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ORIGINAL ARTICLE

Nuclear receptor Nr1d1 alleviates asthma by abating GATA3 gene expression and Th2 cell differentiation

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Abstract

Nuclear receptors are a unique family of transcription factors that play cardinal roles in physiology and plethora of human diseases. The adopted orphan nuclear receptor Nr1d1 is a constitutive transcriptional repressor known to modulate several biological processes. In this study, we found that Nr1d1 plays a decisive role in T helper (Th)-cell polarization and transcriptionally impedes the formation of Th2 cells by directly binding to the promoter region of GATA binding protein 3 (GATA3) gene. Nr1d1 interacts with its cellular companion, the nuclear receptor corepressor and histone deacetylase 3 to form a stable repression complex on the GATA3 promoter. The presence of Nr1d1 also imparts protection against associated inflammatory responses in murine model of asthma and its ligand SR9011 eased disease severity by suppressing Th2 responses. Moreover, Chip-seq profiling uncovered Nr1d1 interactions with other gene subsets that impedes Th2-linked pathways and regulates metabolism, immunity and brain functions, therefore, providing empirical evidence regarding the genetic link between asthma and other comorbid conditions. Thus, Nr1d1 emerges as a molecular switch that could be targeted to subdue asthma.

Keywords Asthma · Nuclear receptor · T lymphocytes · GATA3 · T-bet

Introduction

Asthma is an obstructive pulmonary disease characterized by chronic inflammation of bronchioles and airway mucosa provoking sneezes, breathlessness, coughing, wheezing, and airway remodeling. Asthma is a common illness among all age groups with approximately 339 million affected people worldwide, and occurs as a mixed outcome of genetic susceptibility and environmental influence [1, 2]. There is marked heterogeneity and variability in the pathological features of asthma patients as well as in the responses towards asthma therapy, which have given rise to different forms of asthma, called phenotypes. These phenotypes range from trigger-induced phenotypes, such as allergens, infections or aspirin-exacerbated asthma to phenotypes distinguished by clinical symptoms or biomarkers, such as eosinophilic or non-eosinophilic asthma [3]. However, in the general population of asthma patients, the most commonly observed phenotype is allergic asthma [4]. Allergic asthma presents as a state of eosinophil and lymphocyte infiltration, goblet cell hyperplasia, and degranulation of mast cells. There is also a predominant T helper type 2 (Th2)-linked cellular response with subsequent higher levels of pro-inflammatory cytokines, chemokines, and other mediators [5, 6]. More often than not, asthma exists with numerous other comorbidities, such as viral infections, metabolic diseases and psychopathologies, which mutually influence the clinical expression, disease management and control of asthma [7]. Regardless of the healthcare advancements, asthma is still under-diagnosed and under-treated, creating a huge healthcare burden with clear social implications [8, 9].

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Current asthma therapy recommends corticosteroids as a first line drug along with several other adjunct therapies to provide relief in severe cases [10, 11]. Further, with extensive research, some newer targets have emerged leading to the employment of various cytokine inhibitors, such as anti-Interleukin-4 (IL-4), anti-Interleukin-5 (IL-5) or anti-Immunoglobulin E (IgE) for anti-asthma therapy [12]. However, these therapies fail to address the underlying root cause of the disease and there is still a persistent lack of cleaner medicinal targets that could function as direct molecular switches.

The ability of transcription factors to switch between two modulatory processes upon sensing certain biological signals plays a cardinal role in the development and maintenance of body homeostasis. In higher eukaryotes, the most abundant group of transcription factors is the superfamily of nuclear receptors (NRs). NRs command an extremely vast range of biological processes, including both physiological (development, metabolism, immune responses, reproduction, etc.) and pathophysiological processes (cancer, neurological and cardiovascular diseases) [13–17]. NRs share remarkable structural similarities and carry a historically preserved ligand-binding domain (LBD) which upon ligand binding, carries out the activation or inactivation of their target genes. In current years, NRs have become a significant part of the 'druggable genome' due to their ability to regulate genes through metabolic and nutritional cues [18]. Furthermore, with uncloaking of their significance, NRs are also being extensively researched in the pathology of asthma and its progression [19–21]. Out of all the NRs, the most extensively studied is the glucocorticoid receptor (GR; nuclear receptor subfamily 3, group C, member 1). Glucocorticoids (GCs) are known to have a suppressive effect on interleukin-5 (IL-5), interleukin-13 (IL-13), interleukin-33 (IL-33), and monocyte chemoattractant protein 1 (MCP1) expression, impeding the infiltration of eosinophils, macrophages, neutrophils, and T cells into the lungs [22]. While GCs are the cornerstone of anti-allergic and immunosuppressive therapy, a significant fraction of asthmatics respond poorly to inhaled and systemic steroid treatment [23, 24]. Poorly controlled asthma cases have revealed that use of systemic corticosteroids is conducive to diabetes, obesity, osteoporosis, depression, and enhanced susceptibility to viral infections [25, 26]. Additionally, treatment of GC-resistant patients with GCs produces major health risks, ranging from irreversible tissue damage to the development of Cushing's syndrome, leading to increased morbidity and mortality rates [27]. Further, the GR dictates the sensitive hormonal arm, which upon interference, leads to abnormal steroid pharmacokinetics that substantially impacts the immune system and body homeostasis [28]. Therefore, another class of NRs, the adopted orphan receptors, would make more lucrative drug targets than the endocrine targets as there is minimal associated immune-endocrine interference. As a consequence, pharmacological efforts directed towards the family of adopted orphan receptors would assist in attaining better methods for disease regulation, possibly curing asthma.

Adopted orphan NRs are activated by lower affinity physiological ligands, such as fatty acids, oxysterols, eicosanoids, and other biochemical molecules [29]. Nuclear receptor subfamily 1 group D member 1 (Nr1d1; Rev-erbα) is a member of the adopted orphan category of NRs and has been identified as a heme sensor [30]. Nr1d1 can bind either as a monomer or homodimer to its response element and is a constitutive repressor of transcription [31, 32]. In recent years, various scientific studies have found that Nr1d1 plays a protective role in infectious diseases, Th17 cell-mediated autoimmune diseases, oxidative stress and pulmonary inflammation [33–41]. However, the role of Nr1d1 in asthma has not been mechanistically addressed. There is a lack of fundamental clarity as to how this NR is influencing the predisposition and etiology of asthma along with an absence of knowledge about the underlying molecular pathway. Moreover, other studies from various groups have also stated that Nr1d1 has a protective role in mental ailments [42–44] and other immune diseases [35], which are frequent comorbid conditions found in asthmatics.

Through this study, we sought to explore how the NR Nr1d1 influences the polarization of T helper type 1 (Th1) and T helper type 2 (Th2) cells. We report that Nr1d1 plays a crucial role in Th-cell polarization and inhibits the differentiation of Th2 cells. GATA Binding Protein 3 (GATA3), a master transcription factor for Th2 cells, is directly regulated and suppressed by Nr1d1 at the genomic level. Post-asthma generation, severe inflammation was observed in $Nr1d1^{-/-}$ mice as compared to the $Nr1d1^{+/+}$ mice. The gain of function through overexpression and ligand treatment (SR9011) also conferred protection against asthma in $Nr1d1^{-/-}$/$Nr1d1^{+/+}$ mice. Furthermore, chromatin immunoprecipitation-sequencing (ChIP-seq) data uncovered Nr1d1 interactions with other subsets of genes that impedes Th2-linked pathways and regulates metabolism, immunity, and brain functions. Thus, our current study provides further evidence about how genetically associated pathways may contribute to the phenotypic heterogeneity of asthma. In conclusion, Nr1d1 is a promising target for both asthma and its linked comorbid conditions.

Materials and methods

Animals and ethics statement

C57BL/6 mice ($Nr1d1^{+/+}$ and $Nr1d1^{-/-}$) were obtained from the Jackson Laboratory. Animals were inbred and maintained in a healthy and pathogen-free state in the institute's

animal house facility. Mice experiments were approved by the Animal Ethics Committee of the institute and were carried out as stated by the National Regulatory Guidelines stated by the Committee for the Purpose of Supervision of Experiments on Animals (No. 55/1999/CPCSEA), Ministry of Environment and Forest, Government of India.

Cells, media and reagents

Splenocytes obtained from $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice, Jurkat or EL4 cells were cultured in Roswell Park Memorial Institute Medium-1640 (RPMI-1640) supplemented with fetal bovine serum (FBS) and 1% penicillin/streptomycin that were purchased from Gibco and Invitrogen Life Technologies, respectively. The IMag™ Mouse CD4 T Lymphocyte Enrichment Set and biotin anti-mouse CD44 were purchased from BD Biosciences. Antibodies used for naive T lymphocyte isolation and programing were purchased from BD Biosciences and cytokines were from eBioscience. Nr1d1 ligands GSK4112 and SR9011 were purchased from Calbiochem and Sigma-Aldrich, respectively.

Naive T cell isolation and culture

Naive T lymphocyte ($CD4^+$ $CD62L^+$ $CD44^-$) isolation was performed from the secondary lymphoid organs of 4–6 week-old mice by employing the IMag™ Mouse CD4 T Lymphocyte Enrichment Set, according to the manufacturer's instructions, along with a biotin-tagged anti-mouse CD44 antibody. In vitro differentiation was carried out as described previously [45, 46]. Briefly, in a 24 well plate, cells were seeded at a density of 3×10^5 cells per well. Stimulation was done with plate-bound anti-CD3 (5 µg/ml) and soluble anti-CD28 (2 µg/ml) antibodies. To differentiate the cells into specific subtypes, naive cells were given different combinations of cytokines and antibodies for 3–4 subsequent days. For differentiation to Th0 cells, cells were provided with 5 µg/ml anti-IL4 antibodies, 5 µg/ml anti-IFNγ antibodies, and 100 U IL-2. For differentiation into Th1 cells, 25 ng/ml of IFNγ and IL-12 was added in addition to 5 µg/ml of anti-IL4. For Th2 cell differentiation, 5 µg/ml of anti-IFNγ and 25 ng/ml of IL-4 were provided.

