allowed to recover for 3 h in mast cell culture medium and then were stimulated with 0.5 μ M ionomycin. After 16 h of stimulation, cells were harvested, washed with PBS, and lysed, according to the manufacturers' instructions. Relative luciferase activity was measured using a luminometer (TD20/20; Turner Designs).

Isolation of total RNA

A total of 5×10^6 mast cells was stimulated with ionomycin (1 μ M) in the presence or absence of sialoL (3 μ M) for 24 h. Total RNA was prepared using TRIzol reagent (Invitrogen), and RNA quality (RNA integrity number ≥ 8) was determined on a Bioanalyzer 2100 using a RNA 6000 Nano Chip (Agilent).

Next-generation sequencing and RNA sequencing

Barcoded mRNA sequencing cDNA libraries were prepared from 5 μ g total RNA using a modified version of the Illumina mRNA sequencing protocol.

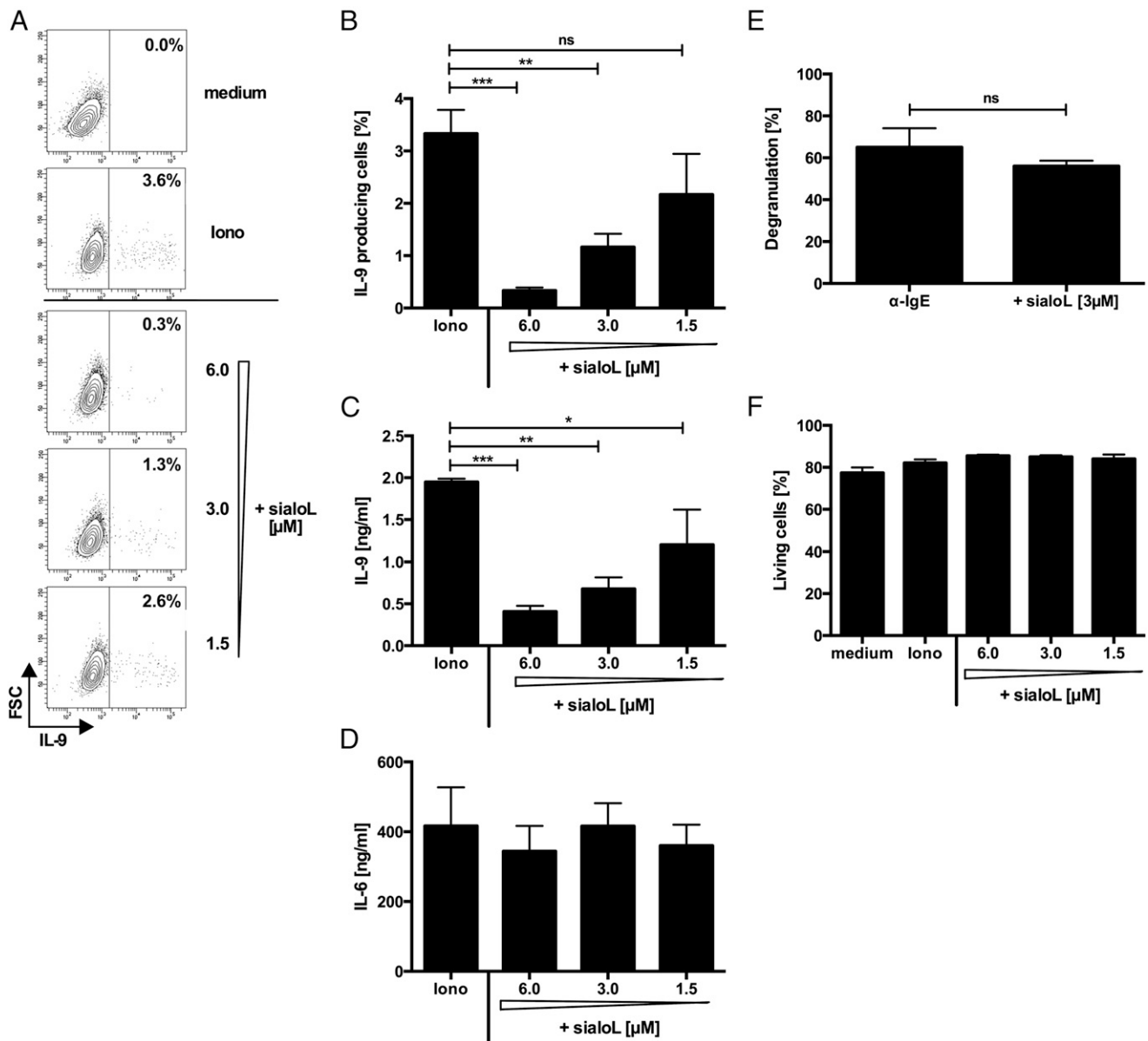


FIGURE 1. SialoL impairs mast cell-derived production of IL-9 without affecting degranulation, IL-6 secretion, or cell viability. Mast cells were stimulated with ionomycin (Iono, 1 μ M) in the presence or absence of different concentrations of sialoL (1.5, 3, and 6 μ M). Production of IL-9 was determined by intracellular flow cytometry (A and B) and ELISA (C) after 48 h of stimulation. (D) IL-6 production was measured by ELISA after 24 h of stimulation. (E) Degranulation of mast cells was assessed after sensitization with IgE and cross-linking with the aid of anti-IgE, as described in *Materials and Methods*. (F) Cell viability was measured via FACS using “Fixable Viability Dye” (eBioscience). With the exception of (A), one representative of three independent experiments, the mean $\pm$ SD of three independent experiments is shown. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, two-tailed unpaired t test. ns, not significant.

With the aid of Sera-Mag Oligo(dT) magnetic beads (Thermo Scientific), mRNA was isolated and subsequently fragmented using divalent cations and heat, resulting in fragments ranging from 160 to 220 bp. Fragmented mRNA was converted to cDNA using random primers and SuperScript II (Invitrogen), followed by second-strand synthesis using DNA polymerase I and RNase H. cDNA was end repaired using T4 DNA polymerase and Klenow DNA polymerase, and was 5' phosphorylated using T4 polynucleotide kinase. Blunt-ended cDNA fragments were 3' adenylated using Klenow fragment (3' to 5' exo minus). 3' single T-overhang Illumina Multiplex-specific adapters were ligated, using a 10:1 molar ratio of adapter to cDNA insert, with T4 DNA ligase. cDNA libraries were purified and size selected at 200–220 bp using E-Gel 2% SizeSelect gel (Invitrogen). Enrichment, adding of Illumina six base index (barcoding), and flow cell-specific sequences were done by PCR using Phusion DNA Polymerase (Finnzymes). Clean-up was done using 1.8× volume of Agencourt AMPure XP magnetic beads. All quality control was done using Invitrogen's Qubit HS assay, and fragment size was determined using Agilent's 2100 Bioanalyzer HS DNA assay. Seven picomolar of barcoded libraries were clustered on the flow cell using cBot and the TruSeq SR Cluster Kit v2.5. Fifty base pairs single reads were sequenced on the Illumina HiSeq 2000 using TruSeq SBS Kit-HS. The raw output data of the HiSeq were preprocessed according to the Illumina standard protocol. This includes filtering for low-quality reads and demultiplexing. Sequence reads were aligned to the reference genomic sequence using bowtie (19). The alignment coordinates were compared with the exon coordinates of the RefSeq transcripts (20), and the counts of overlapping alignments were recorded for each transcript. Sequence reads not alignable to the genomic sequence were aligned to a database of all possible exon-exon junction sequences of the RefSeq transcripts (parameters for the alignment to the splice junction database: default). The counts of reads aligning to the splice

junctions were aggregated with the respective transcript counts obtained after alignment and normalized to the number of reads that map per kilobase of exon model per million mapped reads (21) for each transcript. Alternatively, CLC Genomics Workbench (QIAGEN) was used to analyze the results of RNA sequencing (RNA seq).

