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Toll-like receptor signal is required in maintenance of immune suppressive capacity of regulatory T cells

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ABSTRACT

Dysfunction of immune regulatory cells has been recognized in a variety of immune diseases; the underlying mechanism remains to be further investigated. This study aims to investigate the critical role of Toll-like receptor (TLR) signal in the maintenance of function of regulatory T cells (Tregs). In this study, Tregs were isolated from patients with allergic rhinitis (AR) and healthy control (HC) subjects. The role of TLR signal in the maintenance of Treg's function was tested with experiments of cell culture and an AR mouse model. We observed that the immune suppressive function of AR Treg (Tregs isolated from AR patients) was impaired, although the number of peripheral AR Treg was comparable with HC Treg. Expression of transforming growth factor (TGF)-β was lower in AR Tregs than that in HC Tregs that was positively correlated with expression of Mal in Tregs; the latter was lower in AR Tregs as compared to HC Tregs. TGF-β mRNA in Tregs decayed spontaneously in the culture. Activation of Mal counteracted TGF-β decay and maintained the Treg's immune regulatory function. Mal bound Tristetraprolin (TTP) to prevent TTP from inducing TGF-β mRNA decay. Absence of TLR signals resulted in Treg dysfunctional and worsened experimental AR response in a murine model. In conclusion, TLR signal is required in the maintenance of Treg function. Absence of TLR signal may result in Treg dysfunction and immune intolerance.

1. Introduction

The prevalence of allergic disease, such as allergic rhinitis (AR), asthma, dermatitis and food allergy, increases rapidly worldwide in the recent decades [1]. The underlying mechanism remains to be further investigated. AR is an adverse immune response in the nasal mucosa to innocent antigens. Aberrant higher immune cell activities in the body of patients with allergic diseases are recognized. For example, over-production of T helper (Th)2 cytokines, such as interleukin (IL)-4, IL-5, IL-13, and immunoglobulin (Ig)E; accumulation of eosinophils and mast cells in the local tissues [2]. To date, current therapies for allergic diseases are not satisfactory [3]. Therefore, it is necessary to further understand the mechanism of allergy and to invent novel and more effective remedies for the treatment of allergic diseases.

In general, the immune cell activities are tightly regulated in the body by the immune regulatory system [4]. The immune regulatory system includes immune regulatory cells and immune regulatory mediators. Regulatory T cells (Tregs) are one of the major immune regulatory cells [4]. Tregs produce transforming growth factor (TGF)-β or IL-10 to regulate or suppress other immune cell activities to maintain the immune response within a proper scope to avoid causing tissue injury [5]. There are several other immune regulatory cells, such as regulatory B cells, in the body [4,5]. The dysfunction of Treg, such as mutations of Foxp3 and reduced production of TGF-β, causes severe body inflammation and attracts more attention to the studies of Treg in the recent years [6]. In allergic diseases, the aberrant Th2 activities and

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plasma cell overproduction of IgE mirror the dysfunction of the immune regulatory system in the body. The underlying mechanism remains to be further understood.

Microbial stimuli are required in the development and maintenance of immune regulatory function [7]. Insufficient stimuli of microbe results in impairment of immune regulatory function [7]. Immune cells express Toll-like receptors (TLR) that recognize microbial stimuli [8]. TLRs can be activated upon exposure to microbial products that initiates the response in the signal transduction pathway, in which Mal and MyD88 are the common checkpoints of different TLR signals except TLR3 [9,10]. Although activation of Mal is mainly recorded in microbe-related inflammation in the body, some microbial stimuli, such as those from commensal microbes, are required in the maintenance of Treg's immune regulatory function [11]. Yet, whether TLR signals are required in the maintenance of Treg's function remains to be further investigated. In this study, we examined the immune regulatory function of Treg collected from AR patients. The role of TLR signal in the maintenance of Treg's immune suppressive function was investigated.

2. Materials and methods

2.1. Human subjects

Patients with perennial allergic rhinitis (AR) at the remission stage were recruited into this study in the Affiliated Hospitals at Shenzhen University (Shenzhen, China). The diagnosis and management of AR were carried out by our surgeons following our routine procedures that can be found elsewhere [12]. To obtain nasal mucosa tissue samples, patients with nasal polyps (with or without nasal allergy) were also recruited into this study. Healthy control (HC) subjects were also recruited into this study. The demographic data and AR-related parameters of human subjects are presented in Table S1-3 in supplemental materials. Patients with any of the following conditions were excluded this study: Cancer; severe organ diseases; autoimmune diseases; under immune suppressive therapies for any reasons. The experimental procedures of this study were approved by the Human Ethics Committee at Shenzhen University. An informed written consent was obtained from each human subject.

2.2. Collection of human nasal mucosal samples

Patients with nasal polyps were treated with nasal surgery by our surgeons following the routine procedures. Surgically removed nasal tissues were collected in the operation rooms.