Flow cytometric analysis

For in vitro-differentiated Th1/Th2 cells, the harvested cells were washed with FACS buffer (PBS + 1% FBS) twice and proceeded for staining. Cells were suspended in FACS buffer along with fluorescent-conjugated antibody: anti-CD4 (FITC-conjugated) for 30 min in dark (4 °C). Post-washing, cells were fixed in paraformaldehyde (4%) for 15 min in dark (4 °C). The cells were washed twice with FACS buffer and pelleted cells were re-suspended in saponin for permeabilization (45 min in dark). The cells were pelleted and re-suspended in saponin solution (0.5% in PBS) containing anti-IL4 (Alexa Fluor 647-conjugated)/anti-IFNγ (PE-conjugated) antibodies for 1 h in dark. Post-staining, cells were washed with FACS buffer twice and re-suspended in 300 µl of FACS buffer for acquisition. For lung lymphocytes, mice were killed and lungs were harvested. The minced tissue was then collected and incubated in RPMI-1640 containing collagenase (Stemcell technologies #07912) and DNase I (Stemcell technologies #07900) with gentle shaking at 37 °C for 2 h. The digested lungs were filtered using a 70 µm strainer and RBC were lysed with RBC lysis buffer. T cells were isolated by employing the IMag™ Mouse CD4 T Lymphocyte Enrichment Set [47]. Similar staining procedure was performed as described above using anti-CD4 (PE-Cy7-conjugated) and anti-IL4 (Alexa Fluor 647-conjugated)/anti-IFNγ (PE-conjugated) antibodies. The cells were acquired in FACS Accuri and analyzed using FlowJo software.

ILC2 isolation

The ILC2 cells were isolated employing the EasySep mouse ILC2 enrichment kit (Stemcell technologies #19842) according to the manufacturer's instructions. For ELISA, cells were cultured with IL-7 and IL-33; supernatant was collected after 48 h.

Real-time quantitative PCR (qPCR)

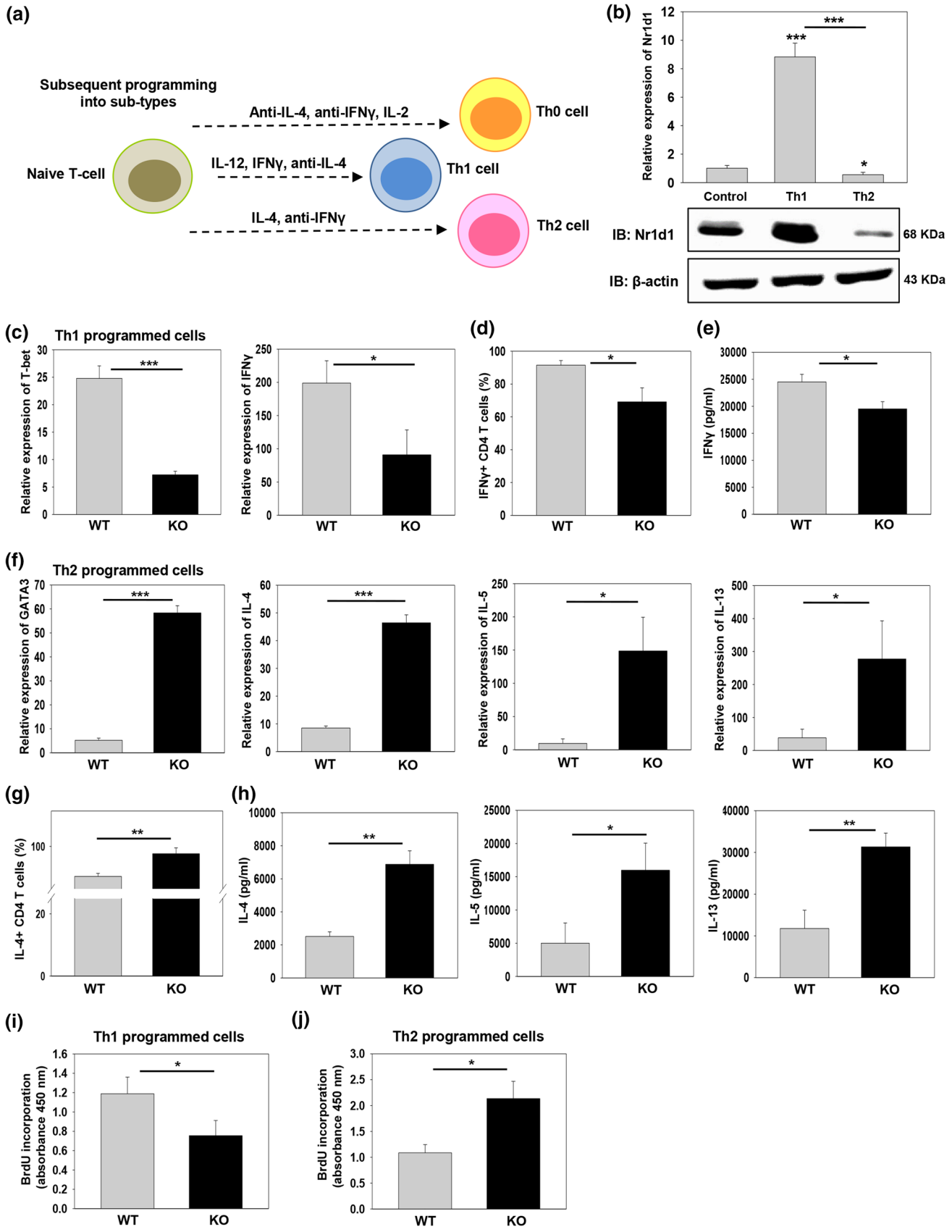
RNA from in vitro differentiated T lymphocytes and lung samples were isolated using the Trizol method and 1 µg of total RNA was synthesized into cDNA using the Verso cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. With the help of gene-specific primers, cDNA was amplified using the DyNAmo ColorFlash SYBR Green R Kit (Thermo Scientific). 18S mRNA was kept as a control and the relative fold change was determined using the formula $2^{-\Delta\Delta Ct}$.

Enzyme linked immunosorbent assay (ELISA)

Cytokine levels in cell-culture supernatants, BALF, and serum were evaluated by a commercially available ELISA kit specific for mouse IFNγ, IL-4, IL-5, IgE (BioLegend) and IL-13 (Invitrogen) in accordance with the manufacturer's instructions.

BrdU assay

In a 96-well plate, naive T cells were seeded at a density of 4×10^4 cells per well and cultured accordingly for subsequent programming to Th1/Th2 cells. The assay was


(a)
Subsequent programming into sub-types
Anti-IL-4, anti-IFNγ, IL-2
Th0 cell
Naive T-cell
IL-12, IFNγ, anti-IL-4
Th1 cell
IL-4, anti-IFNγ
Th2 cell
(b)
Relative expression of Nr1d1
Control
Th1
Th2
IB: Nr1d1
68 KDa
IB: β-actin
43 KDa
(c)
Th1 programmed cells
Relative expression of T-bet
Relative expression of IFNγ
(d)
IFNγ+ CD4 T cells (%)
(e)
IFNγ (pg/ml)
(f)
Th2 programmed cells
Relative expression of GATA3
Relative expression of IL-4
Relative expression of IL-5
Relative expression of IL-13
(g)
IL-4+ CD4 T cells (%)
(h)
IL-4 (pg/ml)
IL-5 (pg/ml)
IL-13 (pg/ml)
(i)
Th1 programmed cells
BrdU incorporation (absorbance 450 nm)
(j)
Th2 programmed cells
BrdU incorporation (absorbance 450 nm)
WT
KO

◂**Fig. 1** Nr1d1 fosters the differentiation of Th1 cells while impeding Th2 cells. **a** Pictorial representation of the naive T-cell programming protocol. **b** Nr1d1 expression (immunoblot/western blot) in differentiated Th1:Th2 cells. Asterisks represent significant differences as indicated. **c** Relative expression levels by qRT-PCR of T-bet and IFNγ in differentiated Th1 cells. **d** Intracellular expression of IFNγ by flow cytometry showing cell percentages and **e** ELISA depicting secreted levels of IFNγ, in differentiated Th1 cells from $Nr1d1^{+/+}$ versus $Nr1d1^{-/-}$ mice backgrounds. **f** Relative expression levels by qRT-PCR of GATA3, IL4, IL5, IL13, **g** flow cytometry profile showing cell percentages for IL-4 expression and **h** ELISA depicting secreted levels of IL-4, IL-5, and IL-13, in differentiated Th2 cells from $Nr1d1^{+/+}$ versus $Nr1d1^{-/-}$ mice backgrounds. BrdU incorporation was measured in differentiated **i** Th1 cells and **j** Th2 cells from $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice backgrounds using BrdU cell proliferation assay. Asterisks (comparison between Th1/Th2 programmed cells of $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$) represent significant differences (* indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$). Th0 cells were taken as control; $2^{-\Delta\Delta Ct}$ was applied. Data shown are representative (**b**; immunoblot/western blot) or average (**b**–**j**) from three independent experiments (mean ± s.d.)

performed using a commercially available BrdU cell proliferation assay kit (#6813, Cell Signaling Technology) in accordance with the manufacturer's protocol. BrdU solution was added for the last 24 h of the cell culture and incorporation was measured at 450 nm.

Chromatin immunoprecipitation (ChIP)

The ChIP and Re-ChIP experiments were carried out as described previously [48]. To isolate chromatin, fixation of cells was performed using 1% (wt/vol) formaldehyde for 30 min. To quench the cross-linking, 100 mM glycine solution was added and cells were subsequently washed with ice-cold phosphate-buffered saline (PBS) solution. To obtain DNA fragments of desired length, cells were sonicated in SDS-lysis buffer. The remaining debris were cleared by centrifugation at 4 °C and the supernatant was proceeded for immunoprecipitation with antibodies to Nr1d1 (sc-47626 X, Santa Cruz Biotechnology), NCoR (sc-1609 X, Santa Cruz Biotechnology), and HDAC3 (sc-11417, Santa Cruz Biotechnology). High-speed centrifugation collected the immune complexes as a pellet, which were washed in order with low-salt buffer, high salt-buffer, LiCl wash buffer, and then twice with 1X TE buffer. The immunoprecipitated DNA was eluted using elution buffer and further amplified with specific primer sets.