RNA seq results were validated using quantitative PCR upon stimulation of 1×10^6 mast cells with 1 μ M ionomycin for 24 h in the presence or absence of 3 μ M sialoL using the following primers: mIL-9.for: 5'-CTGATGATTGTACCACACCGTGC-3', mIL-9.rev: 5'-GCCTTTGCATCTCTGTCTTCTGG-3'; mIRF4.for: 5'-GCCCAACAAGCTAGAAAG-3', mIRF4.rev: 5'-TCTCTGAGGGTCTGGAAACT-3'; mIL-1b.for: 5'-CAACCAACAAGTGATATTCTCCATG-3', mIL-1b.rev: 5'-GATCCACACTCTCCAGCTGCA-3'; mIL-6.for: 5'-CTGCAAGAGACTTCCATCCAG-3', mIL-6.rev: 5'-AGTGTATAGACAGGTCTGTTGG-3'; and mHPRT.for: 5'-GTTGGATACAGGCCAGACTTTGTTG-3', mHPRT.rev: 5'-GAGGGTAGGCTGGCTATAGGCT-3'.

SialoL preparation and LPS decontamination

SialoL protein was expressed in *Escherichia coli*, and the corresponding active protein was purified, as previously described (2). LPS contamination was removed by Arvys Proteins using detergent extraction. The presence of endotoxin was estimated as $<4 \times 10^{-5}$ endotoxin U/ μ g protein (approximately $<3 \times 10^{-14}$ g endotoxin/ μ g protein) with a sensitive fluorescent-based endotoxin assay (PyroGene recombinant factor C endotoxin detection system; Lonza Biologics).

Asthma experimental protocol

Ten to twelve-week-old C57BL/6 mice or Kit^{W-sh/W-sh} mice were sensitized by injection of 20 μ g OVA i.p. (grade V; Sigma-Aldrich) in a total volume

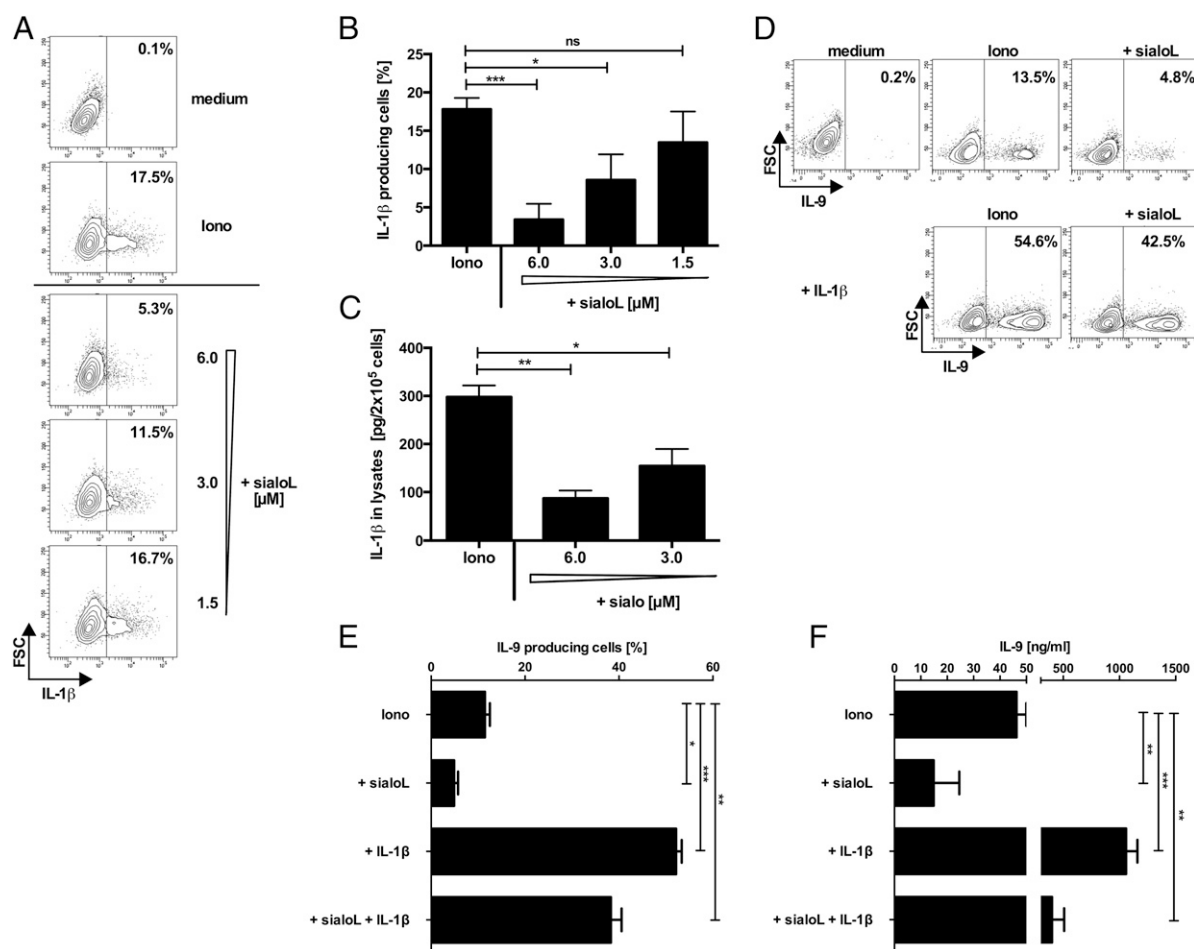


FIGURE 2. SialoL-mediated suppression of mast cell-derived IL-9 can be reversed, in part, by exogenous IL-1 β . Mast cells were stimulated with ionomycin (Iono, 1 μ M) in the presence or absence of different concentrations of sialoL (1.5, 3, and 6 μ M). Production of IL-1 β was determined by intracellular flow cytometry (A and B) or in cell lysates (C) after 48 h of stimulation. IL-9 production by mast cells was measured by intracellular FACS analysis (D and E) and ELISA (F) 48 h after stimulation with ionomycin in the presence of sialoL (3 μ M), IL-1 β (300 pg/ml), or both. With the exception of (A) and (D), one representative of three single experiments, the mean $\pm$ SD of three independent experiments is shown. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, two-tailed unpaired t test. ns, not significant.

of 100 μ l on days 1 and 14. Mice were challenged (20 min) for three consecutive days (days 27–29) with OVA (1% in saline) using ultrasonic nebulization (NE-U17; Omron). SialoL (i.v. 10 μ g/challenge) and IL-9 (intranasal 500 ng/challenge) were applied 30 min before each challenge. Bronchoalveolar lavage fluid was collected, and airway reactivity was measured 24 h after the last challenge. A schematic representation is shown in Supplemental Fig. 1.

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 using trypan blue dye exclusion. Differential cell counts were made from cytocentrifuged preparations that were fixed and stained with the microscopy Hemacolor Set (Merck).

Measurement of airway reactivity

Measurement of airway resistance was performed on anesthetized, intubated, and mechanically ventilated mice (flexiVent; SCIREQ, Montreal, QC, Canada) in response to increasing doses of inhaled methacholine (3.125–50 mg/ml). Measurement of airway resistance was performed every 15 s following each nebulization step until a plateau phase was reached.

Statistical analysis

Statistical evaluations were performed with GraphPad Prism software (version 6.0f) using the unpaired Student *t* test. The *p* values ≤ 0.05 were considered statistically significant.

Results

The tick salivary protein sialoL decisively modulates mast cell effector function

Due to their location beneath interfaces between host and environment, mast cells are probably the first cells to sense ticks upon bite. We demonstrated previously that sialoL possesses immunomodulatory properties (3, 4). To analyze its impact on mast cell effector functions, we stimulated mast cells in the presence and absence of this cystatin and measured their ability to produce IL-9 and IL-6, as well as to degranulate (Fig. 1). IL-9 production by mast cells was found to be severely impaired (Fig. 1A–C), whereas secretion of IL-6 (Fig. 1D) and degranulation (Fig. 1E) in response to IgE sensitization and subsequent anti-IgE treatment were minimally affected. Notably, the viability of mast cells was not reduced in the presence of sialoL, thus excluding nonspecific

toxic effects (Fig. 1F). These findings led us to conclude that sialoL is an immune modulator that selectively blocks mast cell-derived IL-9 production while leaving other mast cell effector functions untouched (IL-6 and degranulation).