2.3. Isolation of Tregs from human nasal tissue

Surgically removed nasal mucosal tissues were cut into small pieces (2 × 2 × 2 mm) and incubated with 1 mg/ml collagenase IV for 2 h at 37 °C with mild agitation. Single cells were passed through a cell strainer (70 μm first, then 40 μm). Mononuclear cells were isolated by Percoll gradient density centrifugation. $CD4^+$ $CD25^+$ $CD127^-$ Tregs were isolated from the mononuclear cells by flow cytometry cell sorting. The purity of isolated Tregs was greater than 98% as checked by flow cytometry. The viability of isolated Tregs was greater than 99% as assessed by Trypan blue exclusion assay.

2.4. Detection of TTP/TGF-β complex in Tregs

HC Tregs were prepared from PBMCs and cultured in RPMI1640 medium for 96 h. The cells were collected and radiated by 0.15 J/cm^2 of 365 nm UV light in a Stratalinker 2400 (Stratagene) to cross-link the RNA/protein in the cytoplasm. The cells were lysed with a lysis buffer. The lysates were then processed with the procedures of the IP. The protein/RNA complexes were eluted from the agarose beads with an eluting buffer. RNA was extracted from the samples with an RNA recovering kit following the manufacturer's instructions and analyzed by RT-qPCR. Proteins were analyzed by Western blotting.

2.5. Assessment of Th2 cytokines in the nasal mucosa

Nasal mucosal tissue was collected at the sacrifice and homogenized in protein extracting buffer containing protease-inhibiting cocktail with a homogenizer in an ice bath. The supernatant was collected and used as protein extracts. Th2 cytokine levels in the extracts were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

2.6. Statistical analysis

The difference between two groups was determined by Student *t* test. ANOVA following by Dunnett's *t* test or SNK test was employed for multiple comparisons. $P < 0.05$ was considered statistical significance.

More experimental procedures are presented in supplemental materials.

3. Results

3.1. AR Tregs show incompetent immune suppressive function

Blood samples were collected from AR patients and HC subjects. Peripheral blood mononuclear cells (PBMCs) were isolated from the samples and analyzed by flow cytometry. The results showed that the frequency of AR Tregs (Tregs isolated from AR patients) in PBMCs was not significantly different from HC Tregs (Fig. 1A–D). These $Foxp3^+$ Tregs did not show CD127 positive (Fig. S1 in supplemental materials). To corroborate the results, Tregs were isolated from PBMCs and analyzed by Western blotting and ChIP. The results showed that protein levels of Foxp3 were comparable between AR group and HC group. Furthermore, Foxp3 and Pol II levels at the *TGFB* promoter locus were also detected and at similar levels between the two groups (Fig. 1E–G). To assess the immune suppressive function of Tregs, Tregs and $CD4^+$ $CD25^-$ effector T cells (Teff; labeled with CFSE; Carboxyfluorescein diacetate succinimidyl ester) were isolated from PBMCs by flow cytometry cell sorting. Tregs and Teffs were cocultured at a ratio of 1:5 for 3 days in the presence of anti-CD3/CD28 Abs. As shown by flow cytometry data, the suppressive effects on Teff proliferation was markedly impaired in the AR group than that in the HC group (Fig. 1H–I). The results indicate that, although the number of AR Tregs in PBMCs is not reduced, their immune suppressive function is impaired.

3.2. Expression of TGF-β is impaired in AR Tregs that is correlated with insufficient expression of Mal

Since TGF-β is the canonical immune regulatory mediator of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, the results shown by Fig. 1 imply that the expression of TGF-β may be impaired in AR Tregs. To test this, Tregs were isolated from PBMCs and analyzed by RT-qPCR and Western blotting. The results showed that the expression of TGF-β was markedly lower in AR Tregs than that of HC Tregs (Fig. 2A–B). In addition, we collected surgically removed nasal mucosa samples from patients with or without nasal allergy. Tregs were isolated from these nasal tissues and analyzed by RT-qPCR and Western blotting. We also found that Tregs from allergic nasal mucosal tissue expressed less TGF-β as compared with the non-allergic nasal tissues (Fig. 2A–B).

Published data show that activation of Toll-like receptors (TLR) can induce TGF-β expression [13,14], Mal (TIR domain-containing adaptor protein, also called TIRAP) is a critical component in the common TLR signal transduction pathway [9]. Therefore, we analyzed the Mal expression in Tregs by RT-qPCR and Western blotting. The results showed that the expression of Mal was lower in AR Tregs as compared with that of HC Tregs (Fig. 2C–D). It is noteworthy that a positive correlation was