ChIP-sequencing

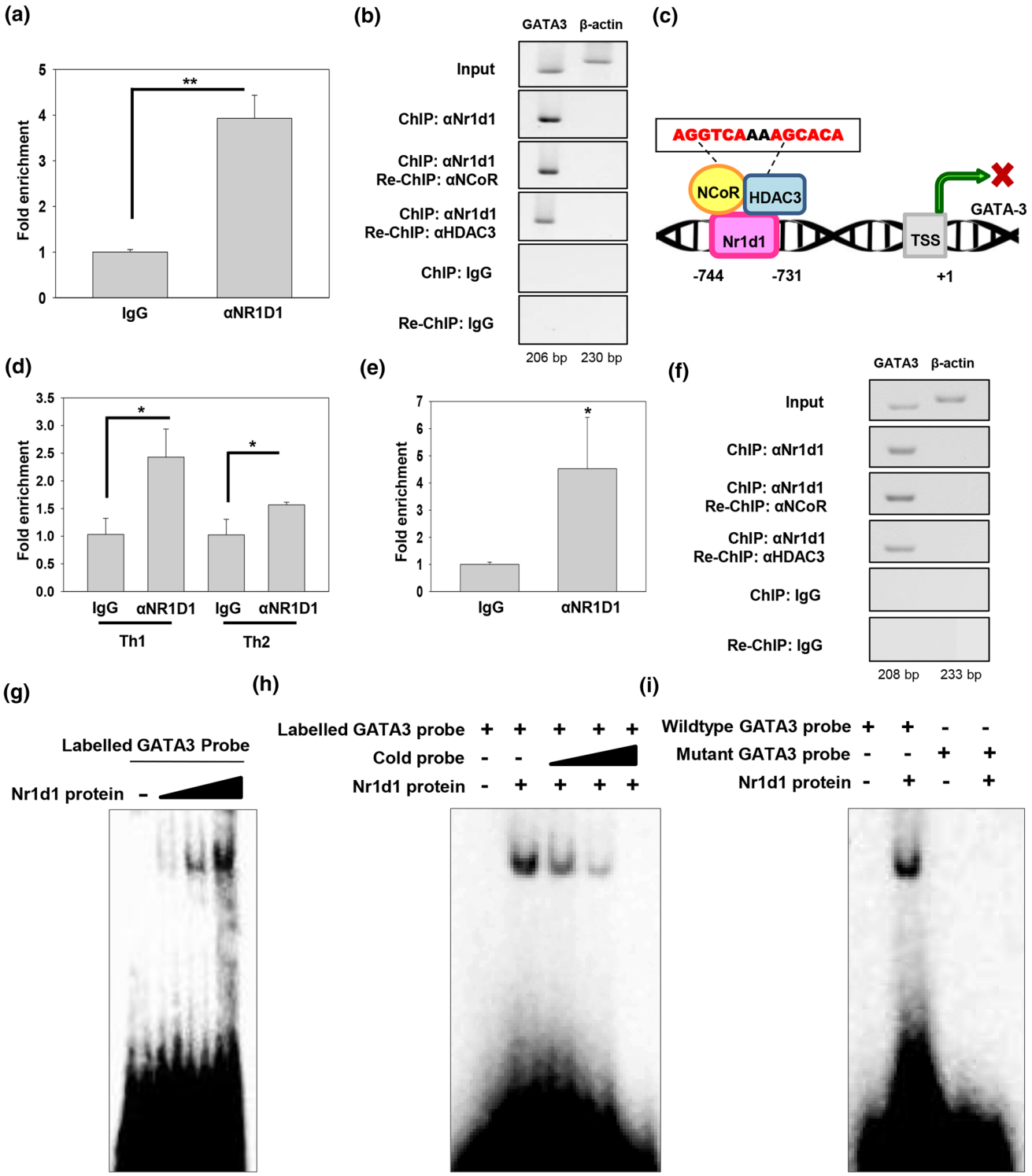
After the naive T-cell programming, Th1 cells were harvested and proceeded for the ChIP experiment. The immunoprecipitated DNA samples were quantified using Qubit DNA BR Assay (Invitrogen, Q32853). DNA fragmentation distribution was checked on HS D1000 Tapes. All samples passed the QC and were proceeded for the DNA library prep. The ChIP samples were further taken for library preparation using NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB, E7645L). First, the fragmented DNA samples with 1 ng of DNA was end repaired and mono-adenylated at 3' end in a single enzymatic reaction. Next, NEB hairpin-loop adapters are ligated to the DNA fragments in a T4-DNA ligase-based reaction. Following ligation, the loop containing Uracil is linearized using USER Enzyme (a combination of UDG and Endo VIII), to make it available as a substrate for PCR-based indexing in the next step. 13 cycles of PCR was performed to incorporate index/barcode sequences using unique primers for each of the samples, thereby enabling multiplexing. Prepared libraries were quantified using Qubit High Sensitivity Assay (Invitrogen, Q32852). The obtained libraries were pooled and diluted to final optimal loading concentration before cluster amplification on Illumina flow cell. Once the cluster generation is completed, the cluster flow cell is loaded on Illumina HiSeq X instrument to generate 60 M, 150 bp paired end reads. The paired-end reads were aligned to the reference Mus Musculus (mm10) release downloaded from Sanger institute database. Alignment was performed using BWA MEM (Ver-0.7.12). The datasets for this experiment have been deposited in GEO (Gene Expression Omnibus) repository under the accession number GSE181065.

Detection of genomic binding site and *de novo* motif prediction

The alignment files after filtration from previous step were considered for peak detection. The Seqpeak function of CisGenome (Ver-v2.0) was used for peak calling analysis. The peaks were considered to be significant if their p-value was less than or equal to 0.05 and peak length greater than 200. The significant peaks were annotated using Homer tool (Ver-v4.10) and annotations were filtered for Promoter-TSS region. GO enrichment and pathway analysis for the final filtered peaks (Promoter-TSS region) were performed using David and Panther database. Further, filtered peaks sequences were subjected to motif prediction using MEME software (Ver-4.12.0). The sequence of 30 bp around each peak call was extracted from the mouse reference genome and this was then provided as an input for the meme-chip module of MEME. The motifs were filtered for AGGTCA motif sequences allowing up to a maximum of 2 mismatches in the motif sequence.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described previously [49, 50]. The annealed oligonucleotides were end-labelled using



T4-polynucleotide kinase and γ^{32}-P-ATP. Protein Nr1d1 was prepared in vitro using the 1-Step Human Coupled IVT Kit (Thermo Scientific) in accordance with the manufacturer's protocol. For competition experiments, molar excess of unlabeled oligonucleotides were added to compete for the DNA binding after the labeled probe. The mutant GATA3 probe in which the binding site was mutated has a sequence: 5' -CACTAATGTTAAAATCAAACATTG- 3'. The DNA–protein binding assay was carried out and samples were run on a native polyacrylamide gel, and bands were visualized using a phosphorimager (Bio-Rad Molecular Imager FX).

◂**Fig. 2** GATA3 is a direct target gene of Nr1d1. ChIP analysis of Nr1d1 binding at the mouse GATA3 promoter region with chromatin isolated from EL4 cells. A ChIP-grade Nr1d1 antibody or isotype control antibody (IgG) was used for immunoprecipitating the crosslinked lysates. **a** Eluted DNA was proceeded for PCR with input and IgG samples to analyze the fold enrichment. **b** Identification of the Nr1d1 repressive complex on the GATA3 promoter region. Re-ChIP assay was performed and cells were analyzed for coimmunoprecipitation of NCoR-HDAC3. **c** Pictorial representation of the GATA3 promoter region showing the conserved motif for Nr1d1. ChIP analysis of Nr1d1 binding at the mouse GATA3 promoter region with chromatin isolated from (**d**) programmed Th1 and Th2 cells from $Nr1d1^{+/+}$ mice background and (**e**) human Jurkat cells. **f** Re-ChIP analysis for coimmunoprecipitation of NCoR-HDAC3 in human Jurkat cells. **g** EMSA analysis using radiolabeled oligonucleotide with the Nr1d1 binding sequence on the GATA3 promoter region in the presence of increasing concentrations of in vitro-translated Nr1d1 protein. **h** A competition experiment performed with 5-, 10-, and 50-fold excess of cold probe carrying the sequence for the consensus GATA3-Nr1d1 response element. **i** EMSA was done using a radiolabeled oligonucleotide containing the sequence for the wildtype GATA3 promoter region and a mutant probe with mutated core motif. Asterisks represent significant differences as indicated (* indicates $P<0.05$; ** indicates $P<0.005$). Data shown are representative (**b**, **f**, **g–i**) or average (**a**, **d**, **e**) from three independent experiments (mean ± s.d.)

Phylogenetic analysis

Phylogenetic tree for GATA3 promoters carrying the conserved motif for Nr1d1 was constructed by MEGA5 (Molecular Evolutionary Genetic Analysis) software. The evolutionary history was inferred using the Neighbor-Joining method with 1000 bootstrap replicates. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 7 nucleotide sequences. Multiple pairwise sequence alignment of GATA3 promoters containing the Nr1d1 binding motif among various mammalian species was carried out using ClustalW2.

Murine asthma model

Asthma induction in mice (6–8 weeks old) was performed as previously described [51] with minor modifications. Mice were sensitized intraperitoneally (i.p.) with OVA (100 µg) emulsified in alum (4 mg alum) in PBS (100 µl). On day 12, a booster dose (OVA + alum through i.p.) was given and for the last 6 days (day 18 to day 23), mice were aerosol challenged with 2% OVA for a duration of 20 min each day. On the final day (day 24), the aerosol challenge with OVA was given for 2 h. Post-48 h of the final challenge, mice were killed. In a similar manner, control mice were sensitized and challenged with PBS + alum and PBS, respectively. For the ligand experiments, mice were divided into two groups (control and SR9011), and the SR9011 treatment was started from day 13. Mice were administered SR9011 at a dose of 100 mg/kg dissolved in 15% cremophor once a day intraperitoneally till the final challenge day (day 24). After the final challenge, mice were killed and samples were collected for lung histopathology, BALF cellularity and ELISA measurements.

Adenovirus production and aerosol delivery to mice

For overexpression studies, recombinant adenovirus-expressing LacZ (Ad-control) and Nr1d1 (Ad-Nr1d1) constructs were made as described previously [34, 52] and in accordance with the user manual provided with Adeno-X Expression System 1 (Clontech, #631513). The experiments were performed on $Nr1d1^{-/-}$ mice which were divided into groups. These groups received aerosol treatment containing Ad-control, Ad-Nr1d1 or Ad-Nr1d1 along with ligand SR9011. The inhalations were given twice a week till the time of killing.

