Respiratory Research

RESEARCH

Open Access

TLR5 activation in respiratory epithelial cells orchestrate mucosal Th17 response through both indirect and direct pathways

Sijian Huang[1†], Xu Li[1,4†], Yuan Cao[1,3], Man Mou[1,5], Jianlun Li[1], Kexing Zhuo[1], Lijuan Wang[1], Zihang Zeng[1], Xianghong Wei[1], Chunlian Tang[1,2*] and Maohua Zhong[1*†]

Abstract

Background Flagellin, a potent mucosal adjuvant administered via the intranasal route, has been widely recognized for its capacity to enhance immune responses against diverse pathogens. However, the effects and the underlying mechanisms by which flagellin modulates $CD4^+$ T cell differentiation remain incompletely understood.

Methods Recombinant flagellin proteins, including full-length flagellin (SF) and a TLR5-binding deficient variant (SFΔ90–97), were produced and purified. An OT-II derived $CD4^+$ T cell adoptive transfer model, a classical intranasal immunization model and dendritic cell (DC)-$CD4^+$ T co-culturing system were used. The proliferation and differentiation of $CD4^+$ T cells were analyzed using flow cytometry analysis. RNA sequencing and neutralizing antibody blocking experiments were performed to determine the essential cytokines involved in flagellin modulated Th17 differentiation.

Results Flagellin preferentially promotes Th17 cells differentiation. Respiratory epithelial cells (RECs), acting as sentinel cells, are the first to encounter exogenous stimuli during intranasal immunization. Flagellin stimulates the secretion of various soluble cytokines by binding to TLR5 on the surface of RECs, with GM-CSF facilitating the functional activation of airway DCs. GM-CSF-conditioned DCs exhibit upregulated IL-6 expression which in turn drives the polarization of naïve $CD4^+$ T cells toward the Th17 phenotype. Furthermore, TLR5-regulated REC-derived IL-6 synergizes with TLR5-modulated DCs to amplify Th17 polarization signals, thereby enhancing the Th17 induction.

Conclusion Flagellin preferentially induced a Th17-enhanced immune response and RECs were highlighted its essential roles during this process through both indirect and direct pathways. For indirect pathway, RECs modulate DC function through GM-CSF. Moreover, RECs directly contribute to Th17 differentiation by secreting IL-6.

Keywords Mucosal adjuvant, Flagellin, Respiratory epithelial cell, Dendritic cells, Th17

†Sijian Huang and Xu Li contributed equally to this work.

†Maohua Zhong: Lead contact

*Correspondence:
Chunlian Tang
tangchunlina@sina.com
Maohua Zhong
zmh@wust.edu.cn
[1]Institute of Infection, Immunology and Tumor Microenvironment, Hubei Province Key Laboratory of Occupational Hazard Identification and Control, Medical College, Wuhan University of Science and Technology, # 2 Huangjiahu West Road, Wuhan, Hubei 430065, China
[2]Wuchang Hospital Affiliated to Wuhan University of Science and Technology, Wuhan, Hubei 430063, China
[3]Analytical & Testing Center, Wuhan University of Science and Technology, Wuhan, Hubei 430065, China
[4]Clinical Laboratory, Wuhan Asia General Hospital, Wuhan University of Science and Technology, Wuhan, Hubei 430056, China
[5]Department of Blood Transfusion, Wuhan Asia General Hospital, Wuhan University of Science and Technology, Wuhan, Hubei 430056, China

Introduction

Most pathogens invade the host via the mucosal route, making mucosal immunity the host's first line of defense [1]. Mucosal vaccines can establish an effective immune response both at the mucosal surfaces and systemically [2]. In general, non-replicating subunit vaccines are safer than live attenuated vaccines but typically require adjuvants for successful antigen-specific immune responses.

As an immune booster, mucosal adjuvants not only enhance the immunogenicity of antigens and promote the host's immune response, but also improve vaccine safety and reduce costs by lowering the required antigen dosage and the number of administrations. Flagellin, the primary structural protein of bacterial flagellar filaments, is a distinct pathogen-associated molecular pattern (PAMP) [3]. The intranasal adjuvant activity of flagellin has been extensively documented, showing that it can significantly enhance both mucosal and systemic antigen-specific antibody responses, as well as increase the protective efficacy of vaccines against various pathogens including *Streptococcus mutans*, influenza virus, respiratory syncytial virus, SARS-CoV-2, *Streptococcus pneumoniae* and Zika virus, etc.

Effective activation of $CD4^+$ T cells is critical for maintaining long-term vaccine-induced immunity. $CD4^+$ T helper (Th) cells, as key components of the immune system, play a critical role in coordinating adaptive immune responses. These cells are a highly heterogeneous population that differentiates into various subsets upon receiving relevant signals from antigen-presenting cells [4]. Th1 cells, defined by the master transcription factor T-bet and the lineage cytokine IFN-γ, can prevent cells from being attacked by intracellular bacteria and viruses. Th2 cells, defined by the master transcription factor GATA3 and the characteristic cytokines IL-4, IL-5, and IL-13, are mainly involved in the immune response against helminths. Th17 cells, defined by the master transcription factor RORγt and the characteristic cytokine IL-17, are responsible for both intracellular and extracellular immune responses against fungi and bacteria [5–7]. Vaccination effectively induces the proliferation and differentiation of specific $CD4^+$ T cells subsets, highlighting their central role in immune defense [8]. There is a notable variation in the Th subsets elicited by flagellin under distinct experimental routes. Systemic immunization with flagellin induce a Th2-polarized immune response against the co-administrated antigen or Th1 polarization targeted the fusion antigen [9]. However, via intranasal immunization route, flagellin promoted Th17 dominated differentiation or a mixed Th1/Th17 immune response [6, 10, 11]. Thus, the effects of flagellin, acted as an intranasal adjuvant, on the differentiation of $CD4^+$ T cells warrant further investigation. Moreover, the underlying mechanism of flagellin modulated Th polarization remains to be elucidated.

The majority of research investigating the adjuvant properties of flagellin focuses on dendritic cells (DCs), which serve as a crucial link between innate and adaptive immune responses [12]. Systemic administration of flagellin promotes the recruitment and activation of $CD103^+$ cDC2, enhancing the secretion of IL-2 and IL-4, which subsequently induces a Th2-type immune response [13]. Moreover, flagellin delivered via the gastrointestinal mucosa directly stimulates TLR5 on DCs, triggering the secretion of IFN-γ to initiate a Th1-type immune response [14]. In contrast, respiratory DCs exhibit significantly low TLR5 expression, rendering them poorly responsive to flagellin administered through the nasal route [15, 16]. Therefore, it remains to be explored how flagellin as an intranasal adjuvant modulate DCs to induce certain Th responses.

Another pivotal role that cannot be ignored when flagellin exerts its unique effects as an intranasal adjuvant is respiratory epithelial cells (RECs). RECs express a variety of pattern recognition receptors and modulates the local immune response in the respiratory tract through cytokine secretion or cell-cell interaction [17–19]. Unlike intestinal DCs, which abundantly express TLR5, respiratory DCs exhibit low levels of TLR5 expression [16]. In our and other's previous studies, it has been evidenced that respiratory DCs were indirectly activated by flagellin via GM-CSF secreted by TLR5-activated RECs [15, 16, 20]. Whether RECs-derived GM-CSF is also essential for flagellin to modulate DCs to induce certain Th response remains unclear. Additionally, since TLR5-activated RECs secret various other kinds of cytokines, whether RECs directly participate in flagellin-modulated Th differentiation needs to be determined.

In this study, we sought to elucidate the effect and mechanism of intranasal adjuvant flagellin modulation on $CD4^+$ T cell response. It was found that intranasal adjuvant flagellin enhance preferentially a Th17 immune response and RECs play essential roles during this process via both indirect and direct pathways. For indirect pathway, TLR5 activated RECs modulate DCs' potential via secretion of GM-CSF. On the other hand, RECs play direct roles in modulating Th17 response through IL-6 production. Furthermore, IL-6 was identified as an essential cytokine for flagellin-modulated DCs-directed Th17 response.

Materials and methods

Mice

C57BL/6 mice aged 6–8 weeks (18–20 g per mouse) were obtained from the Hunan Laboratory Animal Research Center and housed under Specific Pathogen Free (SPF) conditions at the Wuhan University of Science and Technology Laboratory Animal Center. TLR5 knockout (*Tlr5*$^{-/-}$), OT-II, and CD45.1 mice on a C57BL/6 background, sourced from Jackson Laboratory, were also housed under specific pathogen-free (SPF) conditions at the Wuhan University of Science and Technology Laboratory Animal Center. All animals were randomly assigned to groups before experiment.

Expression, purification, and identification of Recombinant proteins

Large-scale expression cultures of E. coli BL21 (DE3) cells containing recombinant flagellin constructs were prepared. These constructs included full-length Salmonella flagellin (SF), its truncated variants SFΔ90–97 (with a deletion in the TLR5-binding domain QRVRELAV), the HIV-1 core antigen P24, and the fusion protein SF-P24. These recombinant proteins containing a 6-His Tag at the C-terminus were prepared and purified using affinity chromatography on a Ni-NTA column (Qiagen) and dialyzed against phosphate-buffered saline (PBS) at 4 °C. Contaminating lipopolysaccharides (LPS) were removed by liquid phase separation using Triton X-114, which preserves the physical integrity and biological activity of the proteins and is safe for experimental animals. The purified proteins were filtered through Acrodisc syringe filters (Pall) and stored at -80 °C. The quantification of purified proteins was performed using the Bradford assay. Residual LPS content was measured using the Limulus assay (Associates of Cape Cod) to ensure that the LPS content in the protein was less than 2 EU/mg. The TLR5 activity of SF and SF-P24 was verified in vitro as in our previous study. Briefly, human intestinal epithelial Caco-2 cells were grown and seeded into 48-well plates and cultured at 37 °C with 5% CO_2 for 5 days. After an overnight culture in medium without fetal bovine serum (FBS), the cells were stimulated with SF and SF-P24 or the control protein SF△90–97 and P24 for 6 h at the indicated concentrations. The supernatants were then collected and quantified for IL-8 with the ELISA kit (BioLegend).

Tissue lymphocyte isolation

Tissue lymphocyte isolation was performed as follows: For cervical lymph nodes (CLNs), CLNs were digested in serum-free RPMI 1640 medium containing 1 mg/mL collagenase D (sigma) and 50 μg/mL DNase I (sigma) at 37 °C for 30 min. The lymphocyte suspensions were then obtained by grinding the tissue through a 70 μm cell sieve at the end of digestion. For spleens, lymphocyte suspensions can be obtained by lysing erythrocytes after grinding the spleen directly through a 70 μm cell sieve. For lungs, the clipped lungs were digested in serum-free RPMI 1640 medium containing 2 mg/mL collagenase type I (sigma) and 0.4 mg/mL DNase I at 37 °C for 35 min. After digestion, the cells were ground through 70 μm cell sieve and resuspended in 40% Percoll (GE Healthcare), which was then slowly added dropwise to 80% Percoll. After centrifugation at 2500 rpm for 30 min, the cells at the interface between 40% and 80% Percoll were carefully and slowly collected as lung lymphocytes.

Adoptive transfer experiment

OT-II-derived $CD44^{-}CD62L^{+}$ naïve $CD4^{+}$ T cells were obtained through negative selection using magnetic bead separation (Miltenyi Biotec). The naïve $CD4^{+}$ T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Thermo Fisher Scientific) were transferred to CD45.1 mice at $3–5 \times 10^{6}$ cells per mouse. The following day, the transferred mice were intranasally (i.n.) immunized with 40 μg OVA in combination with 10 μg SF or SFΔ90–97, in a total volume of 10μL. Five days after immunization, the recipient mice were euthanized, and the CLNs and spleens were isolated and processed into single lymphocyte suspensions for flow cytometry assays. Cells from the CLNs of two mice were combined and divided into two parts. One part was used directly for proliferation detection of OT-II derived $CD45.2^{+}CD4^{+}$ T cells by the flow cytometry assay, and the other part was plated in 48-well plates. After stimulation with the specific peptide $OVA_{323-339}$ and the irrelevant peptide $HBC_{129-140}$ for 48 h, the cell supernatants were collected for IFN-γ, IL-4 and IL-17 A by ELISA (BioLegend, USA).