SialoL inhibits autocrine production of the IL-9-promoting cytokine IL-1

Previously, we demonstrated that production of IL-9 by mast cells is strongly enhanced in the presence of IL-1 β (9). Therefore, we analyzed the influence of sialoL on the expression of IL-1 β by mast cells. Intracellular FACS analyses demonstrated that IL-1 β expression was strongly reduced upon stimulation of mast cells in the presence of sialoL (Fig. 2A, 2B). Because IL-1 β could not be detected in the supernatants of these mast cells, we presumed that its concentration was below the detection level of the applied ELISA. Therefore, IL-1 β was titrated on activated IL-1 α and IL-1 β double-deficient and wild-type (WT) mast cells. Supplemental Fig. 2 shows that 37.5 pg/ml of IL-1 β induced the production of IL-9 in IL-1 α and IL-1 β double-deficient mast cells (white bars), resulting in a concentration in the supernatant that was comparable to the concentration measured in the supernatant of WT mast cells in the absence of exogenous IL-1 β (Iono: black bar). Regardless of cytokine consumption, one can conclude that activated WT mast cells produce ~ 37.5 pg/ml IL-1 β . Alternatively, activated mast cells were lysed, and intracellular IL-1 β was assessed; this revealed that the IL-1 β content of mast cells was reduced considerably in the presence of sialoL (Fig. 2C). To test whether inhibition of IL-1 β production accounts, at least in part, for the observed sialoL-mediated reduction in IL-9 expression, we stimulated mast cells in the presence and absence of sialoL in combination with exogenous IL-1 β . Notably, exogenous IL-1 β almost totally compensated for the sialoL-mediated suppression of IL-9 production by mast cells on a single-cell level (Fig. 2D, 2E), as well as in the resulting supernatants (Fig. 2F).

SialoL inhibits IL-1-mediated signal transduction and IRF4 expression to suppress IL-9 production

In an attempt to identify the molecular mechanism responsible for the sialoL-mediated inhibition of IL-9 production, we performed

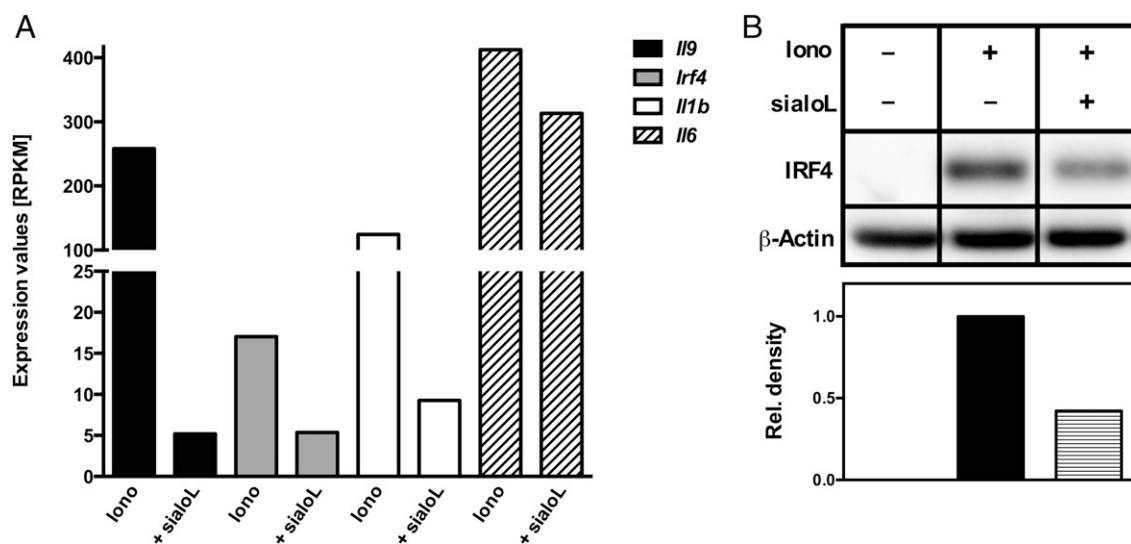


FIGURE 3. Treatment of mast cells with sialoL strongly reduces the expression of *IL9*, *Irf4*, and *IL1b*. Mast cells were stimulated with ionomycin (Iono, 1 μ M) for 24 h in the presence or absence of sialoL (+sialoL, 3 μ M). Isolation of total RNA, library preparation, and RNA seq were conducted as outlined in *Materials and Methods*. (A) Transcript counts obtained after alignment were normalized to the number of reads that maps per kilobase of exon model per million mapped reads for each transcript (single experiment). (B) The expression of IRF4 was assessed by Western blot analysis in parallel to β -actin as a loading control after 24 h of stimulation; densitometric quantification was conducted as outlined in *Materials and Methods*. Data are one representative from three independent experiments.

next-generation sequencing of mRNA (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE66966>). To this end, we stimulated mast cells in the presence and absence of sialoL using ionomycin for 24 h. In silico analyses of the raw data obtained by this approach revealed very strong inhibition of *Il9* and *Il1b* gene expression and a significant reduction in *Irf4* gene expression, whereas *Il6* gene expression was inhibited only marginally (Fig. 3A). These findings were confirmed using quantitative PCR (Supplemental Fig. 3). In analogy to IL-1 and IL-9, the inhibition of *Irf4* gene expression also could be validated on the protein level (Fig. 3B). We showed previously that IRF4 is crucial for IL-9 production by Th9 cells (22). In addition, IRF4 was shown to be involved in the regulation of *Il1b* gene expression in macrophages (23, 24). To test a potential causal correlation between the sialoL-mediated inhibition of IL-9 and IL-1 β expression and the suppression of IRF4 expression, we conducted experiments using mast cells from *Irf4*-deficient (*Irf4*^{-/-}) and *Il1r1*-deficient (*Il1r1*^{-/-}) mice. To this end, we analyzed IL-1 β and IL-9 production of *Irf4*^{-/-} mast cells, *Il1r1*^{-/-} mast cells, and the respective littermate controls (WT). After 24 and 48 h of stimulation, IL-1 β production (Fig. 4A) and IL-9 production (Fig. 4B), respectively, were measured by intracellular FACS analysis, as well as by ELISA of cell lysates (IL-1 β) and supernatants (IL-9). Although WT mast cells produced substantial amounts of IL-1 β and IL-9 upon stimulation, the pro-

duction of these cytokines by *Irf4*^{-/-} and *Il1r1*^{-/-} mast cells was strongly reduced. This indicates that IRF4 and endogenous IL-1 β are required for *Il9* gene expression. In addition, these findings suggest that an IL-1 β -dependent positive-feedback loop largely contributes to activation-induced IL-1 β production of mast cells. In analogy to sialoL-treated mast cells (Supplemental Fig. 4A, 4B), degranulation and IL-6 expression were not affected by *Irf4* or *Il1r1* deficiency (Supplemental Fig. 4C, 4D). Likewise, IRF4-deficient mast cells did not show reduced expression of IL-1R1, thereby excluding an impaired IL-1 responsiveness of such mast cells (Supplemental Fig. 4E). Furthermore, extended comparative analyses of WT and *Irf4*^{-/-} mast cells revealed no differences with regard to the content of β -hexosaminidase and the expression of RANTES (CCL5), eotaxin-2 (CCL24), eotaxin-3 (CCL26), and MCP1 (CCL2), thus excluding a general inability to produce mast cell mediators (data not shown). To analyze whether the strongly reduced ability of *Irf4*^{-/-} mast cells to produce IL-9 is due to the limited capability to produce IL-1 β , we stimulated mast cells of the genotypes depicted in Fig. 5 in the presence and absence of exogenous IL-1 β and measured IL-9 production on the single-cell level by intracellular FACS analyses (Fig. 5A, 5B) and the overall production of this cytokine by ELISA (Fig. 5C). Remarkably, IL-9 production of *Irf4*^{-/-} mast cells, but not of *Il1r1*^{-/-} mast cells, was partially restored in the presence of exogenous IL-1 β , indicating an im-