BALF collection and cell counts

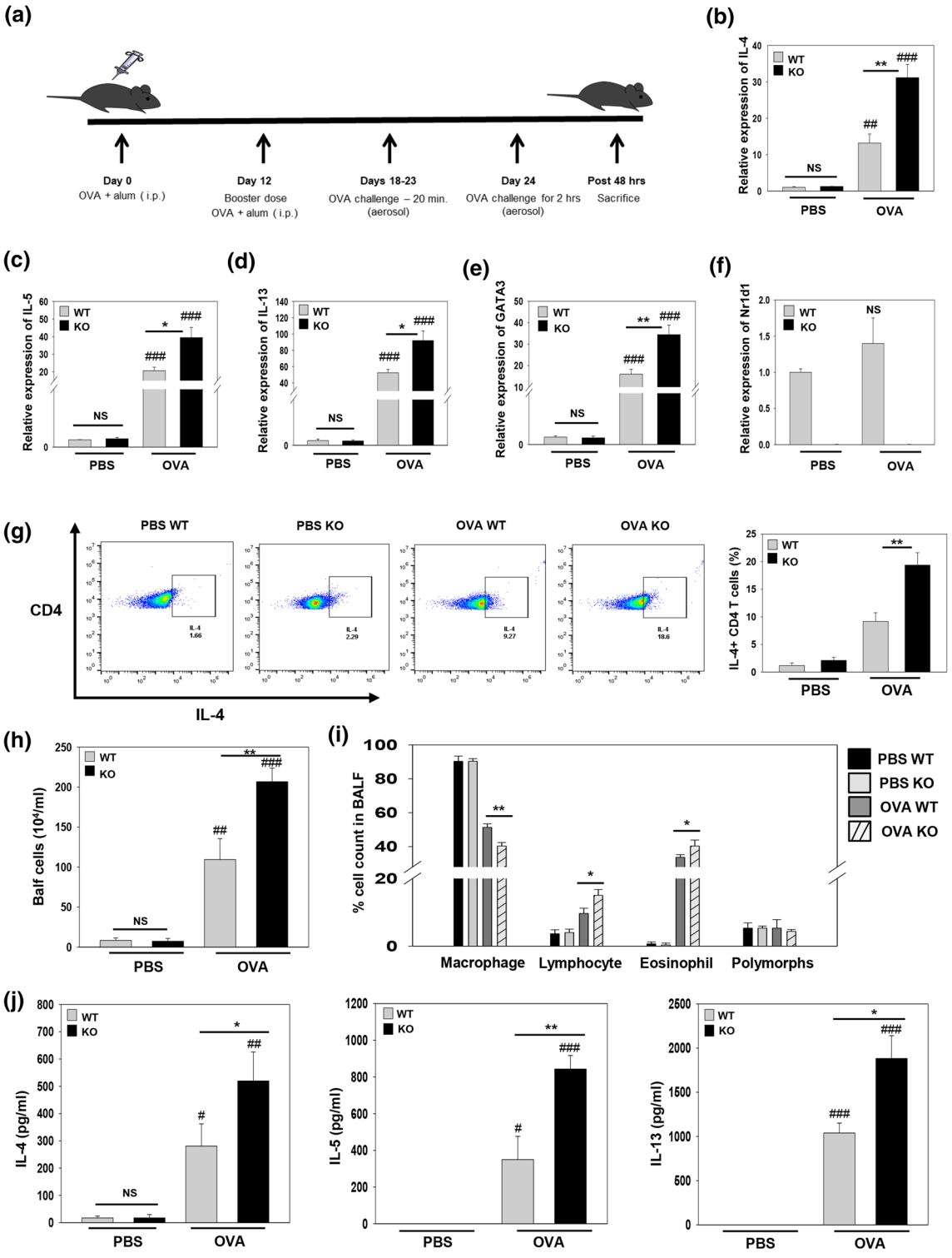
Lungs were flushed twice with cold 1 ml PBS. Post-lavage, the collected BALF was centrifuged and the supernatant was utilized for cytokine analysis. The cell pellets obtained were resuspended and cell counting was done using a hemocytometer. Enumeration of differential cell counts was outsourced to a pathological laboratory and was obtained by conventional Diff-quick staining.

Assessment of OVA-specific cytokines

Post-killing, lungs were harvested from $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice (control and asthmatic) and were minced gently. The minced tissue was then collected and incubated in RPMI-1640 containing collagenase (Stemcell technologies #07912) and DNase I (Stemcell technologies #07900) with gentle shaking at 37 °C for 2 h. The digested lungs were filtered using a 70 µm strainer and RBC were lysed with RBC lysis buffer. The T cells were isolated employing the IMag™ Mouse CD4 T Lymphocyte Enrichment Set according to the manufacturer's instructions. Cells were seeded in a 24-well plate with 100 µg/ml OVA and splenic culture; supernatant was collected for ELISA after three days.

Histopathology

Fixation of lung samples was performed in buffered formalin (10%). Microtome sections of paraffin-embedded lungs were stained with either hematoxylin and eosin (H&E) or periodic acid–schiff (PAS) stain. The images were taken at a magnification of 100X for H&E and 100X and 400X for PAS stained slides. The identity of samples was hidden when given for histopathological analysis.



Statistical analysis

The statistical analysis was performed with SigmaPlot software. The normalization has been performed with the average control value and results are expressed as mean and standard deviation (s.d.), unless otherwise mentioned. *P* values were obtained using two-tailed *t* tests. Statistical significance was established at *, $^{\#}P < 0.05$, **, $^{\#\#}P < 0.005$ and ***, $^{\#\#\#}P < 0.0005$.

◂**Fig. 3** Nr1d1 provides protection against asthma in mice. **a** Pictorial representation of the procedure employed for asthma generation in mice using OVA. Relative expression levels of (**b**) IL-4, (**c**) IL-5, (**d**) IL-13, (**e**) GATA3, and (**f**) Nr1d1, in lung samples of control and diseased $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice. **g** Flow cytometric plots depicting increased percentage of Th2 cells in lungs of asthmatic $Nr1d1^{-/-}$ mice in comparison to the $Nr1d1^{+/+}$ mice. The cell population, as observed through flow cytometry, for intracellular expression of IL-4. Bar diagrams on the right, are representative of flow cytometry data. **h** The total cell counts and (**i**) differential cell counts in BALF isolated from control and diseased $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice. **j** Bar diagrams depicting the levels, by ELISA, of IL-4, IL-5, and IL-13 in the BALF. Asterisks (comparison between asthmatic samples of $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$) and hashtags (comparison between asthmatic sample of $Nr1d1^{+/+}$ or $Nr1d1^{-/-}$ and corresponding non-asthmatic control) represent significant differences (*, # indicates $P<0.05$; **, ## indicates $P<0.005$; ***, ### indicates $P<0.0005$). The data have been normalized to values obtained from control WT mice (**a**–**e**) using the formula $2^{-\Delta\Delta Ct}$. Data shown are average (**b**–**j**) or representative (**g**) from three independent experiments (mean ± s.d.). A total of ten animals per set were used