Classical intranasal immunization experiment

Female wild-type (WT) C57BL/6 or *Tlr5*$^{-/-}$ mice were anesthetized and then immunized intranasally with 5 μg of P24 alone or an equivalent molar amount of SF-P24, administered in a volume of 10 μL, three times at 3-week intervals. One week after the final immunization, mucosal secretions and serum were collected. Following euthanasia, lungs and CLNs were collected to isolate lymphocytes. Samples were collected and prepared as follows. Mouse blood samples were collected from the retro-orbital plexus and then centrifuged to isolate serum. Saliva samples were collected after intraperitoneal injection of carbachol. Vaginal lavage fluid was obtained by rinsing the genital tract three times with 40 μL of sterile PBS. Clipped lung tissue was enzymatically digested, and the supernatant was collected to obtain lung homogenates. All post-immunization sera and mucosal secretions were collected and used to detect P24-specific IgA and IgG. Lymphocytes from lungs and CLNs were plated

in 96-well plates. The cells were stimulated with 2 μg/mL of anti-CD28 and the specific antigen P24 or OVA used as irrelevant antigen control for 48 h, after which the supernatants were collected for IFN-γ, IL-4 and IL-17 A by ELISA (BioLegend, USA). For antigen-specific cytokine data analysis, all test samples were calculated as background subtracted data, where the linear averages of the OVA background signal from duplicate wells of the same sample were subtracted from the stimulated signal (signal–"Neg" OVA background).

Phenotype assays for DCs in CLNs of mice after intranasal immunization

6-8-week-old female C57BL/6 mice were anesthetized and immunized intranasally with 70 μg OVA-AF647 (Thermo Fisher Scientific) alone or in combination with 10 μg SF or SFΔ90–97. Mice were euthanized 18 h after immunization. CLNs were collected, and individual lymphocytes were isolated and assayed by flow cytometry to evaluate antigen-loading and phenotypic changes including activation marker CD80, CD86 and chemokine receptor CCR7 on DCs after Fc blocking via anti-CD16/32 incubation (BioLegend, USA).

DC ex vivo co-culture with Naïve OT-II Naïve $CD4^+$ T cells

Female C57BL/6 mice were anesthetized and immunized intranasally with 40 μg OVA alone or in combination with 10 μg SF or SFΔ90–97. Eighteen hours post immunization, the mice were euthanized. CLNs were collected and processed for positive sorting using magnetic beads to obtain $CD11c^+$ DCs (Miltenyi Biotec, Germany). The sorted $CD11c^+$ DCs were plated in 96-well plates with CFSE-labeled OT-II-derived naïve $CD4^+$ T cells at a ratio of 1:5. The cells were collected after 5 days of co-cultivation, transferred to 96-well plates pre-coated with 1 μg/mL anti-CD3, and cultured at 37℃ with the addition of Brefeldin A (BFA) and monensin. Five hours later, the cells were harvested for flow cytometry analysis of the proliferation and differentiation of $CD3^+CD4^+$ T cells. For IL-6 neutralization, 20 μg/mL of IL-6 specific neutralizing antibody (BioXcell, MP5-20F3) or isotype control antibody (BioXcell, HRPN) were added to the co-culture system.

RECs conditioned medium Preparation

Nasal epithelial cells, serving as surrogates of RECs in our study, were separated and cultured until polarization as previously described [15]. Female WT C57BL/6 mice were euthanized, and the nose was isolated by removing the visible connective and muscular tissues. The turbinate and midline on each side were incised to expose the internal structure of the nose, which was then digested in serum-free DMEM/F12 medium containing 1.4 mg/mL pronase (sigma) and 0.1 μg/mL DNase I at 37 °C with 5% CO_2 for 30 min. At the end of digestion, additional mucosal epithelial cells were dissociated by turning the tissue up and down 12 times. The cells in the supernatant of the digested solution were collected by centrifugation at 21 °C, 120 g, for 5 minutes, resuspended in DMEM/F12 containing 10% FBS, and seeded into 100 mm dishes for 2 h to remove excess fibroblasts. The collected non-adherent cells were then filtered through a 70 μm cell sieve to obtain the RECs. The cells were plated in transwell chambers at 8×10^5 cells per well, and got polarized around 7–10 days after plating. Medium supplemented with PBS, SF, or SFΔ90–97 at a final concentration of 1 μg/mL was added to the apical side of the polarized RECs. Conditioned medium from the apical (REC-M, REC-SF, REC-SFΔ90–97) and basal compartments was collected after 24 h of stimulation and stored at -80 °C for subsequent DC conditioning and cytokine assays. The procedure was illustrated in Fig. S3.

Production of bone marrow DCs (BMDCs)

Female *Tlr5*$^{-/-}$ mice (to exclude the direct stimulation effect of flagellin) were euthanized. Femurs and tibias were isolated to collect bone marrow, and the cells were resuspended in 5 mL of RPMI 1640 containing 100 ng/mL FLT3L (PeproTech) (including 1% penicillin-streptomycin, 50 μM β-mercaptoethanol, 1% non-essential amino acids, 1% sodium pyruvate, and 10% FBS) after filtration through a 70 μm cell sieve. On the third day, the 5 mL of cells were divided into two parts, each supplemented with 2.5 mL of RPMI 1640 containing 100 ng/mL FLT3L. This procedure was repeated on the sixth day. The cells were ready for use on the ninth day when differentiation was completed.

DC-T cell co-culture in vitro

REC apical conditioned medium was pre-incubated with or without 20 μg/mL anti-GM-CSF (BioXcell, MP1-22E9) or isotype Ab (BioXcell, BE0089) for 1 h at 37 °C and 5% CO_2. The apical-conditioned medium was used for the BMDC conditioning assay due to its significantly higher cytokine concentrations compared to basal-conditioned medium, which makes it more appropriate for assessing the impact of epithelial signaling on immune cells. After pre-incubation, BMDCs derived from *Tlr5-/-* mice were exposed to REC apical conditioned medium for 24 h. A portion of the cells was used for DC activation detection by flow cytometry analysis. After extensive washing, the remaining cells were incubated with 2 μg/mL $OVA_{323-339}$ peptide for 2 h. After another round of extensive washing, conditioned BMDCs were co-cultured with CFSE-labeled OT-II mice-derived naïve $CD4^+$ T cells at a ratio of 1:20 in round-bottom 96-well plates. In some instances, additional REC-conditioned medium REC-M or REC-SF were added to the co-culture system to evaluate the direct

effect of these conditioned medium on the $CD4^+$ T cell response. In this setting, to block additional stimulating effect of DCs by GM-CSF in the conditioned medium, the conditioned medium was pre-incubated with anti-GM-CSF neutralizing antibodies at 37℃ for 1 h. For IL-6 neutralization, α-IL-6 neutralizing antibody or isotype control antibody was added to the co-culture. The final concentration of α-GM-CSF, α-IL-6 or control IgG in the co-culture system was 20 μg/mL. The cells were collected after 5 days of co-cultivation, transferred to 96-well plates pre-coated with 1 μg/mL of anti-CD3, and cultured at the presence of BFA and monensin. Five hours later, the cells were harvested for flow cytometry analysis of the proliferation and differentiation of $CD3^+CD4^+$ T cells. The procedure was illustrated in Fig. S3a.

Flow cytometry analysis

Mouse tissue lymphocyte suspensions or in vitro cell cultures were prepared as described above. Fc receptors were blocked with anti-CD16/32 Fc blocker (2.4 G2; BD Biosciences) for 15 min at 4℃ before cell staining. Cells were then stained with a dead cell discrimination marker (Fixable Viability Dye eFluor 506, FVD; Thermo Fisher Scientific) and specific antibodies at 4 °C: CD11c (N418), $I\text{-}A^b$ (M5/114.15.2), CD80 (16-10A1), CD86 (GL-1), CD4 (GK1.5), CD45.2 (104). For cytokine assays, cells were fixed with fixation/permeabilization buffer (eBioscience) overnight, and intracellular cytokine staining was performed after Fc receptor blockade. The intracellular antibodies are as follows: IL-17 A (TC11-18 H 10.1), IFN-γ (XMG1.2), IL-4 (11B11), CD3 (145-2C11) or the corresponding isotype control: mouse IgG2a (MOPC-173), rat IgG2b (RTK4530), rat IgG1 (RTK2071, eBRG1). The fluorescence conjugated antibodies were all from BioLegend (USA). A BD FACSCelesta flow cytometer was used to assess the stained cells and data were analyzed using FlowJo V10.0. The gating strategy was shown in Fig. S4.

RNA sequencing

Polarized RECs were stimulated with PBS or 1 μg/mL SF for 3 h. The cells were then collected, and 1 mL of Trizol was added before sending them for sequencing. RNA sequencing was performed by BGI Genomics (China). Clean reads were mapped to reference sequences using Bowtie2, and gene expression levels for each sample were quantified with RSEM. Differentially expressed genes (DEGs) between samples were identified using DESeq2. KEGG pathway enrichment analysis was conducted on DEGs with a Log_2 fold change ≥ 1, utilizing the web-accessible program Database for Annotation, Visualization, and Integrated Discovery (DAVID). A p-value of < 0.05 was considered statistically significant for pathway enrichment.

Quantitative RT-PCR analysis

Total RNA from cells was extracted with RNA extraction (Takara, Japan). 1 μg of extracted total RNA was reverse transcribed into cDNA fragments (Toyobo, USA). These products were then diluted and amplified using Hieff qPCR SYBR Green Master Mix (Yeasen Biotechnology, China) in the Bio-Rad CFX384 Real-Time PCR Detection System to quantify gene expression. Relative mRNA expression was calculated using the $2^{-\Delta\Delta ct}$ method and normalized to relevant housekeeping gene (β-actin). Primer used as following: IL-6-s 5' TGTATGAACAACG ATGATGCACTT 3' IL-6-as 5' ACTCTGGCTTTGTCT TTCTTGTTATCT 3' IL-1β-s 5' ATGCCACCTTTTGA CAGTGATG 3' IL-1β-as 5' AAGGTCCACGGGAAAGA CAC 3' IL-23-s 5' CACCAGCGGGACATATGAATCT 3' IL-23-as 5' GGAGGTGTGAAGTTGCTCCA 3' IL-27-s 5' TCATTGCCACTTACAGGCTCG 3' IL-27-as 5' TTT AGCATGTAGGGCACCGT 3' β-actin-s 5' CAGCCTTC CTTCTTGGGTAT 3' β-actin-as 5' TGGCATAGAGGT CTTTACGG 3'.

Statistical analysis

Data are presented as mean ± SD. All data analyses were performed with one way ANOVA. When the P value was significant at the 0.05 level, further pairwise comparisons between the experimental group and control conditions were made using Bonferroni's test. Statistical analysis was carried out with GraphPad Instat software, version 10.0. P values of < 0.05 were considered significant.

Results

Intranasal TLR5 activation preferentially promotes Th17 responses

To determine the effects of flagellin as intranasal adjuvants on $CD4^+$ T cell response, both an OT-II mice derived naïve $CD4^+$ T cell transfer model and a classical intranasal immunization model were used. To achieve the aim above, series of recombinant proteins including the full length of flagellin (SF), TLR5-signmalling deficient variant SFΔ90–97, HIV-1 core protein P24, and the fusion protein of SF and P24 (i.e. SF-P24), were obtained using affinity chromatography on a Ni-NTA column. The purity and TLR5 activity of the proteins were verified by SDS-PAGE and Caco-2 stimulation assay, respectively (Fig. S1).