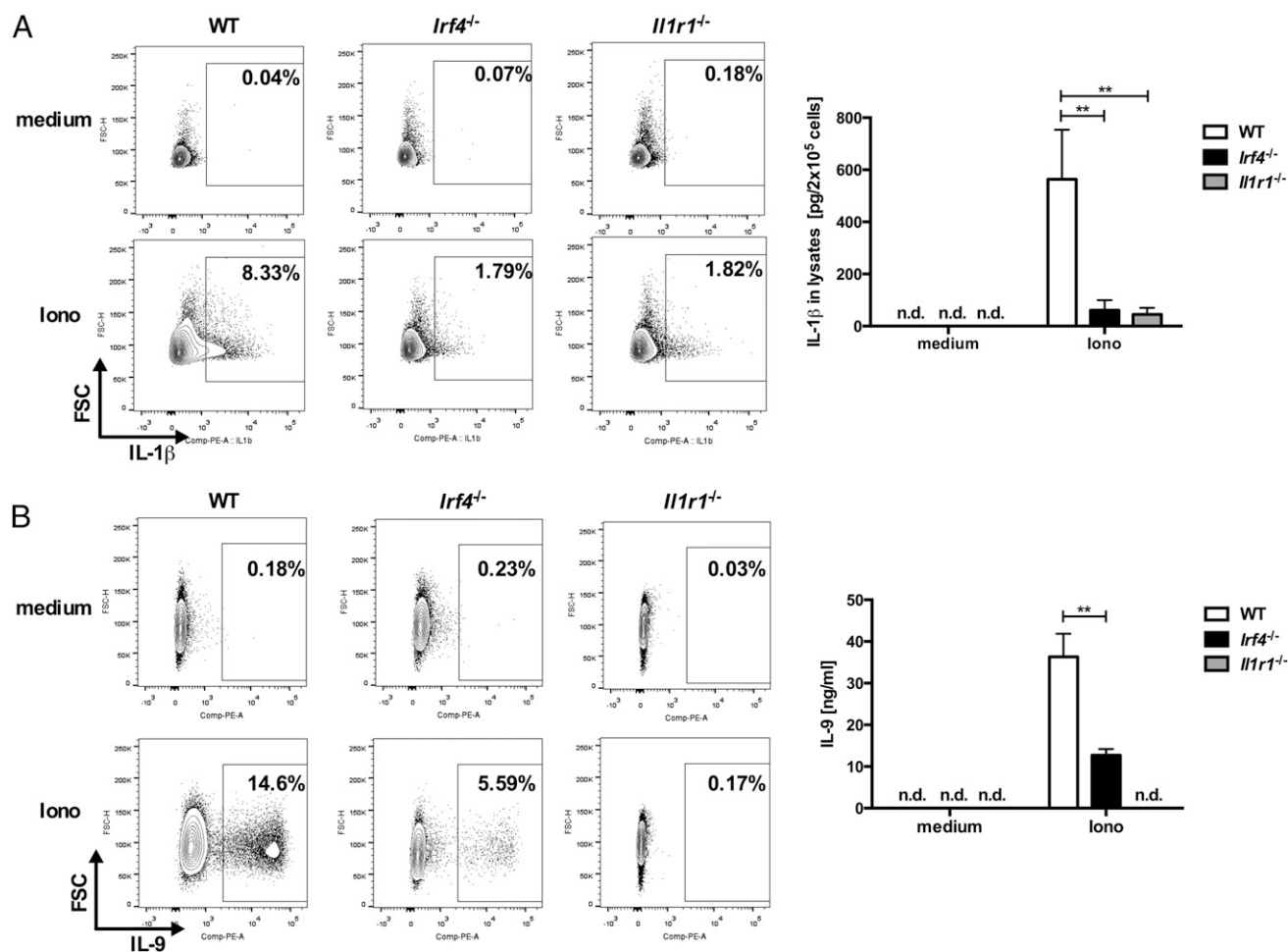


FIGURE 4. Mast cells generated from *Il1r1*^{-/-} or *Irf4*^{-/-} mice produce greatly reduced amounts of IL-1 β and IL-9. Mast cells generated from C57BL/6 (WT), *Il1r1*^{-/-}, or *Irf4*^{-/-} mice were cultured in medium (IMDM + 10% FCS) for 24 h in the presence or absence of 1 μ M ionomycin. Subsequently, the production of IL-1 β (A) or IL-9 (B) was determined by intracellular FACS analysis using anti-IL-9 or anti-IL-1 β Abs and isotype control. With the exception of the FACS blots in (A), one representative experiment of three, or (B), five single experiments, the mean $\pm$ SD of three (A) or five (B) independent experiments obtained by ELISA is shown. ** $p \leq 0.01$, two-tailed unpaired t test. n.d., not detectable.

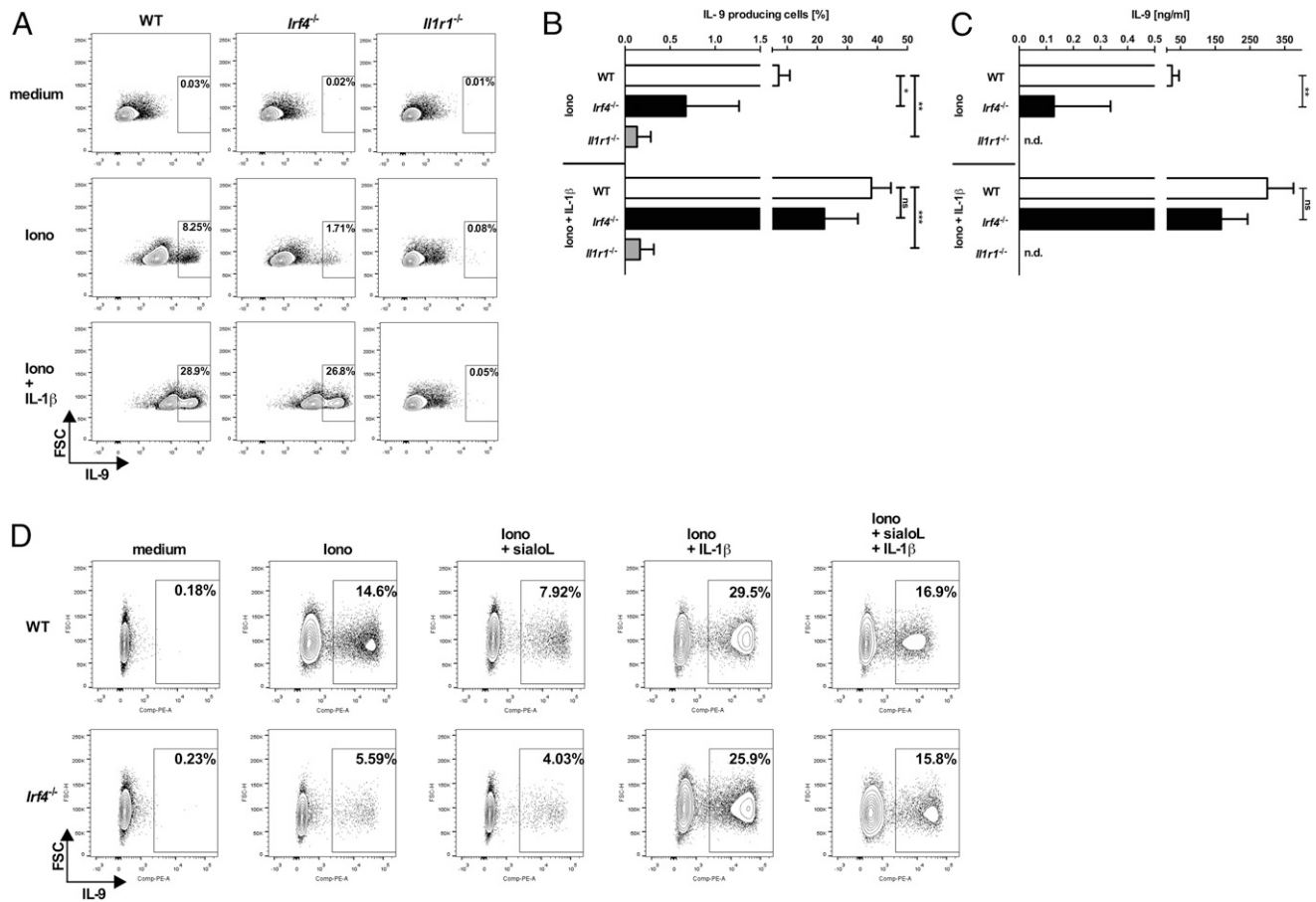


FIGURE 5. Impaired production of IL-9 by mast cells from *Irf4*^{-/-} mice can be reversed, in part, by exogenous IL-1β. Mast cells generated from C57BL/6 (WT), *Il1r1*^{-/-}, or *Irf4*^{-/-} mice were stimulated for 48 h with ionomycin (Iono, 1 μM) in the presence or absence of IL-1β (300 pg/ml) and sialoL (3 μM). Production of IL-9 was assessed by intracellular FACS analysis (A, B, and D) and ELISA in the resulting supernatants (C). With the exception of (A), one representative from three independent experiments, and (D), one representative from two independent experiments, the mean ± SD of at least four independent experiments is shown. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, two-tailed unpaired *t* test. n.d., not detectable; ns, not significant.

portant role for IRF4 in the regulation of the *Il1b* locus and the *Il9* locus. In addition, sialoL only marginally reduced the strongly attenuated IL-9 production of *Irf4*^{-/-} mast cells that could be compensated for in the presence of exogenous IL-1β approximately to the level observed in WT mast cells (Fig. 5D, *Irf4*^{-/-}, 15.8%; WT, 16.9%). Hence, this comparatively weak influence of sialoL on *Irf4*^{-/-} mast cell-derived IL-9, in combination with the strong compensatory potency of exogenous IL-1β, favors a causal involvement of IRF4 and IL-1β in sialoL-mediated inhibition of mast cell-derived IL-9 production.