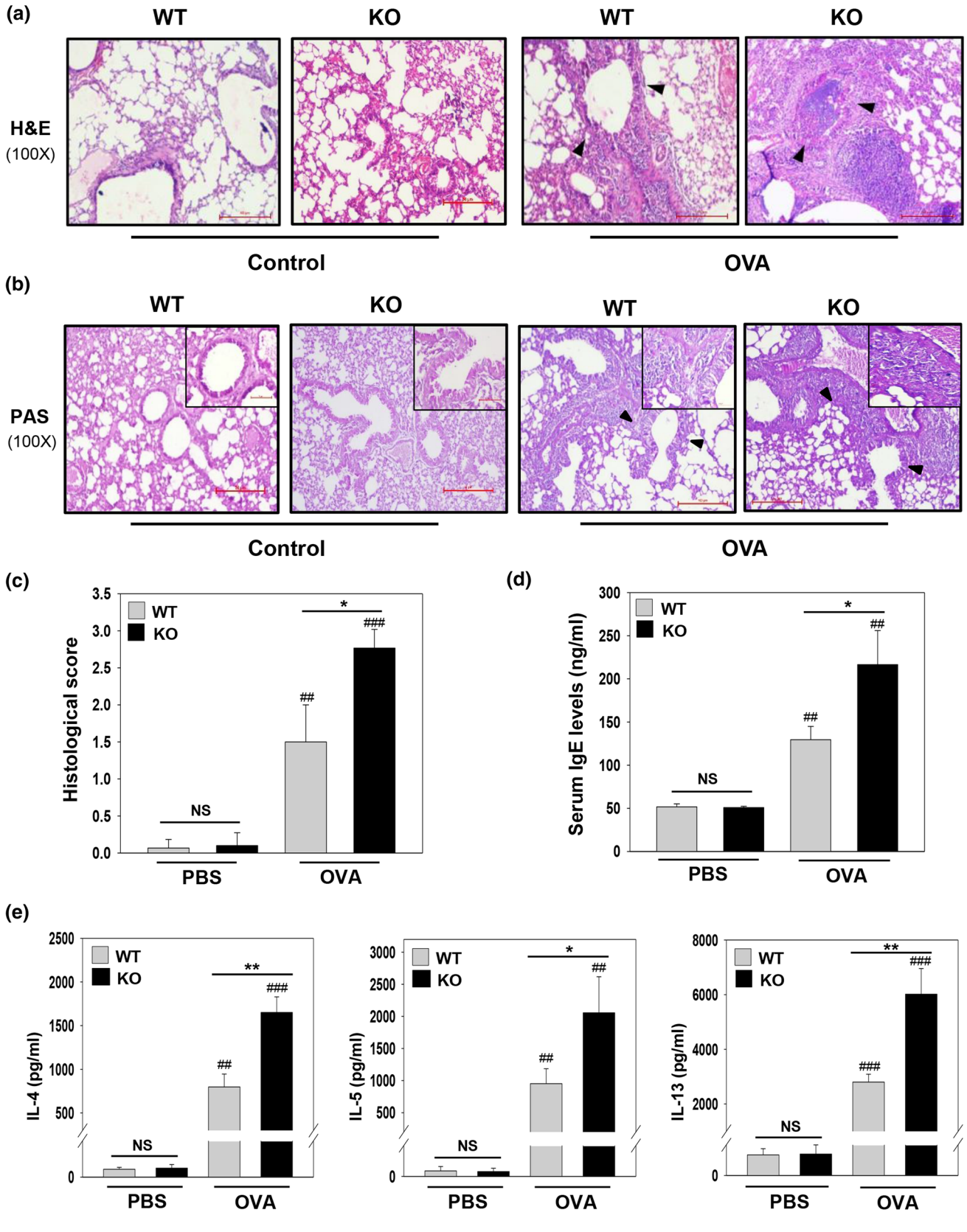
Results

Nr1d1 suppresses differentiation of Th2 cells

Th2 cells play a pivotal role in functioning of the immune system and are also an important factor in asthma pathogenesis [53]. Nr1d1, a transcriptional repressor with immunomodulatory properties, seems a promising target for management of inflammatory disorders [54–57]. Therefore, to elucidate the role of Nr1d1 in asthma, we profiled the transcript levels of Nr1d1 in Th1 and Th2 cells. We isolated naive T cells from the spleen of $Nr1d1^{+/+}$ C57BL/6 mice, and subsequently differentiated them into Th1 and Th2 subtypes (Fig. 1a). The resultant Nr1d1 expression was found to be markedly higher in Th1 than in Th2 cells (Fig. 1b). Similar results were observed upon looking for Nr1d1 expression pattern in a time-dependent manner (Fig. S1). This distinct finding compelled us to further investigate the role of Nr1d1 in Th1 and Th2 differentiation. Thus, we isolated and performed differentiation of naive T cells obtained from $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice. Interestingly, Th1 differentiation was slightly diminished in the $Nr1d1^{-/-}$ mice compared to the $Nr1d1^{+/+}$ mice as observed from lower levels of interferon gamma (IFNγ) and T-bet expression (Figs. 1c and S2a). Similar decrease in intracellular IFNγ expression was also observed through flow cytometry and enzyme linked immunosorbent assay (ELISA) (Figs. 1d, e and S2c). On the contrary, a remarkable increase was seen in Th2 polarization of naive T lymphocytes from $Nr1d1^{-/-}$ mice, displaying higher IL-4 and GATA3 expression levels, compared to $Nr1d1^{+/+}$ mice (Fig. 1f). The expression of other Th2 effector cytokines, IL-5 and IL-13, were observed and as anticipated, the mRNA levels were increased by several folds in $Nr1d1^{-/-}$ compared to $Nr1d1^{+/+}$ mice (Figs. 1f and S2b). Additionally, an increase in intracellular IL-4 expression in Th2 cells of $Nr1d1^{-/-}$ mice background was also observed through flow cytometry (Figs. 1g and S2d). The cytokine levels for IL4, IL5, and IL13 were estimated by ELISA and a notable increase was seen in the $Nr1d1^{-/-}$ mice compared to the $Nr1d1^{+/+}$ mice (Fig. 1h). Furthermore, BrdU cell proliferation assay was performed to investigate the proliferation levels of Th1/Th2 cells. We found that the proliferation rate of Th1 cells was marginally decreased in $Nr1d1^{-/-}$ mice as compared to the $Nr1d1^{+/+}$ mice background (Fig. 1i); however, the Th2 proliferation level was higher in $Nr1d1^{-/-}$ than $Nr1d1^{+/+}$ mice (Fig. 1j). The above results suggest that Nr1d1 influences both the proliferation level and differentiation of Th2 cells as a two-fold increase was observed in its proliferation rate in comparison to the twelve-fold increase in GATA3 mRNA levels. Taken together, these findings demonstrate that Nr1d1 impedes Th2 differentiation and proliferation and associated secretion of effector cytokines, and marginally modulates Th1 differentiation.

GATA3 is a direct target gene of Nr1d1

Since Nr1d1 is a transcriptional repressor which exerts its effect by binding onto the promoter region of a target gene [31], a significant decrease in the levels of Th2 differentiation suggests of a possible gene repression by Nr1d1. To determine whether it modulates the expression of Th2-associated genes at the genomic level, the promoter sequences of various genes were extracted from the Eukaryotic Promoter Database and examined for the Nr1d1 binding site using the NUBIScan database. Interestingly, we found a putative binding site in the proximal region of the GATA3 promoter (Fig. S3a). To validate the binding of Nr1d1 on GATA3 promoter region, we carried out chromatin immunoprecipitation (ChIP) assay. The ChIP experiment confirmed binding of Nr1d1 on this putative site, and polymerase chain reaction (PCR) amplification showed that Nr1d1 interacted with the binding site (Figs. 2a, b and S3b). Along with test primers, control primers were also checked in the pulled-down DNA and no amplification was observed. IgG served as a negative control and showed no amplification, thereby further validating the specificity of binding and amplification (Fig. 2b). It is known that the repressive function of Nr1d1 results from the recruitment of the nuclear receptor corepressor and the histone deacetylase 3 (NCoR-HDAC3) complex [58]; therefore, we looked for this associated transcriptional complex and found that Nr1d1 interacts with NCoR-HDAC3 to carry out its effector function of GATA3 suppression (Fig. 2b and c). We also performed the ChIP experiment in splenocytes isolated from $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice and found Nr1d1 enrichment on GATA3 promoter only in


(a)
WT
KO
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◂**Fig. 4** Histology of lung sections and quantification of cytokines and IgE. Histopathological reflection of the mice lungs from $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice showing representative images of (**a**) H&E staining and (**b**) PAS staining taken at 100X magnification (scale bar: 50 µm). The arrowheads represent areas of inflammation and cellular infiltration. The top-right inset in PAS stained section shows the zoomed image (400X) for the same area. **c** Semiquantitative analysis of the severity of lung inflammation. Peri-bronchial inflammation was scored on a scale of 0–3; grade 0 was designated as no detectable inflammation, grade 1 as mild, grade 2 as moderate, and grade 3 as severe. **d** Estimation of IgE serum levels in control and diseased $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice using ELISA. **e** IL-4, IL-5 and IL-13 levels in culture supernatants of CD4 + T cells isolated from lungs and presented with OVA. Asterisks (comparison between asthmatic samples of $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$) and hashtags (comparison between asthmatic sample of $Nr1d1^{+/+}$ or $Nr1d1^{-/-}$ and corresponding non-asthmatic control) represent significant differences (*, # indicate $P<0.05$; **, ## indicate $P<0.005$; ***, ### indicate $P<0.0005$). Data shown are representative (**a**, **b**) or average (**c**, **d**, **e**) from three independent experiments (mean ± s.d.). A total of ten animals per set were used

$Nr1d1^{+/+}$ splenocytes (Fig. S3c). The binding was further checked in programmed Th1 and Th2 cells from $Nr1d1^{+/+}$ background and we observed enrichment in both cell types (Fig. 2d). Moreover, Nr1d1 enrichment was also observed in human Jurkat cells through ChIP and Re-ChIP experiments, thereby confirming Nr1d1 binding on human GATA3 promoter region along with NCoR-HDAC3 transcriptional complex (Fig. 2e, f). We also examined the effect of Nr1d1 ligand on GATA3 expression and observed a concentration dependent decrease in both EL4 and Jurkat cells (S3d). Additionally, an electrophoretic mobility shift assay (EMSA) was carried out with a double-stranded radiolabeled oligonucleotide containing the Nr1d1 binding motif in the GATA3 promoter region (Fig. 2g). Competition with an unlabeled cold consensus probe was also carried out and the binding was reduced as the cold probe was able to compete out the labeled probe; therefore, substantiating the specificity of binding (Fig. 2h). Furthermore, to determine the functional relevance of the nucleotides, a mutant probe (with mutations in the core motif) was used that obliterated the binding (Fig. 2i). Since GATA3 is critically required for both the differentiation and effector functions of Th2 cells [59, 60], the above findings made us ardent to look for the evolutionary significance of this biology. Interestingly, a conserved motif for Nr1d1 was present in other species as well. We also studied the evolutionary relationship between the GATA3 promoter sequences of seven species that contain a putative Nr1d1 motif by phylogenetic reconstruction (Fig. S4a). Multiple pairwise alignments of the GATA3 gene promoters containing the Nr1d1 conserved motif (response element) from different mammalian species displayed homology in humans, higher primates, and mice. However, in Norway rat, a single base deletion has been found in the Nr1d1 response element, probably indicating that Nr1d1 will fail to bind (Fig. S4b). Interestingly, Norway rats are known to be genetically Th2-predisposed [61], and this mutation in the Nr1d1 response element could be a possible reason for that predisposition. Furthermore, the GATA3 genomic regulation by Nr1d1, in part explains the species specificity observed in NR functionality [52].

Nr1d1 provides protection against asthma in mice

To support the pathological significance of Nr1d1 in asthma, we employed an OVA-based asthma model in mice (Fig. 3a). Post-animal killing, RNA from the lungs (control and asthmatic mice) were isolated and the expression of asthma markers were investigated. A remarkable increase was observed in IL-4, IL-5, IL-13, and GATA3 expression in $Nr1d1^{-/-}$ mice compared to $Nr1d1^{+/+}$ mice (Fig. 3b–e). There was no appreciable change observed in Nr1d1 levels in the asthmatic lung tissue of $Nr1d1^{+/+}$ mice (Fig. 3f); however, a decrease in Nr1d1 expression was observed in T cells from asthmatic $Nr1d1^{+/+}$ mice in comparison to the control $Nr1d1^{+/+}$ mice (Fig. S5a). We also looked for the population of Th2 cells and found higher percentage in asthmatic $Nr1d1^{-/-}$ mice than in the $Nr1d1^{+/+}$ mice (Fig. 3g). Furthermore, we also observed a slight decrease in the IFNγ expression in the cells of $Nr1d1^{-/-}$ mice in comparison to the $Nr1d1^{+/+}$ mice (Fig. S5b). Comparisons of the total and differential bronchoalveolar lavage fluid (BALF) cell counts were done and in comparison to control mice, asthmatic mice displayed an impressive increase in the BALF cellularity levels with a significantly greater amount of BALF cells in the $Nr1d1^{-/-}$ mice than the $Nr1d1^{+/+}$ mice (Fig. 3h, i). Moreover, the macrophage ratio was found to be reduced in the $Nr1d1^{-/-}$ mice than the $Nr1d1^{+/+}$ mice. This could be a consequence of severe asthma present in $Nr1d1^{-/-}$ mice [62]. Along with cell counts, cytokine levels were also examined for IL-4, IL-5, and IL-13 in the BALF. As anticipated, the levels of IL-4, IL-5, and IL-13 were substantially higher in $Nr1d1^{-/-}$ than in $Nr1d1^{+/+}$ mice (Fig. 3j). Conclusively, these findings suggest that asthmatic manifestations were significantly higher in $Nr1d1^{-/-}$ mice compared to $Nr1d1^{+/+}$ mice.