The results in the transfer model showed that the proportion of proliferating $CD4^+$ T cells induced by SF was 1.5-fold higher than that induced by antigen with the addition of SFΔ90–97 (Fig. 1a-d), which indicated that flagellin strongly stimulated the proliferation of antigen-specific $CD4^+$ T cells in a TLR5-dependent manner. To distinguish the influence of flagellin on the differentiation of antigen-specific $CD4^+$ T cells, cells prepared from CLNs were restimulated with the specific peptide

$OVA_{323-339}$ or irrelevant peptide $HBC_{129-140}$ for 48 h and IL-17 A, IFN-γ, IL-4, the surrogate cytokine of Th17, Th1 and Th2 were quantified using ELISA. The antigen-specific cytokine production was calculated as follows: the amounts stimulated by $OVA_{323-339}$ minus those stimulated by $HBC_{129-140}$. As shown in Fig. 1e-g, cells isolated from the CLN of OVA plus SF immunized mice exhibited a marginal IL-17 A and IFN-γ production to the irrelevant peptide $HBC_{129-140}$, whereas they showed a significant response to the specific peptide $OVA_{323-339}$, indicating that cytokine production is highly specific. The results show that IL-17 A production in the OVA + SF group was significantly higher than that in the OVA + SFΔ90–97 groups (Fig. 1e), while the IFN-γ and IL-4 production was comparable between two groups (Fig. 1f-g). The data above suggested that flagellin preferentially enhances Th17 immune response in a TLR5-dependent manner while have little effect on Th1 and Th2 response.

To further confirm the flagellin mediated modulation of flagellin on $CD4^+$ T cell response, a classical intranasal immunization model in which P24, the core antigen of HIV-1 used as model antigen, was performed. In agreement with previous studies, flagellin has shown TLR5-dependent powerful intranasal adjuvanticity as demonstrated by SF-P24 induced much higher P24 specific IgG and IgA titers in serum and mucosal fluids in contrast to P24 alone in WT but not in *Tlr5*$^{-/-}$ mice (Fig. S2a-f). Meanwhile, lymphocytes from draining lymph nodes, CLNs and effector sites, lungs were acquired, restimulated with specific antigen P24 or irrelevant antigen OVA and Th surrogate cytokine were assayed by ELISA. In line with the adoptive transfer model, compared with P24 alone, SF-P24 induced significantly higher production of IL-17 A in both cells from CLNs and lung tissues (Fig. 1h and k). Nevertheless, there was no significant difference of IFN-γ production between SF-P24 and P24 immunized groups (Fig. 1i and l). Besides, IL-4 production was below the low limit of detection (2 pg/mL) (Fig. 1j and m).

Taking the data in the transferred model and classical intranasal immunization model into account, it indicated that as an intranasal adjuvant, flagellin facilitate a Th17 immune response.

TLR5 activated RECs modulate respiratory DCs to facilitate the differentiation of Th17 cells

Previous studies have shown that DCs are essential for the adjuvant activity of i.n. flagellin [16]. After ascertaining that flagellin exhibits a propensity to elicit a Th17 immune response, our next step revolves around elucidating the alterations in respiratory DCs. To this end, we first dissected the migration and activation mode of DCs induced by i.n. flagellin. Mice were i.n. immunized with the fluorescent tracer antigen OVA-AF647 alone or in combination with SF or SFΔ90–97. The proportion of OVA^+ DCs in the CLN of SF-immunized mice was 10-fold higher compared to mice immunized with OVA-AF647 alone or in combination with SFΔ90–97 (Fig. 2a). Correspondingly, the SF significantly upregulated the expression of CCR7, a major chemokine receptor involved in migration of DCs into secondary lymph nodes (Fig. 2b). Meanwhile, in contrast to the OVA alone or with the addition of SFΔ90–97, SF coadministration elevated both CD80 and CD86 expression on CLN DCs (Fig. 2c-d).

To further confirm the ability of flagellin-modulated DCs to promote T cell proliferation and differentiation, $CD11c^+$ DCs were isolated from the CLNs of immunized mice by magnetic sorting and co-cultured with OT-II mice-derived naïve $CD4^+$ T cells. The results demonstrated that DCs from OVA + SF-immunized mice (OVA + SF-DC) significantly enhanced the proliferation of $CD4^+$ T cells compared with those from OVA or OVA + SFΔ90–97 mice (Fig. 2e). In keeping with the results of the transfer and classical i.n. immunization models, OVA + SF-DC induced a higher frequency of IL-17 A^+ cells (Fig. 2f and i). Additionally, the frequency of IFN-γ^+ cells remained similar across the three conditions while IL-4^+ cells were nearly undetectable (Fig. 2g-i).

Taken together, these results suggest that i.n. flagellin promoted the TLR5-dependent migration of activated DCs to the CLN, and these modulated DCs elicit a preferential promotion of the Th17 immune response direction.

TLR5-activated RECs facilitate Th17 cell response by modulation of DCs potential via secretion of GM-CSF

The respiratory epithelium, an immune-active barrier, senses respiratory changes and coordinates with immune cells to fortify frontline defenses [17]. Our and other's previous studies have highlighted the central role of RECs in the intranasal adjuvant effect of flagellin including bridging the activation of respiratory DCs via secretion of GM-CSF [15, 16, 20, 21]. We speculate that RECs indirectly take roles in Th17 differentiation through modulation of DCs via GM-CSF secretion. To clarify this, a BMDC-conditioned and co-cultured model was established (Fig. S3a). In accordance with our previous study, TLR5-activated RECs prompted the secretion of GM-CSF (Fig. 3a) and the activation of BMDCs induced by the conditioned medium from SF-activated RECs was blocked by the addition of GM-CSF neutralizing antibody (Fig. 3b).

Next, we explored the regulatory role of REC-conditioned DCs on the proliferation and differentiation of naive $CD4^+$ T cells. We co-cultured DCs pre-incubated

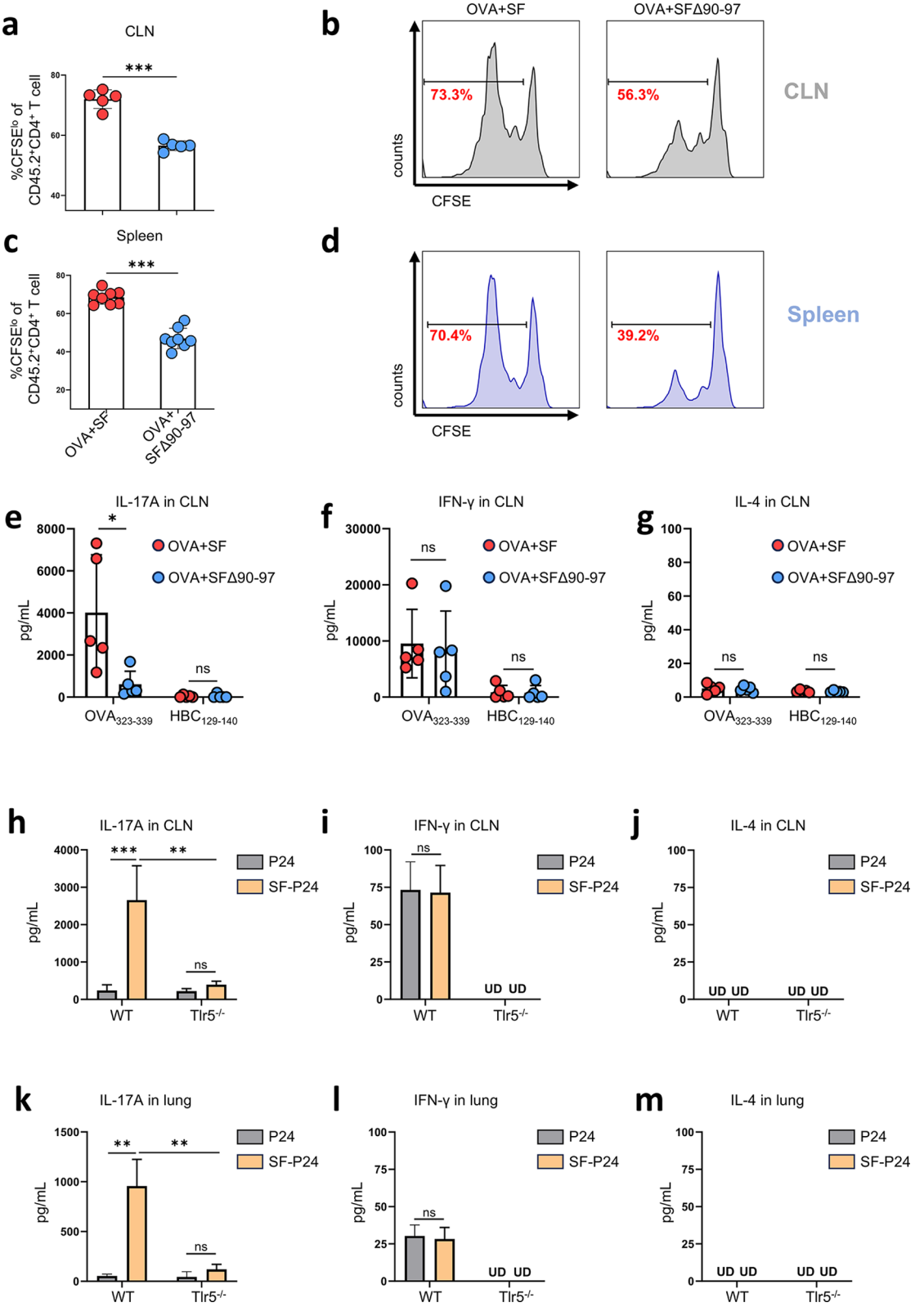

Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 Flagellin as a potent intranasal adjuvant elicits an Th17-enhanced response. OT-II-derived naive $CD4^+$ T cells were sorted and transferred to CD45.1 ($n=10$) mice at $3–5\times10^6$ cells per mouse. The next day, the transferred mice were immunized intranasally with 40 μg OVA in combination with 10 μg SF or SFΔ90–97. After five days of immunization, the spleens and CLNs were collected for flow cytometry assays. Cells from the CLNs of two mice were pooled for one detection. The proliferation of $CD45.2^+CD3^+CD4^+$ T cells from the CLNs (**a**) and spleens (**c**) were analyzed using flow cytometry. (**b**, **d**) The representative dot plots of $CFSE^{low}$ in $CD45.2^+CD3^+CD4^+$ T cells from CLNs and spleens. Lymphocytes from CLNs were plated in 48-well plates. After 48 h of stimulation with the specific peptide $OVA_{323-339}$ and the irrelevant peptide $HBC_{129-140}$, cell supernatants were collected for ELISA (**e**-**g**). C57BL/6 background WT or TLR5 KO (*Tlr5*$^{-/-}$) mice were immunized intranasally with 10 μg P24 or the equivalent molars of SF-P24 three times with three weeks interval. After 7 days of the last immunization, the CLNs (**h**-**j**) and lungs (**k**-**m**) were collected, lymphocytes isolated from the CLNs and lungs ($n=6$) were plated in 96-well plate. Cells were stimulated for 48 h with 2 μg/mL anti-CD28 and the specific antigen P24 or the irrelevant antigen OVA. Subsequently, the cell supernatants were collected for IL-17 A (**h**, **k**), IFN-γ (**I**, **l**) and IL-4 (**j**, **m**) detection by ELISA. Results are expressed as mean ± SD. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, non-significant; UD, undetectable

in REC-conditioned medium with OT-II derived naïve $CD4^+$ T cells and found that REC-SF-conditioned DC (REC-SF-DC) significantly promoted the proliferation of $CD4^+$ T cells as well as Th17 cell differentiation compared to REC-M-conditioned DC (REC-M-DC) and REC-SFΔ90-97-conditioned DC (REC-SFΔ90-97-DC) (Fig. 3c-g). After GM-CSF in REC-SF-conditioned medium was blocked with neutralizing antibody, the proliferation and Th17 differentiation of $CD4^+$ T induced by REC-SF-DC were blunted to the levels similar to REC-M-DC or REC-SFΔ90-97-DC (Fig. 3d and g). However, the addition of α-GM-CSF neutralizing antibody made no difference in the production of Th1 cells (Fig. 3e and g). Simultaneously, Th2 cells remained undetectable in all of the detected settings, irrespective of the types of DCs or the presence and absence of neutralizing antibodies (Fig. 3f-g). The results above show that RECs indirectly dictate flagellin-facilitated Th17 cells immune response by enhancing DCs' potential via GM-CSF.