IL-1R1-mediated induction of IRF4 contributes significantly to Il9 and Il1 gene regulation

To investigate the relationship between IL-1β-mediated signaling and the expression of IRF4 in mast cells, we analyzed the expression of IRF4 in the presence and absence of IL-1β. Although stimulation of mast cells with the calcium ionophore ionomycin induced the expression of IRF4, exogenous IL-1β enhanced IRF4 expression in WT mice, whereas no effect was observed in mast cells from *Il1r1*^{-/-} mice (Fig. 6A). Along with the finding that IL-1β expression by IRF4-deficient mast cells was strongly impaired (Fig. 4A), these data suggested a mutual positive regulation between IL-1β and IRF4.

We performed ChIP analysis to examine whether IRF4 binds to the *Il1b* and *Il9* locus, thereby regulating the transcription of both genes. As shown in Fig. 6B, inducible binding of IRF4 to the promoter regions of both genes is detectable upon stimulation of

mast cells. Therefore, it can be concluded that IRF4 directly regulates the expression of *Il1b* and *Il9*, respectively.

The *trans*-activating capacity of IRF4 for the *Il1b* and *Il9* promoter was demonstrated with the aid of reporter gene assays, and transfection of mast cells with an *Il1b* or *Il9* reporter construct revealed an inducible promoter activity upon stimulation that was enhanced upon ectopic expression of IRF4 (Fig. 6C, 6D). In addition, we performed *in silico* analyses and subsequent site-directed mutagenesis of potential IRF4 consensus binding sites to identify the putative consensus binding site of IRF4 in the *Il1b* and *Il9* core promoters (*Il1b* prom Δ-1060 to -1043 bp and *Il9* prom Δ-243 to -255 bp upstream from the translational start site). As shown in Fig. 6C and 6D, transfection of mast cells with these *Il1b* or *Il9* promoter-deletion mutants strongly impaired *Il1b* or *Il9* promoter activity per se, as well as upon ectopic expression of IRF4, indicating that these sites are essential for both *Il1b* and *Il9* gene expression in mast cells.

Administration of sialoL, as well as mast cell-specific deficiency in Irf4 or Il1r1 in vivo, significantly ameliorates OVA-induced eosinophilia and AHR

To test the *in vivo* relevance of the sialoL-mediated suppression of mast cell-derived IL-9 that is based on inhibition of the expression of IRF4 and IL-1β in mast cells, we used a mast cell-dependent murine model for allergic asthma (25, 26). IL-9-induced allergic symptoms include IL-5- and eotaxin-dependent infiltration of eosinophils into the lung and increased AHR (27). As depicted in

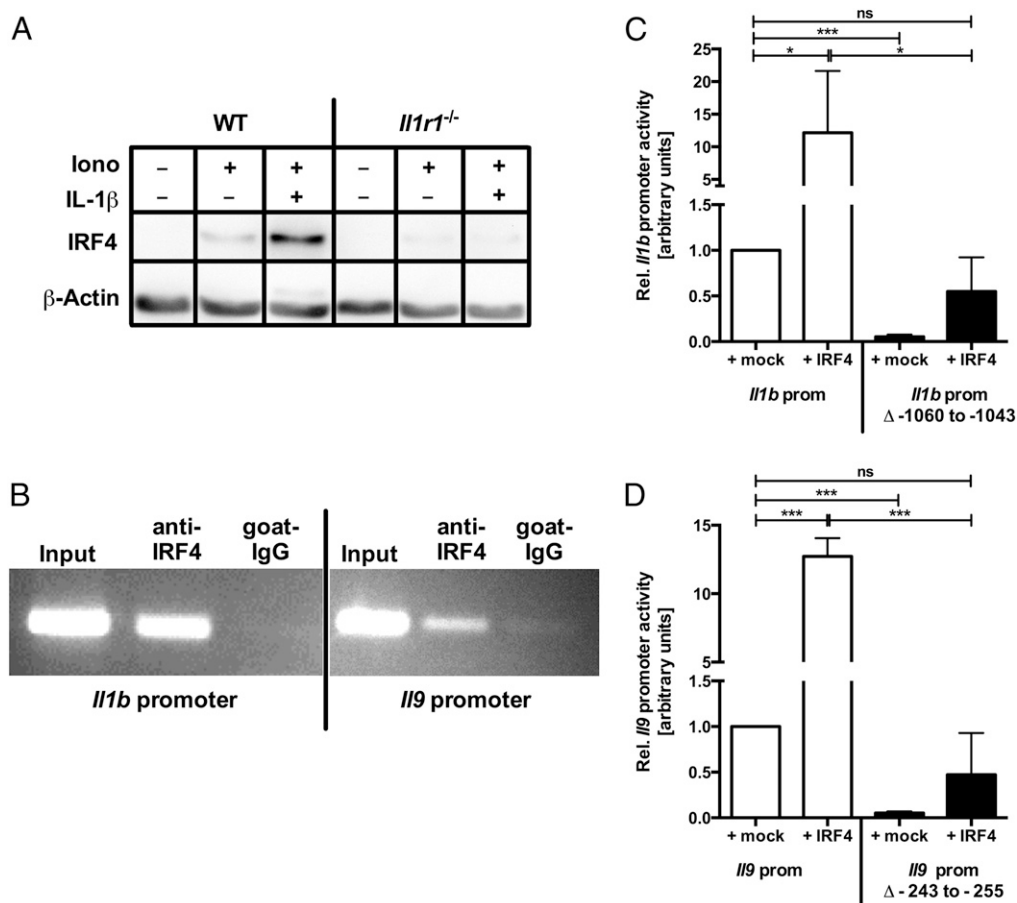


FIGURE 6. IRF4 binds to and transactivates the *Il9* and *Il1b* promoter in mast cells. Mast cells generated from C57BL/6 (WT) or *Il1r1*^{-/-} mice were left untreated or were stimulated for 48 h with ionomycin (Iono, 1 μM) in the presence or absence of IL-1β (300 pg/ml). **(A)** IRF4 expression was assessed by Western blot analyses. **(B)** Binding of IRF4 to the promoters of *Il9* and *Il1b* was assessed by ChIP analyses. For *Il9* and *Il1b* reporter gene analyses, mast cells were transfected with a plasmid coding for IRF4 (+IRF4) or with the corresponding empty vector (+mock), in combination with an *Il1b* **(C)** or *Il9* **(D)** promoter-luciferase reporter or promoter-luciferase reporter with mutated IRF4 binding sites, and stimulated for 16 h with ionomycin (0.5 μM). Cell lysates were prepared as outlined in *Materials and Methods*, and luminescence was measured. Mast cells transfected with promoter-luciferase vector +mock were arbitrarily set to 1. Data represent one representative result of four (A) or three (B) independent experiments. The mean (± SD) of three (C) or five (D) independent experiments is shown. **p* ≤ 0.05, ****p* ≤ 0.001, two-tailed unpaired *t* test. ns, not significant.

Fig. 7A and 7B, sensitization and subsequent challenge (sense/chal) with OVA elicits a strong influx of eosinophils into the lumen of the lungs and a considerable increase in methacholine-induced airway resistance. Addition of IL-9 (sense/chal + IL-9) further increased the number of eosinophils and airway resistance. Notably, both asthma symptoms were substantially reduced by administration of sialoL at the time of challenge (Fig. 7A, 7B; sense/chal + sialoL), and they were restored approximately to the level of the sense/chal approach upon concurrent application of IL-9 (sense/chal + IL-9 + sialoL). To further investigate the in vivo role of IRF4 and IL-1R1 signaling in mast cells, mast cell-deficient *Kit*^{W-sh/W-sh} mice were systemically reconstituted with mast cells derived from WT, *Irf4*^{-/-}, or *Il1r1*^{-/-} mice. Sense/chal with OVA resulted in a robust influx of eosinophils and a strong increase in methacholine-induced airway resistance when *Kit*^{W-sh/W-sh} mice were reconstituted with WT mast cells (Fig. 7C, 7D). These asthma symptoms were barely detectable in the absence of mast cells in *Kit*^{W-sh/W-sh} mice, and they were reduced significantly in *Kit*^{W-sh/W-sh} mice that had been reconstituted with mast cells from *Irf4*^{-/-} or *Il1r1*^{-/-} mice (Fig. 7C, 7D).