Nr1d1 attenuates airway inflammation and serum IgE levels

The overall pathological reflection of Nr1d1 was further confirmed by conducting hematoxylin and eosin (H&E) staining of asthmatic and control lungs. The control group had no signs of epithelial damage other than slight congestion, and the lung parenchyma was clear and devoid of inflammatory cells. However, OVA treatment, which generated asthma in both $Nr1d1^{+/+}$ and $Nr1d1^{-/-}$ mice,

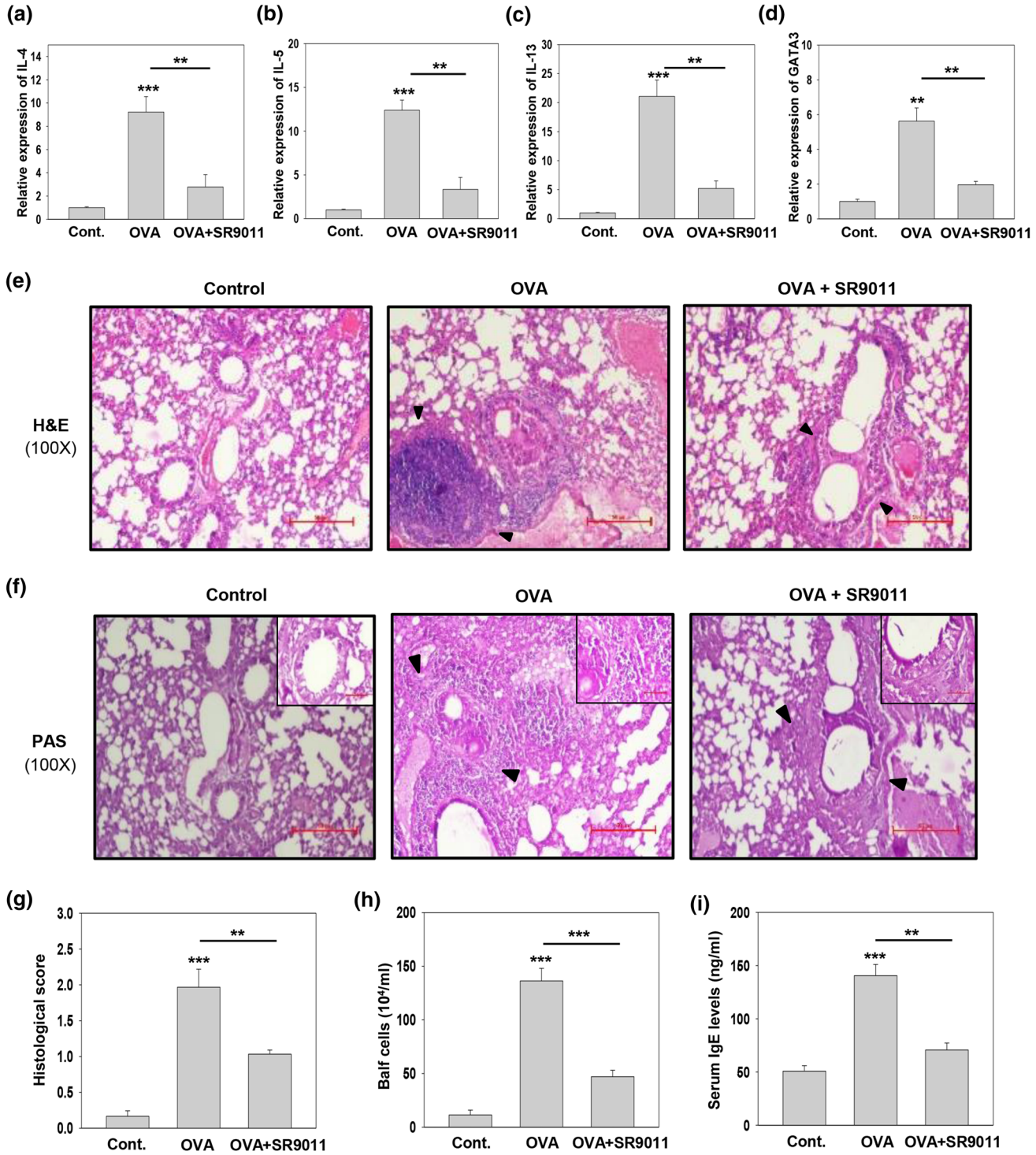


Fig. 5 Nr1d1 ligand SR9011 confers immunity against asthma in Nr1d1$^{+/+}$ mice background. Asthma-induced mice were treated with the ligand SR9011 (100 mg/kg body weight). The mice in control groups were administered with PBS. Post-animal killing, the subsequent Th2 responses were monitored. Relative expression levels of (**a**) IL-4, (**b**) IL-5, (**c**) IL-13, and (**d**) GATA3, as obtained by qRT-PCR in lung samples. Representative images of lung sections stained with (**e**) H&E stain and (**f**) PAS stain taken at 100X magnification (scale bar: 50 µm). The arrowheads denote areas of inflammation and cellular infiltration. The top-right inset in PAS stained section shows the zoomed image (400X) for the same area. **g** Semiquantitative analysis of the severity of lung inflammation. Peri-bronchial inflammation was scored on a scale of 0–3; grade 0 was designated as no detectable inflammation, grade 1 as mild, grade 2 as moderate, and grade 3 as severe. **h** The total cell counts in isolated BALF. **i** Estimation of IgE serum levels by ELISA. Asterisks represent significant differences as indicated (* indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$). Data shown are the average (**a**–**d**, **g**–**i**) or representative (**e**, **f**) from three independent experiments (mean ± s.d.). An overall minimum of ten animals per set were used

led to observed epithelial distortion, peri-bronchial cuffing, and lumen narrowing that are hallmarks of asthma. Further, the microscopic images showed that asthma was more severe in $Nr1d1^{-/-}$ mice compared to $Nr1d1^{+/+}$ mice as grave inflammatory conditions were present at the tissue level in $Nr1d1^{-/-}$ mice. The H&E staining displayed higher infiltration of immune cells in $Nr1d1^{-/-}$ mice when compared with the $Nr1d1^{+/+}$ mice (Fig. 4a). Moreover, lung sections stained by periodic acid-schiff (PAS) support the above data as the stained images exhibited excessive mucus secretion and goblet cell hyperplasia in $Nr1d1^{-/-}$ mice compared to the $Nr1d1^{+/+}$ mice (Fig. 4b). This was accompanied by an overall greater histological score in $Nr1d1^{-/-}$ mice than in $Nr1d1^{+/+}$ mice (Fig. 4c). Furthermore, IgE levels were quantified in the blood serum and $Nr1d1^{-/-}$ mice had an increased proportion of IgE compared to the $Nr1d1^{+/+}$ mice (Fig. 4d). The total population of CD4 + T cells was also isolated from the lungs of both control and asthmatic mice and cultured with OVA. The culture supernatant was harvested and cytokine levels were examined. As anticipated, the levels of IL-4, IL-5, and IL-13 were substantially higher in the culture supernatant of T cells isolated from asthmatic $Nr1d1^{-/-}$ mice in comparison to the $Nr1d1^{+/+}$ mice (Fig. 4e). Furthermore, no significant difference was observed in the group 2 innate lymphoid cells (ILC2) population of $Nr1d1^{+/+}$ mice or $Nr1d1^{-/-}$ mice (Fig. S6a), which confirmed that OVA mounts a predominant Th2 immune response. Thus, in summary, these findings support the protective role of Nr1d1 against asthma.

The Nr1d1 ligand SR9011 alleviates the severity of asthma

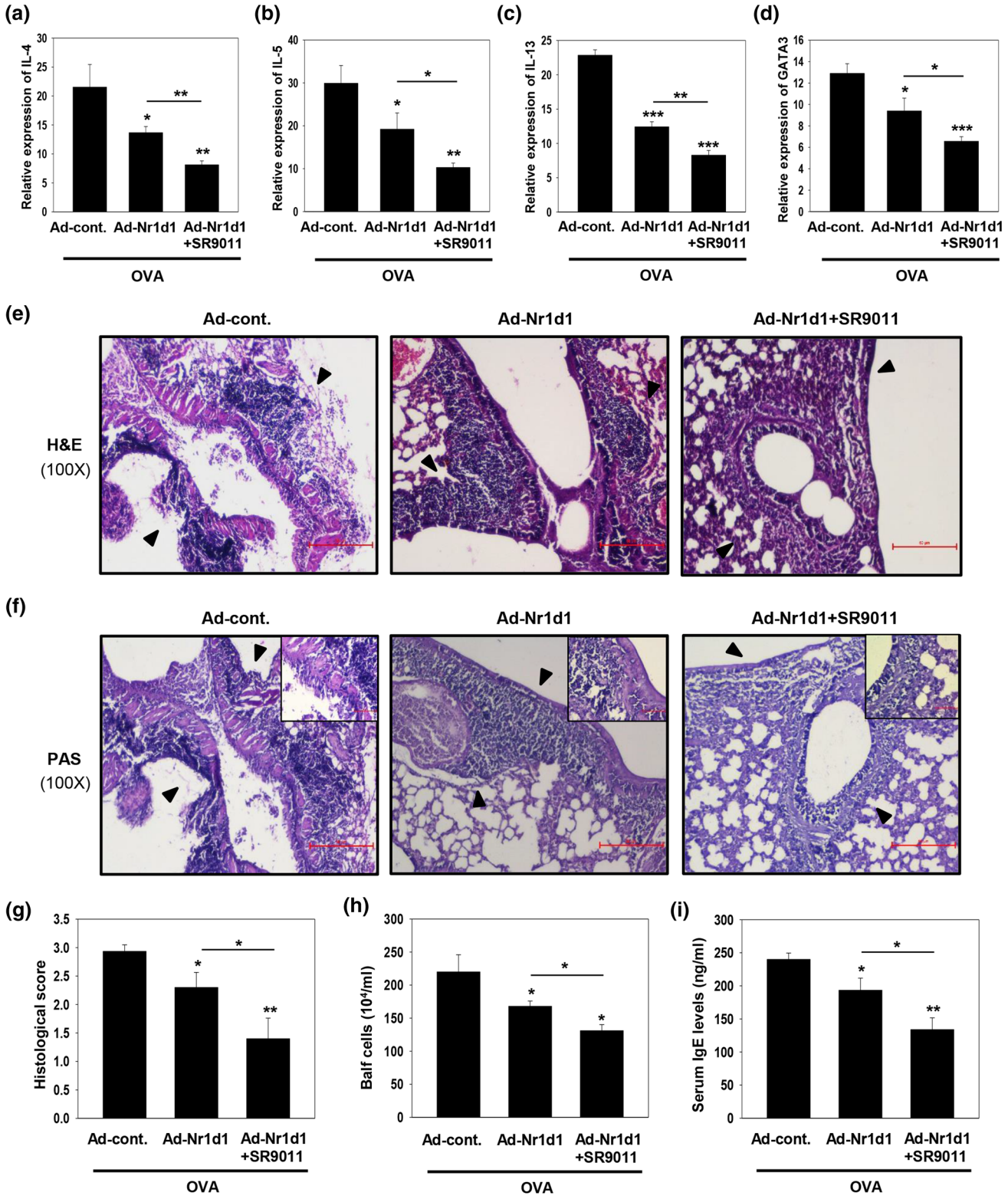
To examine the therapeutic effect of the Nr1d1 ligand SR9011 [63], we assessed its ability to improve asthma symptoms in our murine asthma model ($Nr1d1^{+/+}$ mice). Post-animal killing, RNA from the lungs of control, asthmatic, and asthmatic mice treated with SR9011 were isolated, and the expression of asthma-associated markers was investigated. A significant decline was observed in the expression levels of IL-4, IL-5, IL-13, and GATA3 in SR9011 treated mice compared to the asthmatic mice without ligand treatment (Fig. 5a–d). We also conducted lung histopathology and found that there was a notable decrease in the inflammatory responses of mice treated with the ligand SR9011 (Fig. 5e). The augmented infiltration of immune cells was clearly evident in the H&E stained lung images; however, SR9011 treated mice exhibited reduced infiltration compared to diseased mice with no ligand treatment. The lung sections were also stained by PAS and there was more goblet cell hyperplasia in mice with no ligand treatment as compared to the ligand treated mice (Fig. 5f). This resulted in an overall lower histological score in SR9011 treated mice than mice with no ligand treatment (Fig. 5g). Furthermore, the BALF cellularity and IgE serum levels were also reduced in mice treated with SR9011 (Fig. 5h, i). Hence, together this data affirms that SR9011 ameliorates the severity of asthma and that there is a significant reduction in lung inflammation upon ligand treatment.