TLR5-activated RECs directly orchestrate Th17 differentiation

Given that RECs secrete large amounts of soluble cytokines in response to flagellin stimulation, we hypothesized that RECs may be directly involved in the flagellin-facilitated Th17 cells response besides their indirect roles through modulation of DCs. To this end, additional REC-M or REC-SF medium was supplemented in the in vitro REC-DC-$CD4^+$ T co-culture system after the fulfillment of conditioning, peptide loading and extensive washing of BMDCs (Fig. S3b). To block the activating effect of GM-CSF on DCs, the conditioned medium was pre-incubated with 20 μg/mL α-GM-CSF neutralizing antibody for 1 h at 37℃ before adding to the co-culture system. In comparison to REC-SF-DC without supplementation, the addition of REC-M had no significant influence on the proliferation of $CD4^+$ T while the addition of REC-SF even inhibited the proliferation of $CD4^+$ T cell. With respect to Th17 differentiation, the supplementation of REC-M did not modulate REC-SF-DC-driven Th17 differentiation. In contrast, supplementation with REC-SF significantly elevated the frequency of IL-17 A^+ cells. Furthermore, neither REC-M nor REC-SF supplementation influenced Th1 cell frequencies and Th2 cells consistently remain undetectable (Fig. 4a-e). This observation indicates that flagellin-activated RECs release soluble mediators, which are critically implicated in the direct modulation of Th17 cell differentiation.

IL-6 derived from TLR5-activated RECs play a critical role in the Th17 differentiation

Previous studies have shown that a series of cytokines including IL-6, TGF-β, IL-1β, IL-23 are involved in the Th17 induction [4]. To identify the critical cytokines secreted by flagellin-activated RECs which was directly involved in the Th17 differentiation, a differentially expressed genes (DEGs) analysis by transcriptome sequencing of REC was carried out. By sequencing, a total of 629 DEGs ($|\text{Log}_2$ fold change$|\geq1$ and $p<0.05$) were detected between the SF-REC and M-REC cDNA libraries, of which 445 genes were upregulated (higher expression in SF-REC) and 184 genes were downregulated (Fig. 5a). This result indicated the great alterations in gene expression of RECs during flagellin stimulation. KEGG pathway analysis of the upregulated genes revealed that 13 genes were involved in Th17 differentiation. Among them, IL-6, IL-1β, IL-23 and IL-27, which were classified as soluble factors (Fig. 5b). To confirm the data of the expression profiles generated using the RNA-Seq and DEGs analysis, RT-qPCR was applied to examine the expression levels of the 5 upregulated candidate genes listed above. As expected, the RT-qPCR results matched the RNA-seq results. Among them, IL-6 exhibited the most significant change, being up-regulated approximately 8-fold following SF stimulation compared to mock and SFΔ90–97 stimulation (Fig. 5c-f). Correspondingly, SF induced a 4-fold increase in IL-6 secretion from RECs compared to REC-M and REC-SFΔ90–97 (Fig. 5g).

The results above hinted that IL-6 contributed significantly in the direct modulation of Th17 induction by flagellin-activated RECs. To verify the hypothesis, REC-SF-conditioned medium was pre-incubated with 20 μg/mL α-IL-6 neutralizing antibody or isotype control for 1 h at 37℃ before adding to the co-culture system (Fig. S3b). GM-CSF specific neutralizing antibody

was also included during the pre-incubation process to block its' activation of BMDCs. Actually, IL-6 blocking in REC supernatants markedly attenuated Th17 differentiation induced by REC-SF-DC (REC-SF-DC + REC-SF + α-IL-6 vs. REC-SF DC + REC-SF). Specifically, the inhibition of IL-6 resulted in a substantial reduction in the Th17-inducing capacity of REC-SF supplement (REC-SF DC + REC-SF + α-IL-6 vs. REC-SF DC + REC-SF + Ctrl IgG). Unexpectedly, the frequency of IL-17 A^+ cells in the IL-6 neutralization group was even lower than that in the REC-M-DC group (REC-M-DC vs. REC-SF DC + REC-SF + α-IL-6) (Fig. 5h-i). This led us to propose that IL-6 blocking may influence the Th17 production contributed not only by RECs but also by DCs. In other words, IL-6 contributes for flagellin-modulated DCs could potentially drive Th17 differentiation.

In a nutshell, flagellin-stimulated RECs directly regulate Th17 differentiation and IL-6 contributed significantly in this process.

IL-6 secreted from DCs modulated by TLR5-activated RECs is also critical for the Th17 differentiation

As suggested above, IL-6 may be important for flagellin-modulated DCs-enhanced Th17 production. To elucidate this, we first conducted RT-qPCR on REC-conditioned DCs. The data revealed that IL-6 expression was markedly upregulated in REC-SF-DC, showing 2-fold increase relative to REC-M-DC and REC-SFΔ90-97-DC. Following GM-CSF blockade in the supernatant, IL-6 expression in DCs was significantly downregulated, even falling below REC-M-DC levels and exhibiting 4-fold reduction compared to REC-SF-DC (Fig. 6a). To confirm the reliability of the expression profiles generated using the RT-qPCR, ELISA was applied to examine the expression levels of IL-6. REC-conditioned DCs were prepared and stimulated with LPS (10 ng/mL) for 24 h. It was observed that, consistent with RT-qPCR data, BMDC conditioned with REC-SF medium secreted more IL-6 than those conditioned with medium from REC-M or REC-SFΔ90–97 and neutralization of GM-CSF blunted this enhanced production of IL-6 (Fig. 6b).

Combined the data of GM-CSF blocking induced Th17 induction attenuation (Fig. 3d and g) with the IL-6 production decrease of DCs (Fig. 6a-b), it was reasoned that IL-6 is an essential cytokine for flagellin-educated DCs mediated Th17 enhancement. Thus, the neutralizing antibody against IL-6 or the isotype control were added in vitro REC-DC-$CD4^+$ T co-culture system (Fig. S3c). Indeed, neutralization of IL-6 led to a pronounced reduction in the frequency of Th17 cells (REC-SF-DC + Ctrl IgG vs. REC-SF-DC + α-IL-6) (Fig. 6c-d). We then tested whether IL-6 was also required for the differentiation of Th17 in the ex vivo DC-$CD4^+$ T co-culture system. Consistent with the REC-SF-conditioned DC, DCs isolated from the CLNs of OVA + SF-DC immunized mice produced significantly more IL-6 in comparison with OVA-DC or OVA + SFΔ90-97-DC (Fig. 6e). Moreover, similar to what we observed in the in vitro REC-DC-$CD4^+$ T co-culture system, blocking IL-6 signaling in the in vitro co-culture system also suppressed the induction of Th17 cells (Fig. 6f-g).

All together, these results suggested that IL-6 derived from flagellin-modulated DCs is essential for the differentiation of Th17 cells.

Discussion

In this study, we aimed to investigate the effects and the underlying mechanisms of intranasal adjuvant flagellin modulating $CD4^+$ T cell response. In experimental systems involving adoptive transfer and classical immunization model, we determined flagellin facilitated a Th17 differentiation. Furthermore, we highlighted the essential roles of RECs in this process. RECs synchronize flagellin-mediated Th17 differentiation by not only modulating DCs' potential via GM-CSF but also directly participating through IL-6 production (Fig. 7).

Various mucosal adjuvants exhibit distinct mechanisms and advantages in promoting $CD4^+$ T cell responses. For instance, STING agonists bolster immune defense against viral and cellular pathogens by inducing Th1 responses through the activation of the STING-IRF3 pathway [22]. Protein-based mucosal adjuvants, such as Escherichia coli heat-stable toxin and cholera toxin; nucleic acid-based adjuvants, such as CpG oligodeoxynucleotides; and natural product-based adjuvants, such as chitosan, can promote Th1, Th2, or Th17-type cellular responses by activating antigen-presenting cells that secrete a diverse array of cytokines [23–25].

Our previous study has reported that flagellin induced mixed Th1/Th17 response with the production of both IFN-γ and IL-17 A following intranasal immunization with P-KFD1, a chimeric protein of RSV phosphoprotein (P) and the E.-coli-K12-strain-derived flagellin variant KFD1. Moreover, these P-specific $CD4^+$ T cells either reside in or migrate to the respiratory tract and mediate protection against RSV infection [10]. Two most recent studies by two independent research groups both found that flagellin promoted Th17-biased response via i.n. administration of chimeric protein of flagellin and pathogen virulence factor and conferred immune-protection against SARS-CoV-2 and pneumococcal, respectively [6, 11]. In this work, two mice models, adoptive transfer model and classical i.n. model were used. We found that i.n. immunization of antigen alone would induce antigen-specific IFN-γ and IL-17 A production but without IL-4. However, irrespective the formulation of antigen and flagellin, mixed or chimeric, flagellin preferentially enhanced IL-17 A production while had no effect on