In summary, we demonstrate that the inhibition of mast cell-derived IL-9 by the tick salivary protein sialoL is based on an altered IL-1R1 signaling and a partial inhibition of IRF4 expression, leading to a severely impaired self-induction of mast cell-

derived IL-1β. The relevance of these findings is emphasized by the fact that a mast cell-specific deficiency in *Il1r1* and *Irf4* prevents IL-9 production by mast cells in a murine asthma model, thereby diminishing the influx of eosinophils and the accompanying airway resistance, both cardinal features of allergic asthma.

Discussion

Adaptation of parasites to a hematophagous life cycle has led to the development of parasitic immune-evasion strategies that are based, to a great extent, on immunosuppressive agents (28). Such agents enable the survival of parasites by preventing an effective immune response, and they are supposed to represent a fertile source of naturally occurring immune modulators for the development of novel drugs to treat allergic and autoimmune diseases. This discovery of novel drugs from parasites and other pathogens is called the “drugs from bugs” approach (29).

SialoL is one of these immunosuppressive molecules that was characterized from tick saliva several years ago (2). Meanwhile, we demonstrated that, among other immunosuppressive activities, sialoL strongly inhibits the development of Th9 cells, which produce the asthma-promoting cytokine IL-9 (4). Initially, it was reported that IL-9 production depends on IL-2, is synergistically enhanced by a combination of IL-4 and TGF-β, and is inhibited by IFN-γ (30). SialoL was found to inhibit the production of IL-9 and

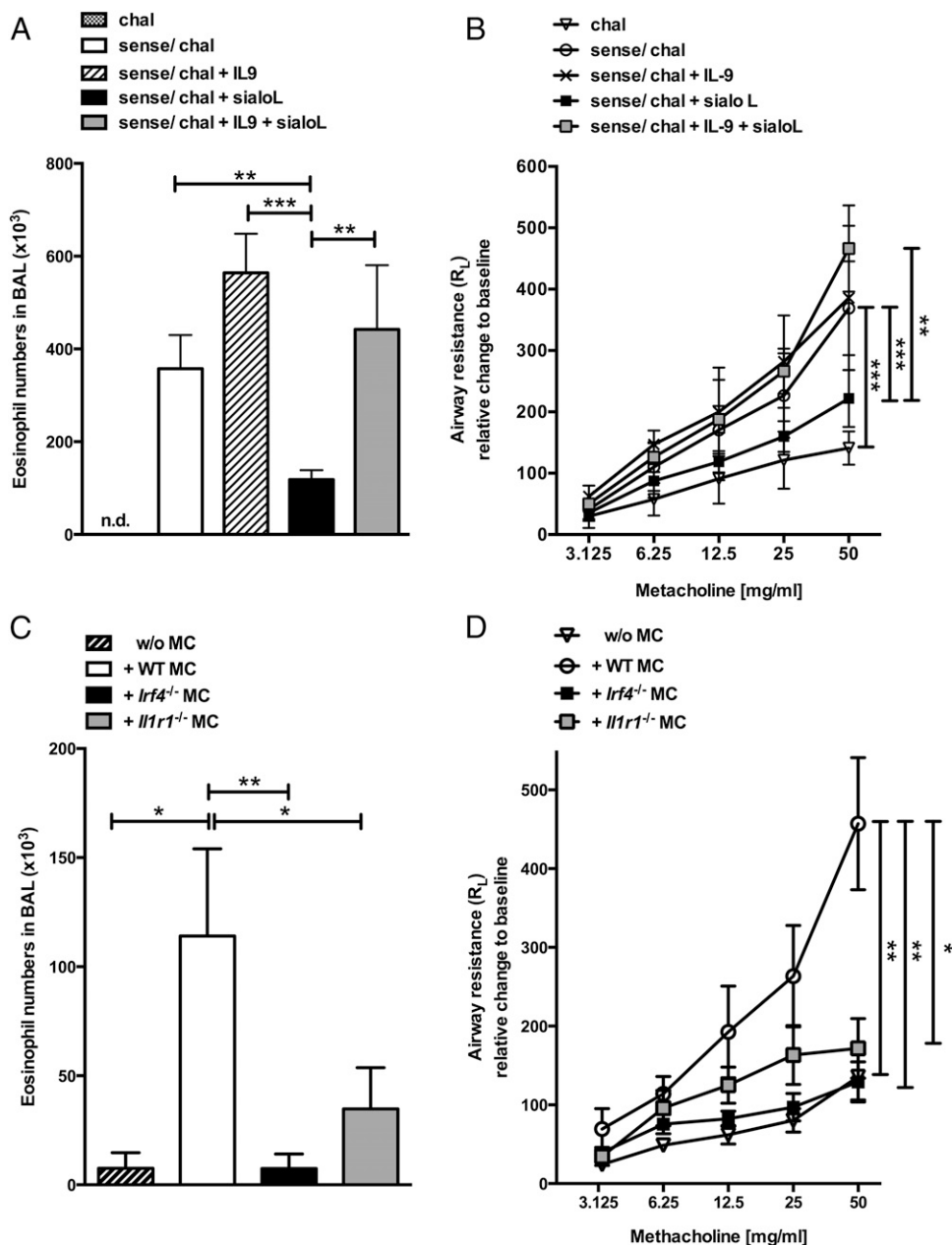


FIGURE 7. Administration of sialoL, as well as mast cell-specific deficiency in *Irf4* or *Il1r1*, significantly ameliorates eosinophilia and AHR. (**A** and **B**) Eosinophilia and AHR were induced by sensitizing and challenging C57BL/6 mice with OVA in the absence (sense/chal) or presence (+sialoL) of sialoL or sialoL in combination with IL-9 (+IL-9 + sialoL). Mice challenged without sensitization (chal) served as a negative control. Mast cell-deficient Kit^{W-sh/W-sh} mice were reconstituted with mast cells (MC) generated from WT, *Il1r1*^{-/-}, or *Irf4*^{-/-} mice. Nonreconstituted Kit^{W-sh/W-sh} mice (without MC) served as control. (**C** and **D**) Eight weeks after reconstitution, mice were immunized as described in *Materials and Methods*. Numbers of eosinophils in bronchoalveolar lavage fluid (**A** and **C**) and airway resistance (**B** and **D**) were determined 24 h after the last challenge on day 31. Data are mean $\pm$ SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, unpaired t test.

IL-2 by CD4⁺ T cells. Therefore, we assumed that the suppressive mechanism of sialoL is based on the reduced production of T cell-derived IL-2. However, the addition of exogenous IL-2 could not reverse the sialoL-mediated inhibition of IL-9 production, suggesting a different suppressive mode of action. Th9-derived IL-9 was shown to be a major pathogenic factor in an experimental model of asthma, and treatment of mice suffering from asthma with sialoL significantly reduced their asthma symptoms. Upon in vivo administration to treat asthma, sialoL can certainly have an impact on several cell types; mast cells represent prominent candidates because they were shown to produce considerable amounts of IL-9, as well as to induce asthmatic symptoms (9, 31). Therefore, it is conceivable that sialoL-mediated inhibition of mast cell-derived

IL-9 contributes greatly to the amelioration of asthmatic symptoms. Consequently, we analyzed the influence of sialoL on mast cell-derived IL-9 in detail.

In general, we found that sialoL strongly reduced the production of IL-9 by mast cells after activation using ionomycin. This inhibitory effect of sialoL could be compensated for, in part, by IL-1 β , which represents a potent costimulator of mast cell-derived IL-9 production (see below) (10). In addition, IRF4 was identified as a crucial driver of ionomycin-induced IL-9 expression by mast cells. These findings were corroborated by the fact that *Il1r1*^{-/-} mast cells showed only marginal IL-9 production, and *Irf4*^{-/-} mast cells exhibited greatly reduced IL-9 production that could be compensated for, in part, by IL-1 β . In contrast, ionomycin-

induced IL-6 was not affected by sialoL, IL-1 β , or a combination of both. Finally, comparable results were obtained when IgE was applied as a classical mast cell activator (data not shown).