Nr1d1 overexpression and its ligand SR9011 treatment diminishes asthma severity

To further validate our findings, we overexpressed Nr1d1 using an adenoviral overexpression system in the lungs of the $Nr1d1^{-/-}$ mice through the aerosol route. The ectopic overexpression of Nr1d1 was confirmed by western blotting (Fig. S6b). The asthma was induced through OVA in adeno-control (Ad-control), Nr1d1-overexpression (Ad-Nr1d1) and Nr1d1-overexpression with SR9011 ligand (Ad-Nr1d1 + SR9011) treated mice groups and they were further examined for disease severity. Post-animal killing, RNA from the lungs were isolated and expression level of the asthma markers were examined. We observed a notable decrease in the expression levels of IL-4, IL-5, IL-13 and GATA3 in Ad-Nr1d1 and Ad-Nr1d1 + SR9011-treated mice groups in comparison to the Ad-control group (Figs. 6a–d, S6b). The H&E staining of lungs was conducted and we found a notable decrease in tissue inflammation and much less infiltration of immune cells in Ad-Nr1d1 and Ad-Nr1d1 + SR9011-treated mice when compared with the Ad-control group (Fig. 6e). The PAS-stained sections also exhibited diminished mucus production in the Ad-Nr1d1 and Ad-Nr1d1 + SR9011 treated mice group (Fig. 6f); resulting in a reduced histological score (Fig. 6g). Further, the BALF cellularity and serum IgE levels were examined and we found reduced levels of BALF cells (Fig. 6h) and serum IgE (Fig. 6i) in mice treated with Ad-Nr1d1 and Ad-Nr1d1 + SR9011 in comparison to the control group. Although Nr1d1 overexpression (Ad-Nr1d1) also decreased the asthma severity, the level of reduction was less in comparison to the Nr1d1 overexpression + SR9011 treatment group (Ad-Nr1d1 + SR9011). Thus, together these results assert that Nr1d1 provides defense against asthma.

Genome-wide mapping of Nr1d1 binding sites in differentiated Th1 cells

Since Nr1d1 is a transcription factor, we hypothesized that it carries out this process of cellular programing via binding to the promoter region of its various target genes. To characterize the regions bound by Nr1d1, we carried out genome-wide ChIP-seq using an Nr1d1 antibody. Short reads from IgG were used as a control for peak detection. HOMER analysis identified that the majority of the



genes were in intergenic and intronic regions. The peaks that were found enriched in the promoter-transcription start site (TSS) region corresponded to 5.5% of the total peaks (Fig. 7a). Since we conceptually wanted to identify the interaction of Nr1d1 protein with gene promoters, the putative role of transcriptional regulation with chromatin regions other than the promoters was not addressed. Additionally, the enriched peaks (promoter-TSS region) were subjected to

◂**Fig. 6** Nr1d1 overexpression along with SR9011 ligand treatment provides defense against asthma in Nr1d1$^{-/-}$ mice background. Post-killing, the subsequent Th2 responses were monitored. Relative expression levels of (**a**) IL-4, (**b**) IL-5, (**c**) IL-13, and (**d**) GATA3, as obtained by qRT-PCR in lung samples. Histopathological reflection of the mice lungs showing representative images of (**e**) H&E staining and (**f**) PAS staining taken at 100X magnification (scale bar: 50 µm). The arrowheads represent areas of inflammation and cellular infiltration. The top-right inset in PAS-stained section shows the zoomed image (400X) for the same area. **g** Semiquantitative analysis of the severity of lung inflammation. Peri-bronchial inflammation was scored on a scale of 0–3; grade 0 was designated as no detectable inflammation, grade 1 as mild, grade 2 as moderate, and grade 3 as severe. **h** The total cell counts in isolated BALF. **i** Estimation of IgE serum levels by ELISA. Asterisks represent significant differences as indicated (* indicates $P<0.05$; ** indicates $P<0.005$; *** indicates $P<0.0005$). Data shown are representative (**e**, **f**) or average (**a**–**d**, **g**–**i**) from three independent experiments (mean ± s.d.)

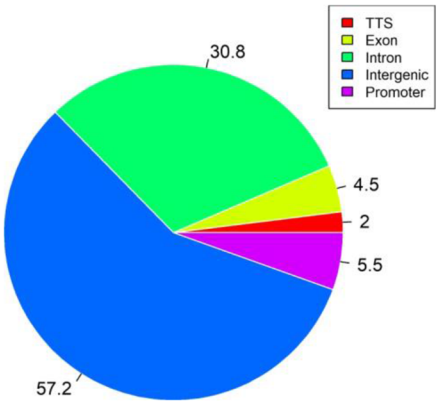
gene ontology (GO) and pathway analysis using the DAVID and PANTHER databases. Twelve of the most enriched biological processes (GO terms) included: cellular processes (162 genes), metabolic processes (114 genes), biological regulation (111 genes), localization (61 genes), multicellular organismal processes (58 genes), stimulus responses (26 genes), developmental processes (17 genes), cellular adhesion (14 genes), reproduction (5 genes), immune system (4 genes), cellular proliferation (4 genes), and rhythmic processes (2 genes) (Fig. 7b) that are reported to be involved in 67 different pathways (Fig. S7). Moreover, to determine the type of motifs contained in the filtered peaks, these DNA sequences were evaluated using MEME software. The enriched motif sequence displayed strong similarity to the consensus binding sequence (AGGTCA) of Nr1d1 (Fig. 7c), suggesting a probable interaction of Nr1d1 with these genes. From our overall ChIP data, few of the genes having functions pertinent to specifically asthma and immunity have been listed (Fig. 7d) [64–81]. This association of Nr1d1 with other genes also partly explains the diverse physiological role of this receptor in metabolic, psychological, and immune disorders. Thus, together these findings indicate that Nr1d1 globally associates with genes of different physiologies and aids in their proper functioning, thereby maintaining the body homeostasis. Any disruption in the expression of Nr1d1 interrupts this coordination, thereby leading to asthma and other linked health comorbidities (Fig. 8).

Discussion

Type-2 immune responses comprising of inflammation, enhanced serum IgE, mucus production, and cellular infiltration are imperative for the body's immunity; however, undesirable and dysregulated activation of such responses, triggered by certain allergens, can lead to the development of asthma or other allergic disorders [82]. The current study has attempted to address the underlying molecular pathways producing susceptibility to asthma, as well as the genetic link between asthma and subsequent comorbidities. We identify a new druggable target, the NR Nr1d1, and demonstrate for the first time that Nr1d1 provides defense against asthma by decreasing the differentiation of Th2 cells (Fig. 1). Although the Nr1d1 expression is found to be low in Th2 cells (Fig. 1), its functional relevance in impeding Th2 differentiation is clearly evident from the experiments performed in knockout mice. We also discovered that Nr1d1 suppresses Th2 cell differentiation via the direct suppression of GATA3, the master transcription factor of Th2 cells, by binding onto the promoter region of the GATA3 gene and inhibits its transcription in both mice and human cells (Fig. 2). NRs mainly activate expression of their target genes via binding to a natural or pharmaceutical ligand, which promotes a conformational switch from an earlier suppressed state to an activated one [83]. However, Nr1d1 is unique among all of the NRs as it is a constitutive repressor of transcription and lacks the C-terminal helix, H12 [31]. Nr1d1 binds strongly to its cellular companion, NCoR, which then recruits the class I histone deacetylase HDAC3, forming a stable repression complex on their endogenous target genes [84, 85]. In light of the above scientific facts, we also observed that Nr1d1 interacted with NCoR and HDAC3 to carry out its effector function of GATA3 suppression (Fig. 2).

Activated Th2 cells have been reported to produce IL-4, IL-5, and IL-13 cytokines that not only act as signature markers for the Th2 response but also mediate effector functions, such as antibody class switching to IgE, mucus production, as well as activation and recruitment of eosinophils [82]. Additionally, few reports have shown that IL-13 can account for overall disease intensification and resistance to corticosteroid therapy [86]. Therefore, we examined whether Nr1d1 can modulate the Th2 cytokine milieu and we discovered that Nr1d1 significantly impaired the production of IL-4, IL-5, and IL-13 cytokines (Figs. 1 and 3). Furthermore, the evolutionary significance of Nr1d1 binding to the GATA3 promoter sequences from various species was also studied by phylogenetic reconstruction (Fig. S4). The binding motif of Nr1d1 is well conserved in humans and higher mammals; however, in Norway rat, phylogenetic analysis unveiled the presence of a single base deletion in the core binding site. This single deletion likely indicates that Nr1d1 would fail to bind to the region, and perhaps, naturally, this could form one plausible explanation behind the genetic Th2-predisposition of Norway rats [61]. Moreover, to corroborate our in vitro results, an OVA induced asthma model in mice was employed and it led to the undeniable observation that Nr1d1 has the forte to rescue animals from asthma and associated inflammatory responses (Figs. 3 and 4). Although there are several experimental models which are being routinely used to generate asthma in mice, every

(a)



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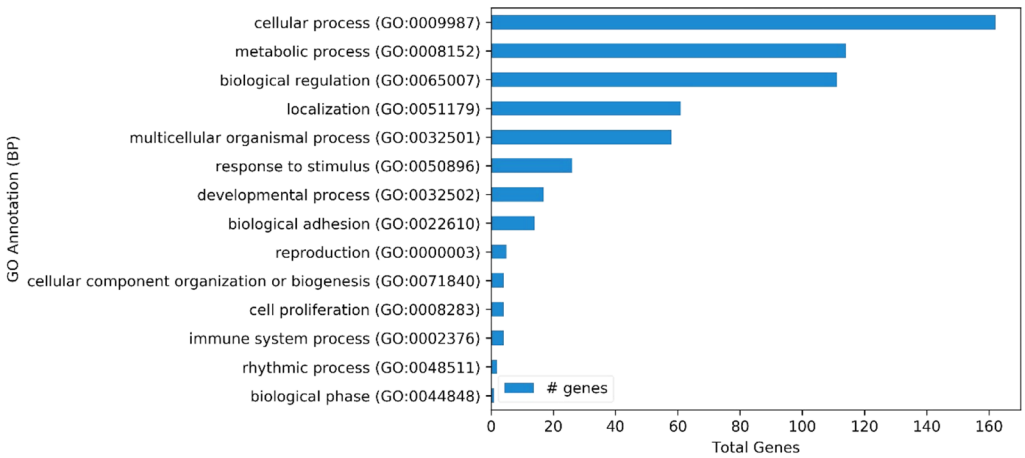