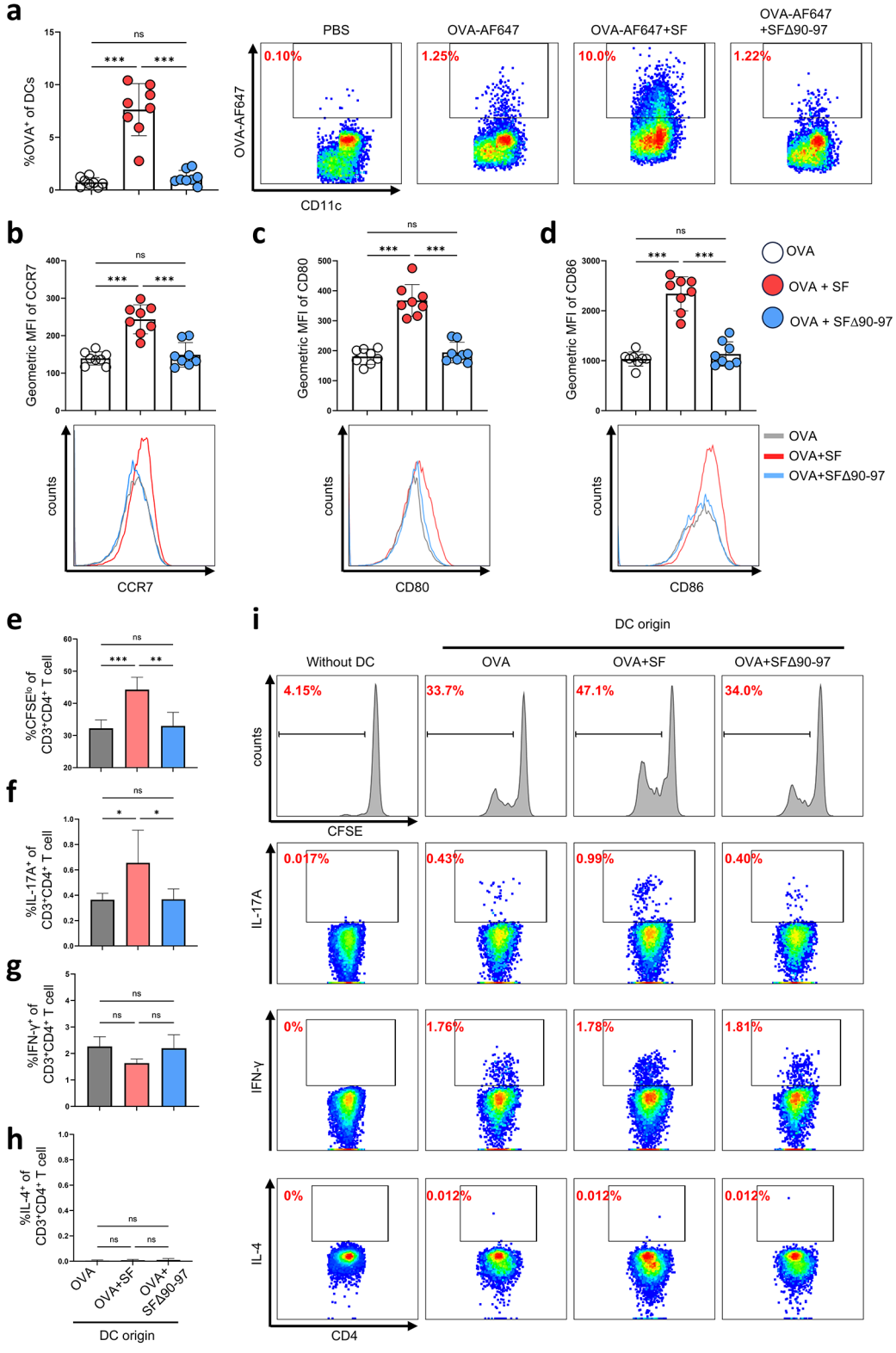

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Flagellin facilitates the differentiation of $CD4^+$ T cells through the modulation of DCs function. (**a**-**d**) C57BL/6 (WT, $n=8$) mice were immunized intranasally with PBS, 70 μg OVA-AF647 alone, or in combination with 10 μg SF or SFΔ90–97. Mouse CLNs were isolated 18 h after immunization, digested and milled to prepare cells for flow cytometry assay. The proportion of OVA^+ DCs in the total DC population (**a**) and the expression of CCR7 (**b**), CD80 (**c**), and CD86 (**d**) on the DCs were analyzed using flow cytometry. The upper panel of B-D, the MFI on DC population; the lower panel of b-d, representative histograms. (**e**-**i**) C57BL/6 mice were immunized intranasally with 40 μg OVA alone or in combination with 10 μg SF or SFΔ90–97. 18 h later, the CLNs were isolated, pooled, digested. $CD11c^+$ DCs were sorted using magnetic beads, and then co-cultured with OT-II-derived naive $CD4^+$ T cells at a ratio of 1:5. After five days of co-culture, the cells were transferred to another 96-well plates pre-coated with 1 μg/mL anti-CD3 and stimulated for 5 h with BFA and monensin. Flow cytometry assay was performed to assess the proliferation (**e**) and differentiation of $CD3^+CD4^+$ T cells (**f**-**h**). (**i**) The representative dot plots of $CFSE^{low}$, IL-17 A^+, IFN-γ and IL-4^+ in $CD4^+$ T cells. Results are expressed as mean ± SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, nonsignificant; UD, undetectable

IFN-γ or IL-4 production against cognate antigen. Our data suggested that i.n. immunization of antigen alone induces mixed Th1/Th17 response, which may be determined by the characteristics of respiratory DCs. Actually, respiratory DCs drive naïve $CD4^+$ T cells differentiate into both Th1 and Th17 as illustrated in OVA-DC in Fig. 2f-h. Moreover, the intranasal adjuvant flagellin have the capacity to promote Th17 response uniquely but do not affect Th1 and Th2. Th1 and Th17 are important for the control of respiratory infection [6, 10, 26–32], but a mass of studies showed that Th2 was blamed for allergy and asthma [33]. Therefore, the unique Th subtype immune-modulation feature of flagellin strengthen its adjuvant effect and safety again, which pave the way for its application used as an intranasal adjuvant.

Th17 cells are a distinct subset of $CD4^+$ T helper cells characterized by the specific expression of the transcription factor RoRγt and the secretion of IL-17. Th17 cells have been demonstrated to play a crucial role in protecting mucosal surfaces against fungal, bacterial and certain virus such as respiratory syncytial virus infections [6, 10, 26–31]. Vaccines based on Th17 cells provide robust protection against RSV, influenza and secondary bacterial infections [6, 34, 35]. The IL-17 cytokine family includes members such as IL-17 A and IL-17 F, with IL-17 A being the most prominent. IL-17 A enhances the recruitment and activation of neutrophils to the site of infection by inducing the expression of chemokines CXCL1 and CXCL2 in epithelial and other immune cells. Moreover, IL-17 amplifies the localized inflammatory response, extends neutrophil survival, and effectively establishes a robust local immune defense barrier.

It is well known that Immunoglobulin A (IgA) is a critically important effector molecule at the mucosal surface. In addition to its significant function in defense against mucosal pathogen infection, Th17 also plays an important role in mucosal IgA induction and secretion. As the most abundant Th subtype in the intestine, through upregulation of IgA isotype switch and polymeric Ig receptor (pIgR) expression, Th17 contribute to intestinal homeostasis [36, 37]. Similar to the intestine, intranasal administration of IL-17 upregulates epithelial pIgR expression and promotes influx of B cells and IgA into the lungs [38]. Correspondingly, vaccine induced cognate antigen-specific Th17 cells are recruited to the lung parenchyma where they transform into resident lymphocytes that persist in the lung tissue and facilitate the accumulation of IgA^+ B cells and rapidly increasing IgA levels. Accordingly, depletion of IL-17 abrogates the recruitment of T cells to the lungs and significantly reduces the local IgA response [7]. As an intranasal adjuvant, flagellin promotes robust IgA induction in serum and mucosal secretions and conferred protection against pathogens [14]. A recent study showed that flagellin-promoted vaccine-specific IgA response in serum and mucosal fluid exhibited a robust positive correlation with the Th17 response [11]. Further studies will be carried out to ascertain the direct roles of flagellin promoted Th17 response in its enhanced antibody especially IgA production.

DCs are highly specialized antigen-presenting cells that provide $CD4^+$ T cells with dual activation signals through the presentation of antigens via MHC-II molecules and the provision of co-stimulatory signals. Additionally, DCs orchestrate $CD4^+$ T cell differentiation through the secretion of cytokines. Our previous study has demonstrated that flagellin does not impact antigen uptake by DCs. However, flagellin enhances the activation and migration of antigen-loaded respiratory DCs to the draining lymph nodes in a TLR5-dependent manner [39]. Due to the low expression of TLR5, respiratory DCs were indirectly activated by flagellin [15, 16]. RECs, which act as a protective barrier between external and internal environments, express a wide range of toll-like receptors and secrete numerous soluble factors upon exposure to exogenous stimuli. Consistently, our transcriptomic analysis revealed that RECs significantly upregulated the expression of various cytokines and chemokines following SF stimulation (Fig. 5a). Among these, GM-CSF, a hematopoietic growth factor that promotes the maturation of DCs, was evidenced as a key cytokines in flagellin mediated indirect action of DCs by our and other's previous work [15, 20]. Moreover, multiple genes associated with Th17 differentiation were also upregulated in RECs. We hypothesize that RECs take essential roles in flagellin-induced Th17 differentiation through both direct and indirect regulatory pathways. Using an in vitro co-culture system, we observed that DCs educated by conditioned

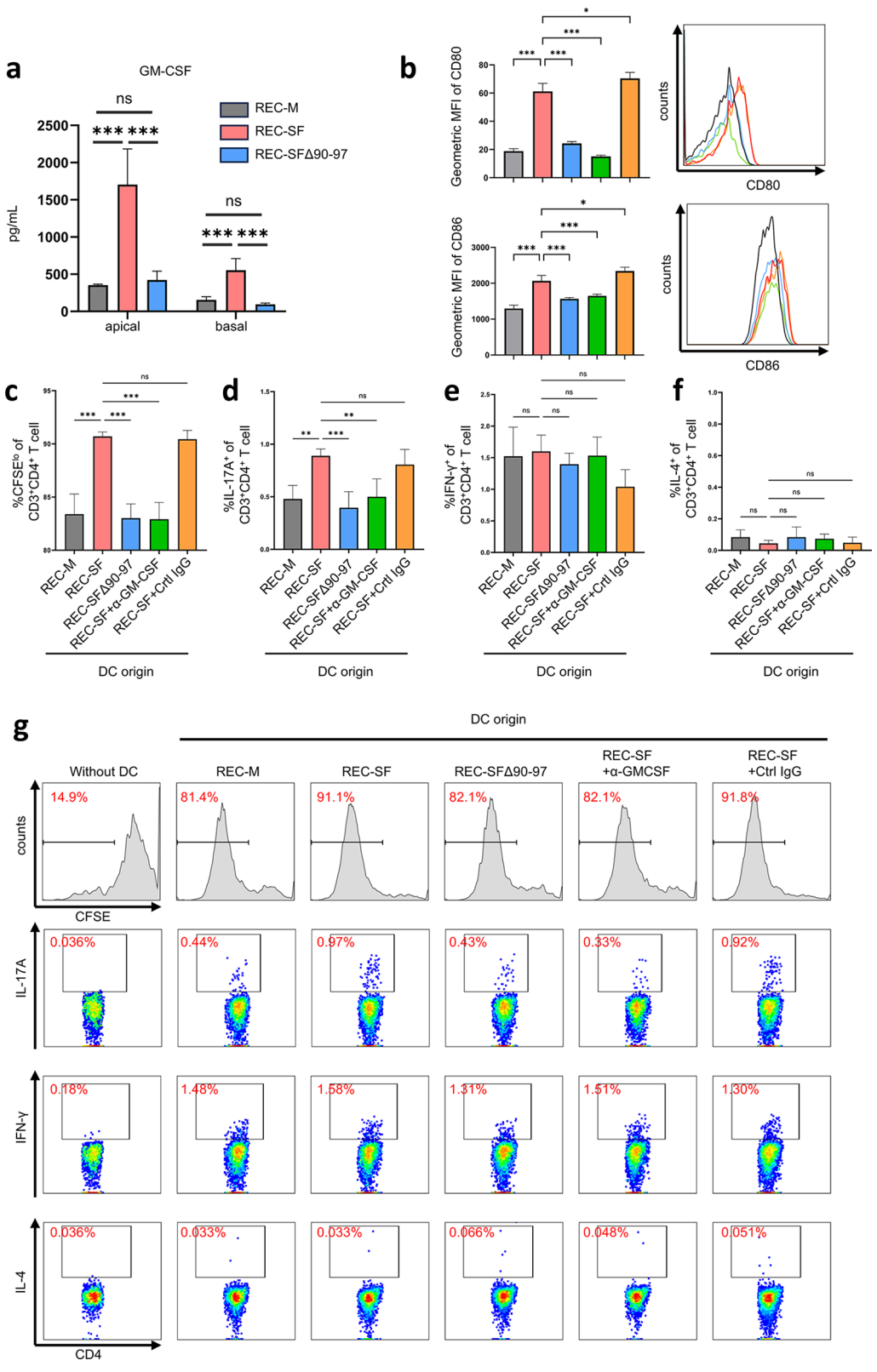

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 TLR5-activated RECs indirectly modulate Th17 differentiation. RECs were cultured in the transwell chambers for two weeks. After polarization, the cells were stimulated with medium supplemented with PBS or with a final concentration of 1 μg/mL SF or SFΔ90–97 for 24 h. Apical and basal media were then collected for ELISA (**a**). *Tlr5*$^{-/-}$ BMDCs were stimulated with REC-apical conditioned medium for 24 h, and CD80/CD86 expression (**b**) was subsequently detected by flow cytometry assay. After treating with REC-derived conditioned medium and $OVA_{323-339}$ peptide loading, BMDCs were co-cultured with OT-II-derived naive $CD4^+$ T cells at a ratio of 1:20. Five days later, the cells were collected and transferred to another 96-well plates pre-coated with 1 μg/mL anti-CD3, then stimulated with BFA and monensin for 5 h. The proliferation (**c**) and differentiation (**d**-**f**) were detected by flow cytometry assay. (**g**) The representative dot plots of $CFSE^{low}$, IL-17 A^+, IFN-γ and IL-4^+ in $CD4^+$ T cells. Results are expressed as mean ± SD. *, $P < 0.05$: **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant; UD, undetectable

medium from flagellin activated RECs enhanced Th17 cells production and this effect was blunted with the addition of α-GM-CSF neutralizing antibody (Fig. 3). Furthermore, REC-SF-conditioned medium supplement experiments indicated that RECs also directly participate in Th17 differentiation (Fig. 4). Taken together, our findings suggest that flagellin activated RECs synergize the direct and indirect pathways to promote Th17 induction.