Asthma-associated eosinophilia was greatly reduced after treatment with sialoL, suggesting a considerably effective suppression of endogenous IL-9 and IL-1, and both were described to have a profound eotaxin-inducing capacity by preferentially affecting airway smooth muscle and epithelial cells. This assumption was substantiated by the finding that nonresponsive mast cell-deficient mice developed clear-cut asthma-associated eosinophilia and AHR after reconstitution with WT mast cells, whereas reconstitution with *Irf4*^{-/-} or *Il1r1*^{-/-} mast cells led to a strong reduction in the eosinophilic influx. Hence, a mast cell-specific inability to produce maximal amounts of IL-9 either caused by *Irf4* deficiency or IL-1 unresponsiveness is crucial to the development and maintenance of asthma symptoms in the mast cell-dependent preclinical asthma model used in this study.

Comparable to T cells, it was shown that the expression of IL-9 by mast cells could be enhanced by NF- κ B-inducing stimuli like IL-1 (10) or LPS (11). In addition, a combination of IL-10 or Kit ligand (KL) with IL-1 strongly enhanced the production of IL-9 by mast cells (10). Although stimulation of mast cells with a combination of IL-1 and IL-10 or KL increased *Il9* promoter activity, only IL-10 enhanced the half-life of IL-9 mRNA, suggesting posttranscriptional modifications by this cytokine. Nevertheless, IL-10 and KL exerted only a minimal effect in the absence of IL-1, indicating that IL-1-induced transcription factors are obviously master regulators of the *Il9* locus in mast cells. The essential relevance of IL-1 also was emphasized by results showing that an IL-9-induced increase in IL-9 production by mast cells depends on endocrine IL-1 β production (32). These findings prompted us to assess the role of autocrine IL-1 β production when we analyzed the molecular mechanisms underlying the sialoL-mediated inhibition of mast cell-derived IL-9. The simultaneous reduction in IL-9 and IL-1 β secretion in mast cells in the presence of sialoL, as well as the fact that the addition of exogenous IL-1 β substantially reversed the suppressive activity of sialoL, indicated that impairment of autocrine IL-1 β production represents a central inhibitory mechanism of sialoL. This result was confirmed by transcriptome analysis that indicated a strong inhibition of the expression of *Il9* and *Il1b* in sialoL-treated mast cells. In addition, these analyses revealed that sialoL can inhibit expression of the *Irf4* gene, suggesting that, in analogy to Th9 cells, IRF4 positively regulates mast cell-derived IL-9. Using *Irf4*^{-/-} mast cells, we demonstrated that this transcription factor is of crucial importance for the production of IL-9 in Th9 cells, as well as mast cells [this study and (22)]. However, this does not apply to PU.1, which is a transcription factor known to substantially contribute to *Il9* gene expression in Th9 cells (33). In contrast, *Il9* promoter reporter analyses revealed that ectopic expression of PU.1 led to a silencing of the *Il9* locus in mast cells (data not shown), whereas IRF4 strongly enhanced the activity of this reporter gene construct. Another important positive regulator of *Il9* expression in mast cells is GATA-1, which was shown to be activated by p38 MAPK-mediated phosphorylation (12). In this context, PU.1 was found to inhibit GATA-1 function by physical interaction, leading to PU.1/GATA-1 heterodimers that prevent the binding of GATA-1 to DNA (34). Thus, PU.1 inhibits the expression of *Il9* in mast cells, whereas T cells are not negatively affected because they lack GATA-1 expression (35). Taken together, regulation of the *Il9* promoter in T cells and mast cells appears to share similarities, but it also differs in several aspects. Nevertheless, sialoL inhibits IL-9 production of mast cells and Th9 cells, indicating that it acts on

molecules essentially involved in both cell types in the production of this cytokine (i.e., IRF4).

In light of these findings, our observation that IRF4 is involved in the IL-1-dependent production of IL-9 by mast cells might be of pathophysiological importance, because it was demonstrated that IL-9 is a central mediator in allergic asthma and is involved in an effective antimelanoma immune response (36). Therapeutic approaches for the treatment of asthma on the basis of IL-9 neutralization in preclinical mouse models showed promising results (37, 38). However, neutralization of human IL-9 using the Ab MEDI-528 could not alleviate asthma symptoms in adults with uncontrolled asthma, suggesting that simply blocking IL-9 in chronic asthma is not sufficient to cure such a complex disease (39). Nevertheless, the curative influence of sialoL in our preclinical asthma model is most likely based on the manipulation of various functions in distinct cell types. In addition to inhibition of IL-9 production by Th9 cells and mast cells, sialoL impaired the maturation and Ag-specific activation of dendritic cells, as well as the expansion of CD4⁺ T cells. Therefore, further comparative studies will be required to elucidate the influence of sialoL on the transcriptional mechanisms that are responsible for the differential regulation of the *Il9* locus in mast cells and Th9 cells, as well as on dendritic cell functions and the proliferation of CD4⁺ T cells in general. The resulting knowledge will presumably serve as a basis to develop innovative strategies for the treatment of asthma by exploiting the suppressive mechanisms of sialoL.

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Disclosures

The authors have no financial conflicts of interest.

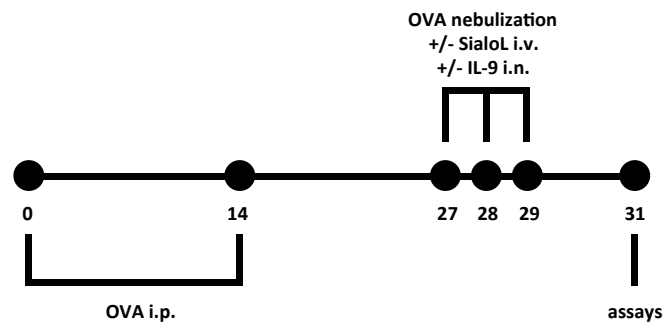
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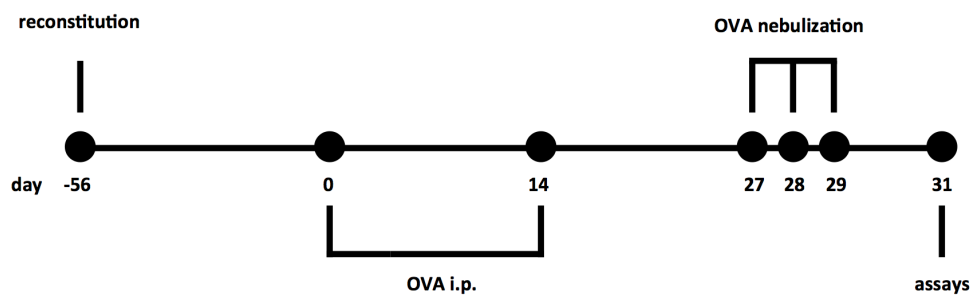
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Supp. Fig. 1

A



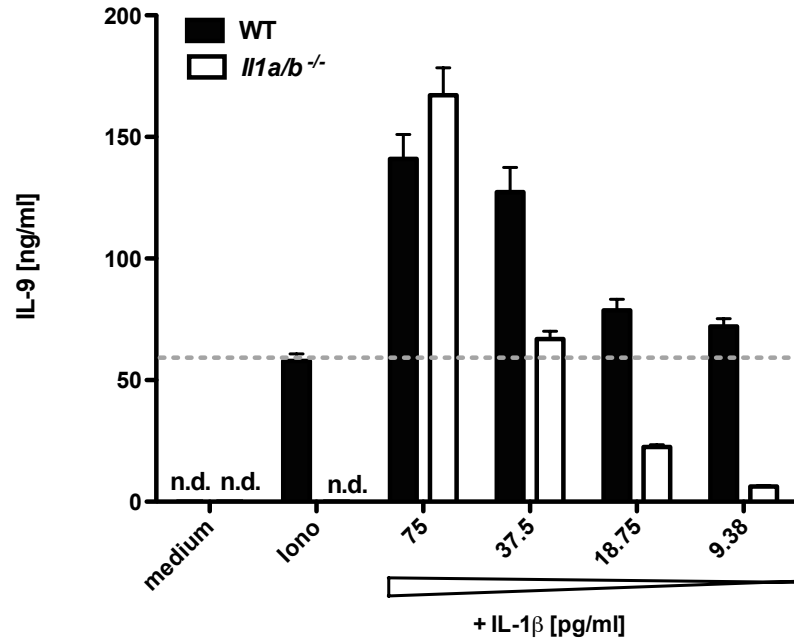
B



Supp. Figure 1: Schematic overview of the asthma models.