(c)

de novo binding motif

(d)

Gene symbol	Role in asthma or immunity (references)	Cell type studied
Phlpp1	64	T cells
Hrh1	65	Blood sample
Fbxl3	66, 67	SCN (hypothalamus), immune cells, lung tissue
Tnfrsf1a	68	Various Immune cells
Emp2	69	Polymorphonuclear neutrophils
Timp3	70	HEK-293H cell line
Rhoa	71	Bronchial Smooth Muscle
Nox4	72	Human bronchial epithelial cells
Arnt2	73	Murine lungs & human subjects
Jag1	74, 75	Pulmonary leukocytes, Alveolar macrophages
Wdr1	76	Blood sample
Sox11	77	Embryonic lung fibroblast (HEK293T and MRC-5 cells)
Stk40	78	Lung tissue
Gata3	79	Th2 cells
Ryr3	80, 81	Airway smooth muscle cells

Fig. 7 Genomic distribution of Nr1d1 binding sites and their gene ontology (GO) analysis. **a** Distribution of the overall Nr1d1 binding sites as obtained by ChIP-seq experiments. **b** GO analysis of genes with Nr1d1 binding sites in the promoter-TSS region and **c** the enriched sequence motif. The DAVID and PANTHER databases were used to determine the biological processes regulated by the enriched genes. **d** List of selected genes that are specifically linked to asthma and immunity

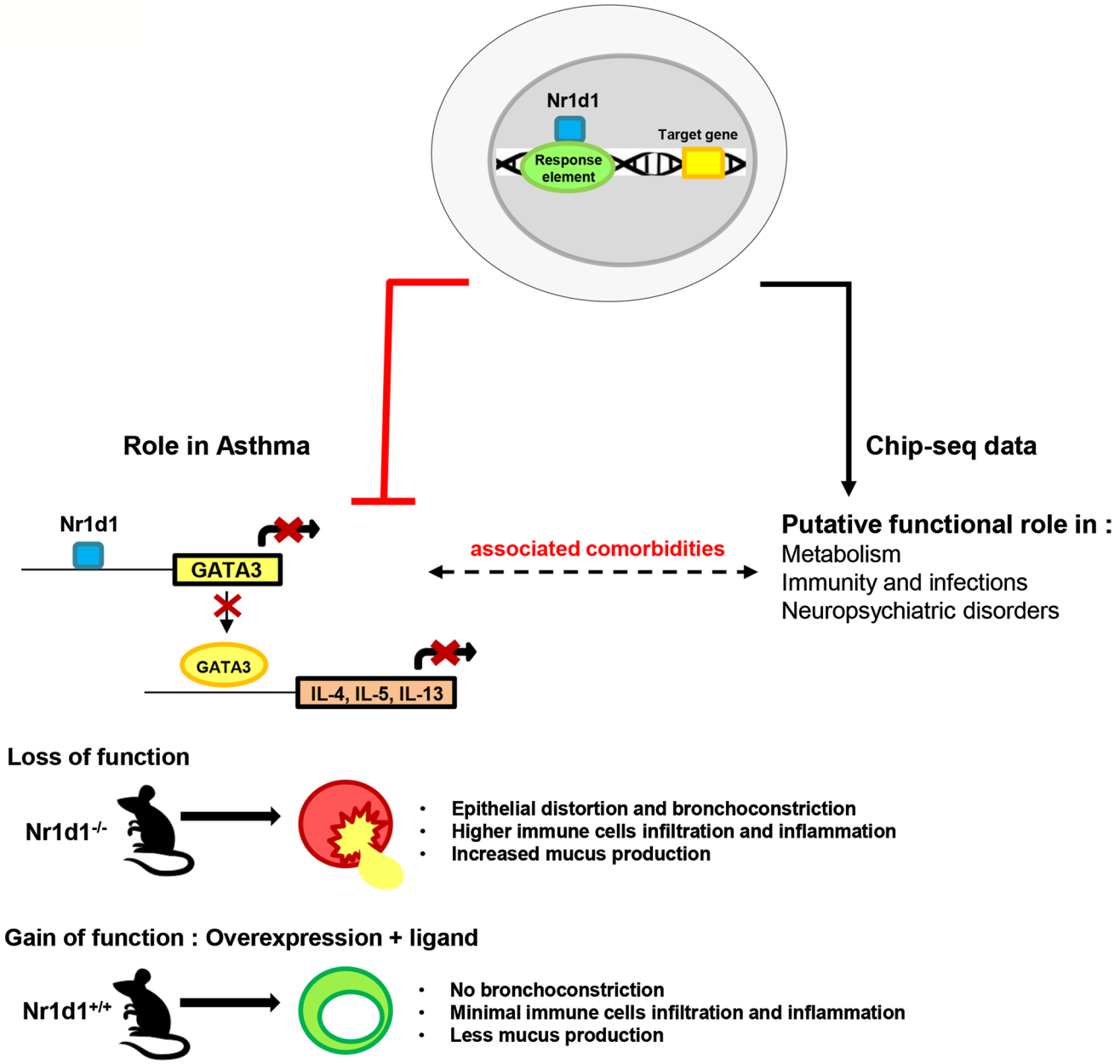

Fig. 8 Schematic representation featuring the role of Nr1d1 in providing protection against asthma and its possible interaction with genes involved in asthma-associated comorbidities. Nr1d1 transcriptionally impedes GATA3 gene, the master transcription factor required for the differentiation of Th2 cells. The murine model of asthma showed that the presence of Nr1d1 provides protection against asthma by reducing the disease severity and inflammation as observed from both the loss of function and gain of function experiments. Additionally, the ChIP-seq data discovered the interaction of Nr1d1 with various other subsets of genes involved in metabolism, immunity and brain disorders, which are also the asthma-associated comorbidities

model has its own benefits and limitations. The OVA challenge models are particularly advantageous in generating a predominant Th2-biased immune response and unlike environmental allergens, it is a relatively well defined antigen and possess molecular purity (homogeneous). This helps in administering controlled dosages, thereby avoiding complications in the interpretation of experimental results [87]. Apart from Th2 cells, ILC2s in lungs are capable of secreting signature type-2 cytokines and several studies have shown their role in influencing pulmonary immunity and asthma [88]. Therefore, to specifically look for the effect of Th2 cells, we have chosen an asthma model generated

through systemic administration of OVA which mounts a predominant Th2 immune response [89, 90]. In addition to this, we examined the therapeutic angle of the Nr1d1 ligand SR9011 and observed that ligand treatment alleviated the severity of asthma by suppressing the Th2 response in both $Nr1d1^{+/+}$ mice or $Nr1d1^{-/-}$ mice (treated with Ad-Nr1d1) (Figs. 5 and 6). Furthermore, in this study, we have elucidated the role of Nr1d1 in Th2 cells as these cells are the main trigger behind allergic asthma; leading to other pathophysiological responses like homing of inflammatory cells, increased mucus production, smooth muscle contraction and subsequent lung damage. Regardless of the approach, we believe that a successful remedy for the allergic asthma would comprise of targets that cause allergen-specific Th2 cell inhibition. However, we do not rule out the possibility of other cellular targets and it would be further intriguing to study the role of Nr1d1 in other immune cells.

It is interesting to observe a slight decrease in the Th1 proliferation and an increase in Th2 proliferation in $Nr1d1^{-/-}$ as compared to the $Nr1d1^{+/+}$ mice background (Fig. 1). This study adequately addresses the role of Nr1d1 in Th2 cell differentiation, but given the high expression of Nr1d1 observed in Th1 cells, its role in Th1 polarization needs to be further investigated. Earlier, basal expression of Nr1d1 has also been reported in Th1 cells by another group [40]. While our study specifically addresses the role of Nr1d1 in Th2 cell differentiation and asthma disease as a model, their focus was on Nr1d1 role in Th17 cell-mediated autoimmune diseases [40, 41]. Recently, many scientific reports have highlighted Nr1d1 as a therapeutic molecule for psychological, metabolic, and immune disorders [34, 35, 42–44, 91], which are common comorbidities associated with asthma. Our current ChIP-seq data are also in accordance with these different scientific investigations and emphasizes the involvement of Nr1d1 with various genes related to metabolism, immunity and neuronal diseases, as well as other cellular signaling pathways (Fig. 7). The findings indicate that Nr1d1 globally associates with genes of different physiologies and aids in their proper functioning. The disruption of Nr1d1 function disturbs this coordination, thereby leading to asthma and other linked health comorbidities. However, these are merely speculations and require strong experimental evidence. This forms the foundation for future research which would provide new insights into immune responses.

In conclusion, our study is the first to describe the indispensable role of Nr1d1 in influencing the differentiation of Th1:Th2 cells and its protective effect in asthma (Fig. 8). As the current asthma therapies fail to achieve disease control in a significant proportion of patients, there arises a strong need for the development of improved asthma treatment. Over the years, various NRs have been studied in inflammatory diseases [92–94]. The NR Nr1d1 is deemed to have a therapeutic role in various inflammatory conditions like Th17 cell-mediated diseases, LPS-induced type-1 immune responses [38, 40, 41] and also in asthma as shown in our study. Few scientific studies have also indicated towards the possibility of Nr1d1 involvement in asthma pathogenesis and the role of circadian rhythm in influencing the asthmatic respiratory system [95–97]. Another study has reported that healthy patients have higher levels of Nr1d1 expression than asthmatic patients, suggesting of a similar protective role as depicted in our study [98]. Since Nr1d1 is a major regulator of both clock function and energy metabolism, it aids in shielding the organism from any major disturbances in circadian and metabolic physiology [91]. Therefore, Nr1d1 seems to be a promising target for maintaining body homeostasis. Future investigations into Nr1d1 as a drug target for asthma will create significant improvements for the majority of asthmatic patients. Considering the byzantine role of Nr1d1 in immune responses, it will also be interesting to study its upstream signaling mechanisms and associated post-translational modifications.

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Author contributions DT and PG conceived the idea and designed the research, DT, NA, SK, RK, RN, SG, AKK, EB and RA performed the experiments, DT and PG analyzed the data, DT and PG interpreted the data and wrote the manuscript, and PG supervised the project.

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Data availability The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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