REC sequencing results regarding Th17 differentiation revealed that SF significantly upregulated a spectrum of Th17 differentiation-related genes in RECs, including IL-6, IL-1β, IL-23 and IL-27. Among these upregulated genes, IL-6, a critical mediator in the initiation of Th17 differentiation, facilitated the enhanced expression of RoRγT in $CD4^+$ T cells, while IL-1β and IL-23 contributed to the sustained maintenance of the Th17 phenotype. IL-27 is recognized for its role in promoting Treg cell expansion, thereby suppressing Th17 differentiation. However, in the absence of IL-27, Th17 hyperactivity is observed, highlighting its pivotal role in regulating Th17 homeostasis and preventing excessive Th17-mediated responses [40]. Our RT-qPCR data indicated that IL-6 expression was most markedly upregulated (Fig. 5c), leading us to infer that IL-6 is likely a pivotal cytokine directly involved in REC-mediated Th17 differentiation. In the conditioned medium supplement experiments, neutralization of IL-6 in REC-SF markedly impaired the Th17 inducing capacity of REC-SF-DC, with the frequency of IL-17 A^+ cells falling even below those observed in REC-M-DC (Fig. 5f-g). We propose that IL-6 neutralization in REC-SF may not only attenuate the regulatory influence of REC-SF on Th17 differentiation but may also inhibit the role of DC-derived IL-6 in modulating this process.

GM-CSF, functioning as a critical factor in flagellin-secreted cytokines to modulate DCs function, initiates the downstream JAK2-STAT5 signaling cascade by binding to the GM-CSF receptor (GM-CSFR) on the surface of DCs. This activation enhances the expression of co-stimulatory molecules CD80 and CD86, while also triggering the production of inflammatory signaling molecules including IL-6 [41]. Both ex vivo and in vitro models demonstrated a marked elevation in IL-6 secretion by DCs upon SF stimulation (Fig. 6). Notably, the blockade of IL-6 impaired the Th17 inducing ability of flagellin-modulated DCs, underscoring the pivotal role of DC-derived IL-6 as a key cytokine in this process. IL-6, a crucial determinant in Th17 differentiation, not only initiates the transcriptional machinery necessary for Th17 lineage commitment through STAT3 activation but also plays an essential role in sustaining the Th17 cell phenotype. Specifically, IL-6 augments the responsiveness of Th17 cells to IL-23 by upregulating IL-23R expression, thereby reinforcing its role in the preservation of the Th17 phenotype [42]. Collectively, our findings elucidate a dual role of RECs in synchronizing flagellin-mediated Th17 response. On one hand, TLR5-activated RECs modulate DC function via GM-CSF secretion, which amplifies IL-6 production by DCs, thereby indirectly promoting Th17 differentiation; on the other hand, TLR5-induced REC-derived IL-6 is directly involved in facilitating Th17 differentiation.

The ability of flagellin to function as a potent mucosal adjuvant makes it a promising candidate for mucosal vaccines due to its easy preparation, high potential for modification, and low toxicity. Here, we identified the Th17 enhancing capacity of flagellin. Taking the instrumental roles of Th17 for protecting the respiratory mucosa against bacterial, fungal and viral infection into account, flagellin is a favorable vaccine scaffold which not only harbor excellent adjuvanticity but also enhance the immunogenicity of immunogen especially for low-molecular weight molecules by fusion. Furthermore, we also shed some light on the underlying mechanism by highlighting the essential roles of RECs, which will contribute to effectively harnessing and regulating the Th17 response in the design of flagellin-based mucosal vaccines.

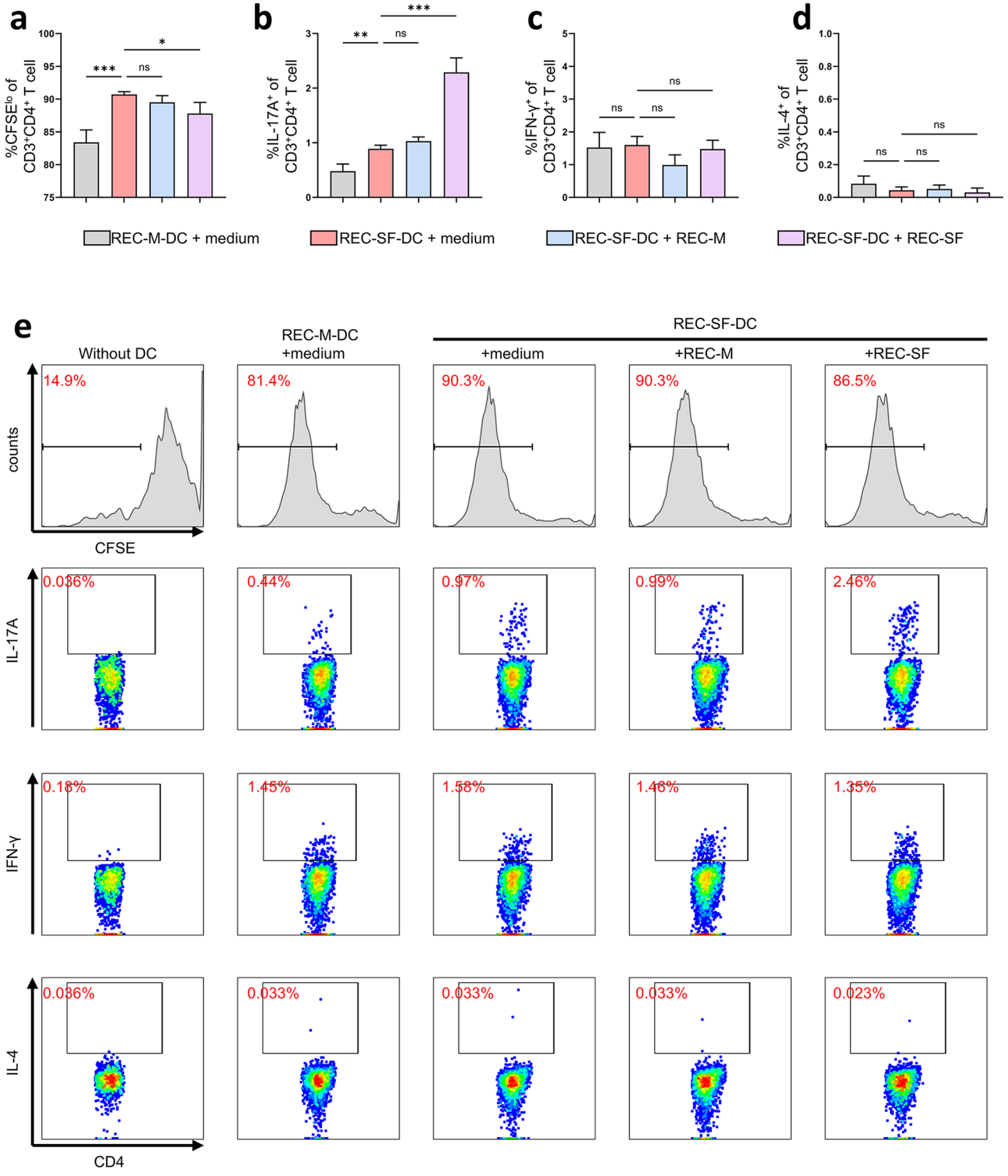

Fig. 4 TLR5-activated RECs directly orchestrate Th17 differentiation. REC-M and REC-SF-conditioned medium were supplemented into the *Tlr5*$^{-/-}$ BM-DCs-CD4^{+} T co-culture system. Five days later, cells were collected, restimulated with plate-coated anti-CD3 in the addition of BFA and monensin. The proliferation (**a**) and differentiation (**b**-**d**) of CD4^{+} T cells were analyzed by flow cytometry. (**e**) The representative dot plots of CFSE^{low}, IL-17 A^{+}, IFN-γ and IL-4^{+} in CD4^{+} T cells. Results are expressed as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant; UD, undetectable

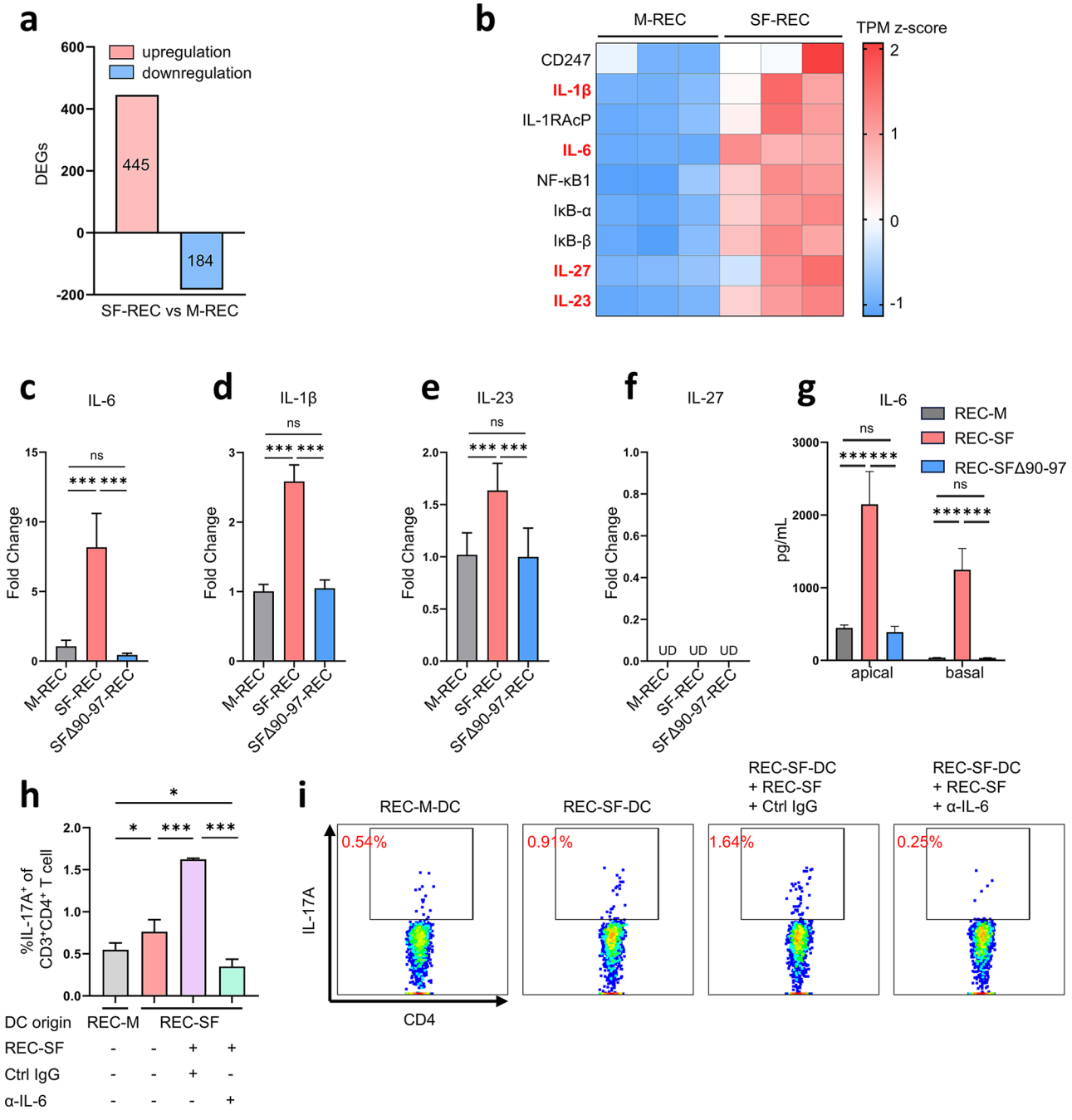

Fig. 5 Mechanistic insights into the regulation of Th17-enhanced immune responses by TLR5-activated RECs. (**a**-**b**) RNA-seq data of polarized RECs. RECs were cultured in the transwell chambers for two weeks. After polarization, the cells were stimulated with medium supplemented with PBS or with a final concentration of 1 μg/mL SF for 3 h. Then cells were collected for RNA sequencing. Differential gene counts were analyzed between SF-REC and M-REC (**a**). A heatmap of REC sequencing data illustrates the upregulation of genes involved in Th17 differentiation (**b**) Fluorescence quantitative PCR of Th17 differentiation-related genes (**c**-) The polarized RECs were stimulated with medium supplemented with PBS or a final concentration of 1 μg/mL SF or SFΔ90–97 for 24 h, and apical and basal medium was collected for IL-6 detection by ELISA (**g**). (**h**-**i**) Influence of IL-6 blockade on DC induced $CD4^+$ T cell proliferation and differentiation. *Tlr5*$^{-/-}$ BMDCs pre-stimulated with REC-SF-conditioned medium were co-cultured with naïve OT-II $CD4^+$ T cells with the addition of REC-SF-conditioned medium in the absence or presence of IL-6 neutralizing antibody or isotype control. 5 days later, the co-cultures were collected, restimulated with plate-coated anti-CD3 in the addition of BFA and monensin. The production of Th17 cells were analyzed by flow cytometry. The frequencies (**h**) and representative dot plots (**i**) of IL-17 A^+ in $CD4^+$ T cells. Results are expressed as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant; UD, undetectable