C57BL/6 mice were sensitized by two injections (i.p.) of OVA (20mg) on day 0 and day 14 and subsequently challenged with nebulized OVA in the absence or presence of sialoL (10µg, i.v.) or IL-9 (500ng, i.n.) (**A**). *Kit*^{W-sh/W-sh} mice on a C57BL/6 background were reconstituted with 5x10⁶ mast cells derived from *Irf4*^{-/-}, *Il1r1*^{-/-} or WT mice and sensitized by two injections (i.p.) of OVA (20mg) and subsequently challenged with nebulized OVA (**B**).

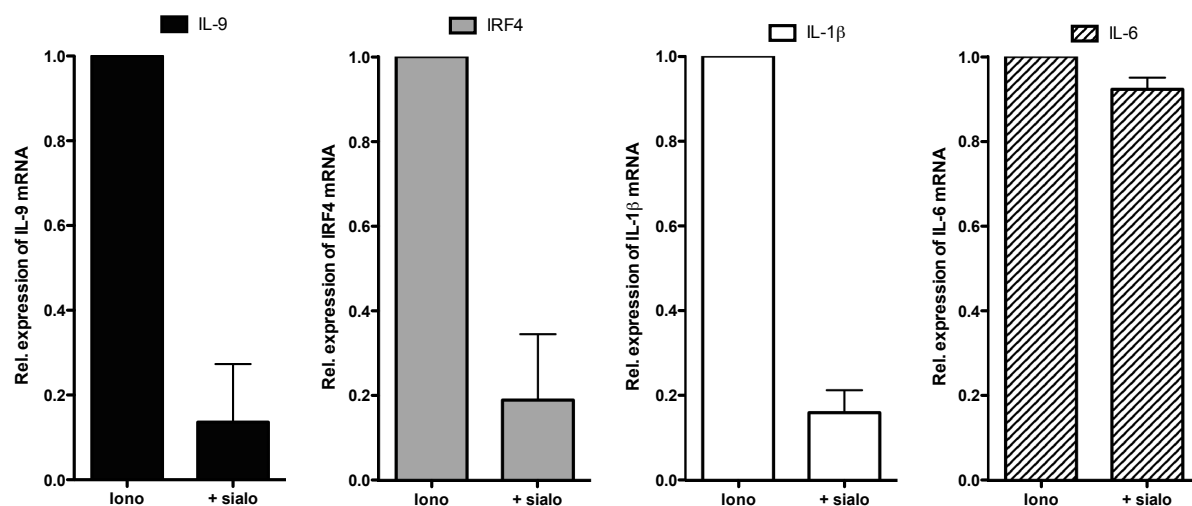
Supp. Fig. 2



Supp. Figure 2: Impaired production of IL-9 by mast cells from *Il1a* and *Il1b* double-deficient mice can be restored by exogenous IL-1 β .

Mast cells generated from C57BL/6 (WT) or *Il1a/b* double-deficient mice were stimulated for 48h with ionomycin (Iono, 1 μ M) in the presence or absence of different concentrations of IL-1 β (75, 37.5, 18.75, 9.38 pg/ml). IL-9 production was measured by ELISA after 48h of stimulation. Shown is the mean ($\pm$ SD) of three independent experiments.

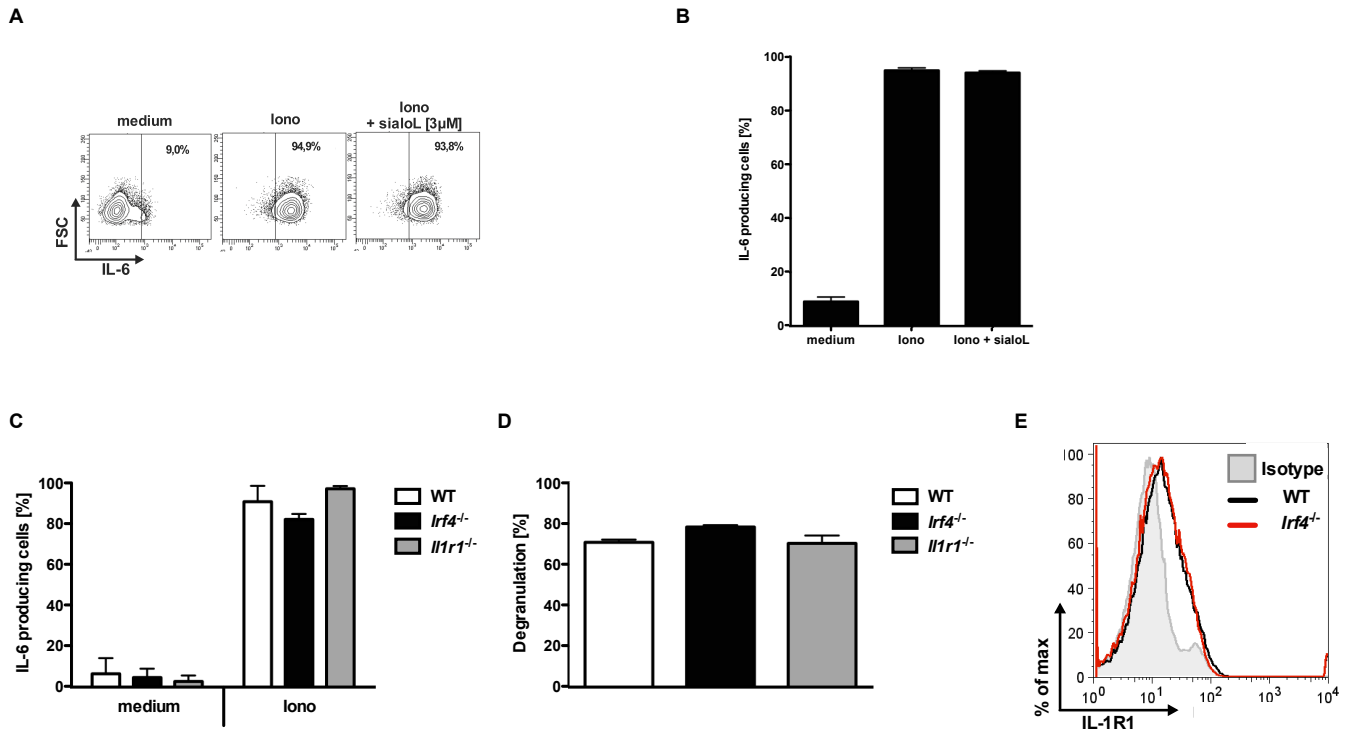
Supp. Fig. 3



Supp. Fig. 3: Validation of mRNA-Seq results by qRT-PCR.

Mast cells were stimulated with ionomycin (1 μ M) in the presence or absence of sialoL (3 μ M) for 24h. After isolation of total RNA and reverse transcription, qRT-PCR was performed as outlined in the materials and methods section to determine IL-9, IRF4, IL-1 and IL-6 mRNA expression levels. IL-9, IRF4, IL-1 β and IL-6 mRNA levels of ionomycin stimulated mast cells was set to 1. Shown is the mean ($\pm$ SD) of three independent experiments.

Supp. Fig. 4



Supp. Figure 4: IL-6 production and degranulation are not impaired by sialoL, *Irf4*- or *Il1r1*-deficiency.

Mast cells generated from C57BL/6-WT, *Il1r1*^{-/-} or *Irf4*^{-/-} mice were cultivated for 6h in medium (IMDM + 10% FCS) in the absence or presence of ionomycin (Iono, 1μM) and sialo L (3μM; as indicated) and the production of IL-6 was assessed by intracellular FACS analysis (**A-C**). Degranulation (beta-hexosaminidase release) of mast cells was assessed as outlined in the materials and methods section (**D**). (**E**) Mast cells generated from C57BL/6 (WT) or *Irf4*^{-/-} mice were stimulated with ionomycin (1μM) for 24h and IL-1R1 expression was determined by flow cytometry. In **B**, **C** and **D** the mean of three independent experiments (±SD) is shown. Panel **A** and **E** both show one representative of three independent experiments.