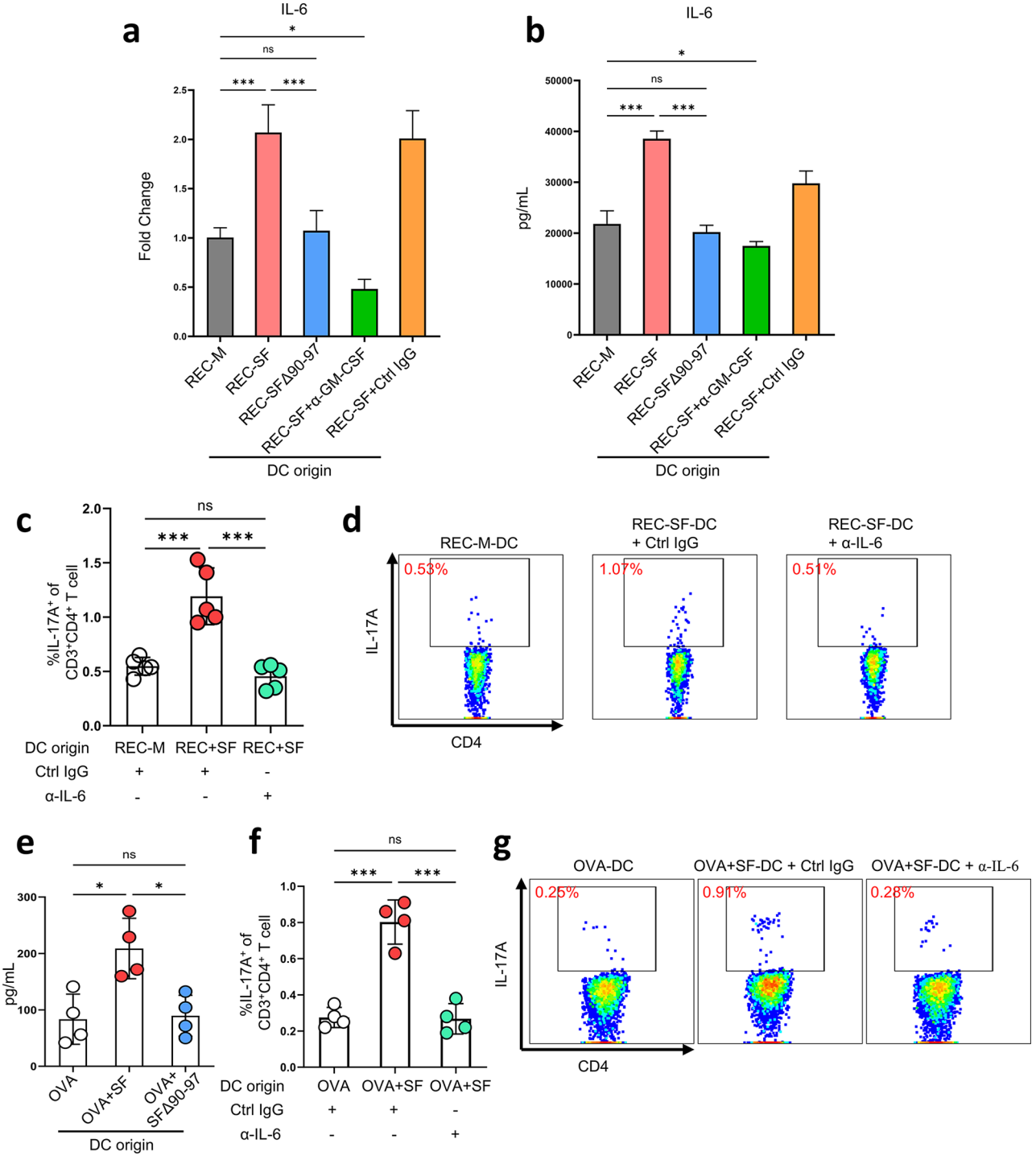

Fig. 6 (See legend on next page.)

(See figure on previous page.)

Fig. 6 Regulatory pathways governing flagellin-modulated DC-driven Th17-enhanced immune responses. (**a**) RT-qPCR assay of REC-conditioned DCs. BMDCs derived from *Tlr5*$^{-/-}$ mice were stimulated in either REC-M or REC-SF for 24 h. Prior to stimulation, REC-SF was pre-incubated with α-GM-CSF or Ctrl IgG at 37 °C for one hour. After stimulation, BMDCs were harvested, and total cellular RNA was extracted and reverse-transcribed into cDNA for subsequent RT-qPCR analysis. (**b**) IL-6 produced by REC-conditioned BMDCs. *Tlr5*$^{-/-}$ BMDCs stimulated with conditioned medium alone or in the presence of GM-CSF neutralizing antibody or isotype control were stimulated with 10 ng/mL LPS for 24 h and IL-6 in cell supernatants was determined by ELISA assay. (**c**-**d**) Influence of IL-6 blockade on DC induced Th17 production in an in vitro co-culture system. *Tlr5*$^{-/-}$ BMDCs stimulated with conditioned medium were co-cultured with naïve OT-II CD4$^+$ T cells in the absence or presence of IL-6 neutralizing antibody or isotype control. Five days later, the co-cultures were collected, restimulated with plate-coated anti-CD3 in the addition of BFA and monensin. The production of Th17 cells were analyzed by flow cytometry. The frequencies (**c**) and representative dot plots (**d**) of IL-17 A$^+$ in CD4$^+$ T cells. (**e**) IL-6 produced by CLN-DCs. DCs magnetically separated from CLNs of mice intranasally immunized with OVA alone or in the presence of SF or SF△90–97 were stimulated with 10 ng/mL LPS for 24 h and IL-6 in cell supernatants was determined by ELISA assay. (**f**-**g**) Influence of IL-6 blockade on DC induced Th17 production in an ex vivo co-culture system. DCs magnetically separated from CLNs of mice intranasally immunized with OVA alone or in combination with SF were co-cultured with naïve OT-II CD4$^+$ T cells in the absence or presence of IL-6 neutralizing antibody or isotype control. 5 days later, the co-cultures were collected, restimulated with plate-coated anti-CD3 in the addition of BFA and monensin. he production of Th17 cells were analyzed by flow cytometry. The frequencies (**f**) and representative dot plots (**g**) of IL-17 A$^+$ in CD4$^+$ T cells. Results are expressed as mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant; UD, undetectable

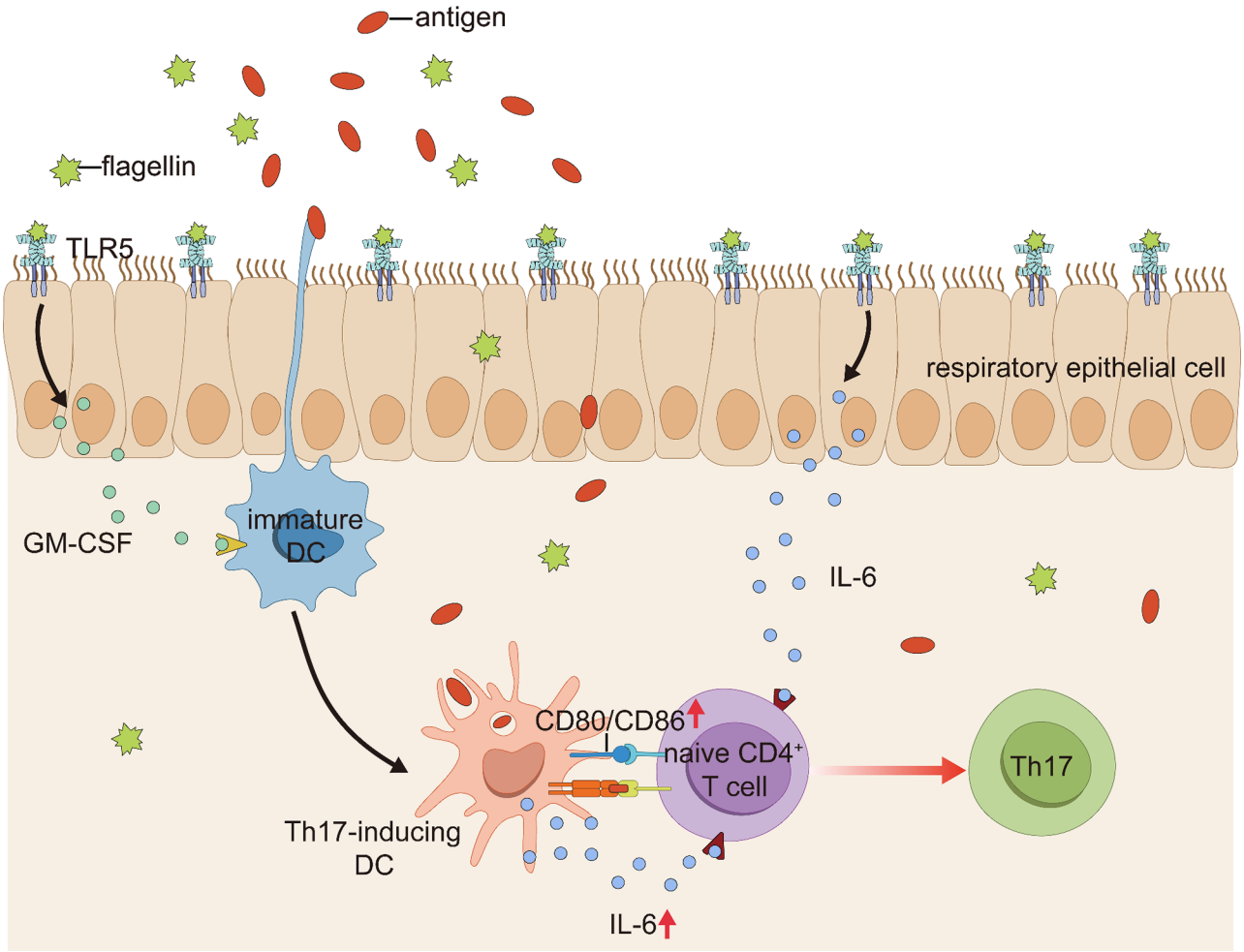

Fig. 7 The mechanistic map of the Th17 response triggered by the nasal mucosal adjuvant flagellin. Following intranasal administration, flagellin binds to the TLR5 receptor on the surface of RECs, thereby initiating the TLR5 signaling cascade. This signaling promotes the release of soluble cytokines such as GM-CSF and IL-6. GM-CSF secreted by RECs modulates the activation and function potential of respiratory DCs, evidenced by the upregulation of co-stimulatory molecules CD80/CD86 and IL-6, ultimately transforming them into Th17-inducing DCs. These specialized DCs capture and present antigens to naïve CD4$^+$ T cells through MHC-II interactions, delivering the primary activation signal. The co-stimulatory molecules CD80/CD86 on DCs provide a secondary signal, while the secretion of IL-6 by Th17-inducing DCs directs the polarization of naïve CD4$^+$ T cells toward the Th17 lineage. Additionally, IL-6 released by RECs acts synergistically to enhance Th17 differentiation, further amplifying the immune response initiated by DCs

Abbreviations

REC	Respiratory epithelial cell
DC	Dendritic cell
PAMP	Pathogen-associated molecular pattern
Th cell	T helper cell
SPF	Specific pathogen-free
WT	Wild-type
BMDC	Bone marrow dendritic cell
DEGs	Differentially expressed genes
DAVID	Database for Annotation, Visualization, and Integrated Discovery
IgA	Immunoglobulin A
pIgR	Polymeric Ig receptor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-025-03186-w.

Supplementary Material 1

Author contributions

S.H. and X.L. contributed equally to the conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing—review and editing. Y.C., M.M., and J.L. contributed to the investigation and writing—review and editing. K.Z., L.W., Z.Z., and X.W. contributed to the conceptualization, validation, and writing—review and editing. C.T. contributed to the conceptualization, supervision, validation and writing–review & editing. M.Z. contributed to the conceptualization, project administration, supervision, validation, writing—original draft, and writing—review and editing. All of the authors gave final approval and agreed to be accountable for all aspects of the work.

Funding

This work was supported by the National Natural Science Foundation of China (Nos. 81971568, 81302609 and 82070810), the Scientific research plan of Education Department of Hubei Province (No. B2023016), "The 14th Five Year Plan" Hubei Provincial advantaged characteristics disciplines (groups) project of Wuhan University of Science and Technology (No. 2023C0307) and Innovation research Fund of Wuchang Hospital (No. WCYY2022G01).

Data availability

The datasets of RNAseq were uploaded to the SRA database. The project ID is PRJNA1198122.

Declarations

Ethical approval

This study was approved by the Institutional Animal Care and Use Committee of Wuhan University of Science and Technology (approval number 202179) and adhered to Regulations for the Administration of Affairs Concerning Experimental Animals in China and the Guidelines for Animal Care and Use, Wuhan University of Science and Technology.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 7 October 2024 / Accepted: 10 March 2025
Published online: 17 March 2025

References

1. Lavelle EC, Ward RW. Mucosal vaccines - fortifying the frontiers. Nat Rev Immunol. 2022;22(4):236–50.
2. Fujimoto K, Uematsu S. Development of prime-boost-type next-generation mucosal vaccines. Int Immunol. 2020;32(9):597–603.
3. Kaur A, Baldwin J, Brar D, Salunke DB, Petrovsky N. Toll-like receptor (TLR) agonists as a driving force behind next-generation vaccine adjuvants and cancer therapeutics. Curr Opin Chem Biol. 2022;70:102172.
4. Saravia J, Chapman NM, Chi H. Helper T cell differentiation. Cell Mol Immunol. 2019;16(7):634–43.
5. Sun L, Su Y, Jiao A, Wang X, Zhang B. T cells in health and disease. Signal Transduct Target Therapy. 2023;8(1):235.
6. Liu X, Van Maele L, Matarazzo L, Soulard D, Alves Duarte da Silva V, de Bakker V, Denereaz J, Bock FP, Taschner M, Ou J et al. A conserved antigen induces respiratory Th17-mediated broad serotype protection against pneumococcal superinfection. *Cell host & microbe* 2024, 32(3):304–314 e308.
7. Amezcua Vesely MC, Pallis P, Bielecki P, Low JS, Zhao J, Harman CCD, Kroehling L, Jackson R, Bailis W, Licona-Limon P, et al. Effector T(H)17 cells give rise to Long-Lived T(RM) cells that are essential for an immediate response against bacterial infection. Cell. 2019;178(5):1176–e11881115.
8. Rydyznski Moderbacher C, Kim C, Mateus J, Plested J, Zhu M, Cloney-Clark S, Weiskopf D, Sette A, Fries L, Glenn G et al. NVX-CoV2373 vaccination induces functional SARS-CoV-2-specific CD4 + and CD8 + T cell responses. *The Journal of clinical investigation* 2022, 132(19).
9. Zhao L, Wang X, Li Z. A novel chimeric Recombinant FliC-Pgp3 vaccine promotes Immunoprotection against chlamydia muridarum infection in mice. Int J Biol Macromol. 2024;258(Pt 1):128723.
10. Zhao B, Yang J, He B, Li X, Yan H, Liu S, Yang Y, Zhou D, Liu B, Fan X, et al. A safe and effective mucosal RSV vaccine in mice consisting of RSV phosphoprotein and Flagellin variant. Cell Rep. 2021;36(3):109401.
11. Li X, Xu M, Yang J, Zhou L, Liu L, Li M, Wang S, Liu MQ, Huang Z, Zhang Z, et al. Nasal vaccination of triple-RBD scaffold protein with Flagellin elicits long-term protection against SARS-CoV-2 variants including JN.1. Signal Transduct Target Therapy. 2024;9(1):114.
12. Wang S, Zhang G, Cui Q, Yang Y, Wang D, Liu A, Xia Y, Li W, Liu Y, Yu J. The DC-T cell axis is an effective target for the treatment of non-small cell lung cancer. Immun Inflamm Dis. 2023;11(11):e1099.
13. Flores-Langarica A, Cook C, Müller Luda K, Persson EK, Marshall JL, Beristain-Covarrubias N, Yam-Puc JC, Dahlgren M, Persson JJ, Uematsu S, et al. Intestinal CD103(+)CD11b(+) cDC2 conventional dendritic cells are required for primary CD4(+) T and B cell responses to soluble Flagellin. Front Immunol. 2018;9:2409.
14. Cui B, Liu X, Fang Y, Zhou P, Zhang Y, Wang Y. Flagellin as a vaccine adjuvant. Expert Rev Vaccines. 2018;17(4):335–49.
15. Cao Y, Zhang E, Yang J, Yang Y, Yu J, Xiao Y, Li W, Zhou D, Li Y, Zhao B, et al. Frontline science: nasal epithelial GM-CSF contributes to TLR5-mediated modulation of airway dendritic cells and subsequent IgA response. J Leukoc Biol. 2017;102(3):575–87.
16. Fougeron D, Van Maele L, Songhet P, Cayet D, Hot D, Van Rooijen N, Mollenkopf HJ, Hardt WD, Benecke AG, Sirard JC. Indirect Toll-like receptor 5-mediated activation of conventional dendritic cells promotes the mucosal adjuvant activity of Flagellin in the respiratory tract. Vaccine. 2015;33(29):3331–41.
17. Hewitt RJ, Lloyd CM. Regulation of immune responses by the airway epithelial cell landscape. Nat Rev Immunol. 2021;21(6):347–62.
18. Paplinska-Goryca M, Misiukiewicz-Stepien P, Nejman-Gryz P, Proboszcz M, Mlacki M, Gorska K, Krenke R. Epithelial-macrophage-dendritic cell interactions impact alarmins expression in asthma and COPD. Clin Immunol (Orlando Fla). 2020;215:108421.
19. Shouib R, Eitzen G. Cdc42 regulates cytokine expression and trafficking in bronchial epithelial cells. Front Immunol. 2022;13:1069499.
20. Vijayan A, Van Maele L, Fougeron D, Cayet D, Sirard JC. The GM-CSF released by airway epithelial cells orchestrates the mucosal adjuvant activity of Flagellin. J Immunol (Baltimore Md: 1950). 2020;205(10):2873–82.
21. Van Maele L, Fougeron D, Janot L, Didierlaurent A, Cayet D, Tabareau J, Rumbo M, Corvo-Chamaillard S, Boulenouar S, Jeffs S, et al. Airway structural cells regulate TLR5-mediated mucosal adjuvant activity. Mucosal Immunol. 2014;7(3):489–500.
22. Rezabakhsh A, Sadaie MR, Ala A, Roosta Y, Habtemariam S, Sahebnasagh A, Khezri MR. STING agonists as promising vaccine adjuvants to boost immunogenicity against SARS-related coronavirus derived infection: possible role of autophagy. Cell Communication Signaling: CCS. 2024;22(1):305.
23. Duan Q, Xia P, Nandre R, Zhang W, Zhu G. Review of newly identified functions associated with the Heat-Labile toxin of enterotoxigenic Escherichia coli. Front Cell Infect Microbiol. 2019;9:292.
24. Li Z, Li Y, Wang Y, Hou Y, Cao H, Wu X, Hu S, Long D. Intranasal immunization with a rNMB0315 and combination adjuvants induces protective immunity

against neisseria meningitidis serogroup B in mice. Int Immunopharmacol. 2021;93:107411.
25. Wang Y, Liu S, Li B, Sun X, Pan Q, Zheng Y, Liu J, Zhao Y, Wang J, Liu L, et al. A novel CpG ODN compound adjuvant enhances immune response to Spike subunit vaccines of Porcine epidemic diarrhea virus. Front Immunol. 2024;15:1336239.
26. Curtis MM, Way SS. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. Immunology. 2009;126(2):177–85.
27. Dubin PJ, Kolls JK. Th17 cytokines and mucosal immunity. Immunol Rev. 2008;226:160–71.
28. Gaffen SL, Moutsopoulos NM. Regulation of host-microbe interactions at oral mucosal barriers by type 17 immunity. Sci Immunol 2020, 5(43).
29. Hajishengallis G. Delving into the adytum of the IL-17 defense pathway. Cell Host Microbe. 2023;31(10):1599–601.
30. Kolls JK, Khader SA. The role of Th17 cytokines in primary mucosal immunity. Cytokine Growth Factor Rev. 2010;21(6):443–8.
31. Mills KH. Induction, function and regulation of IL-17-producing T cells. Eur J Immunol. 2008;38(10):2636–49.
32. Finn CM, McKinstry KK. Ex pluribus unum: the CD4 T cell response against influenza A virus. Cells 2024, 13(7).
33. Turner MC, Radzikowska U, Ferastraoaru DE, Pascal M, Wesseling P, McCraw A, Backes C, Bax HJ, Bergmann C, Bianchini R, et al. AllergoOncology: biomarkers and refined classification for research in the allergy and glioma nexus-A joint EAACI-EANO position paper. Allergy. 2024;79(6):1419–39.
34. Li Z, Cai H, Li Z, Ren L, Ma X, Zhu H, Gong Q, Zhang H, Gu Z, Luo K. A tumor cell membrane-coated self-amplified nanosystem as a nanovaccine to boost the therapeutic effect of anti-PD-L1 antibody. Bioact Mater. 2023;21:299–312.
35. Raeven RHM, Rockx-Brouwer D, Kanojia G, van der Maas L, Bindels THE, Ten Have R, van Riet E, Metz B, Kersten GFA. Intranasal immunization with outer membrane vesicle pertussis vaccine confers broad protection through mucosal IgA and Th17 responses. Sci Rep. 2020;10(1):7396.
36. Cao AT, Yao S, Gong B, Elson CO, Cong Y. Th17 cells upregulate polymeric Ig receptor and intestinal IgA and contribute to intestinal homeostasis. J Immunol (Baltimore Md: 1950). 2012;189(9):4666–73.
37. Hirota K, Turner JE, Villa M, Duarte JH, Demengeot J, Steinmetz OM, Stockinger B. Plasticity of Th17 cells in Peyer's patches is responsible for the induction of T cell-dependent IgA responses. Nat Immunol. 2013;14(4):372–9.
38. Jaffar Z, Ferrini ME, Herritt LA, Roberts K. Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. J Immunol (Baltimore Md: 1950). 2009;182(8):4507–11.
39. Li X, Cao Y, Mou M, Li J, Huang S, Zhang E, Yan H, Yang J, Zhong M. Enhanced TLR5-dependent migration and activation of antigen-loaded airway dendritic cells by Flagellin. J Leukoc Biol. 2023;113(6):567–76.
40. Chen X, Deng R, Chi W, Hua X, Lu F, Bian F, Gao N, Li Z, Pflugfelder SC, de Paiva CS, et al. IL-27 signaling deficiency develops Th17-enhanced Th2-dominant inflammation in murine allergic conjunctivitis model. Allergy. 2019;74(5):910–21.
41. Lotfi N, Thome R, Rezaei N, Zhang GX, Rezaei A, Rostami A, Esmaeil N. Roles of GM-CSF in the pathogenesis of autoimmune diseases: an update. Front Immunol. 2019;10:1265.
42. Harbour SN, DiToro DF, Witte SJ, Zindl CL, Gao M, Schoeb TR, Jones GW, Jones SA, Hatton RD, Weaver CT. T(H)17 cells require ongoing classic IL-6 receptor signaling to retain transcriptional and functional identity. Sci Immunol 2020, 5(49).

Publisher's note