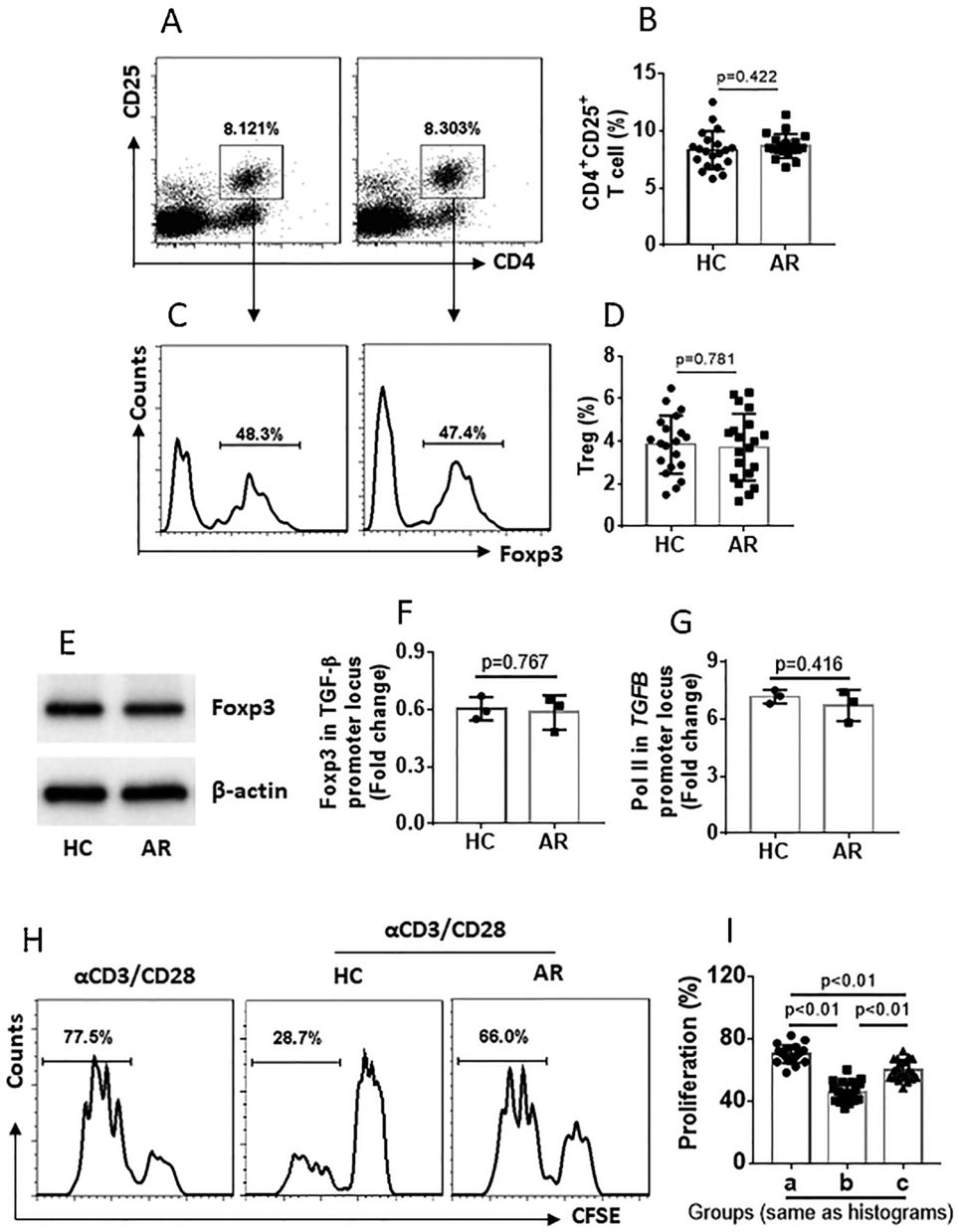

Fig. 1. Immune suppressive function of AR peripheral regulatory T cell (Treg) is impaired. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples collected from AR patients (n = 20) and HC subjects (n = 20). PBMCs were analyzed by flow cytometry. A, gated dot plots indicate frequency of $CD4^+$ $CD25^+$ T cells. B, bars indicate summarized $CD4^+$ $CD25^+$ T cells in PBMCs. C, gated histograms indicate frequency of $Foxp3^+$ Tregs. D, bars indicate summarized frequency of $Foxp3^+$ Tregs. E, Tregs were isolated; the protein extracts were pooled and analyzed by Western blotting. The immunoblots indicate protein levels of Foxp3. F–G, a portion of isolated Tregs were analyzed by ChIP. The recovered DNA were pooled and analyzed by qPCR. The bars indicate levels of Foxp3 (F) and Pol II (G) at the *TGFB* promoter locus. H, gated histograms indicate frequency of Teff proliferation. I, bars indicate summarized data of Teff proliferation. Data of bars are presented as mean ± SEM. Each dot inside bars presents data of an independent experiment. The data of E are from one experiment which represent results obtained from 3 independent experiments.

detected between levels of TGF-β and Mal in Tregs (Fig. S2). The expression of TLR2, 3, 4, 7 and 9 was detectable in Treg extracts, which did not show a marked difference between the AR group and the HC group (Fig. S3). The results demonstrate that AR Tregs express lower levels of TGF-β than that of HC Tregs and the expression of Mal is impaired in AR Tregs. The expression of TGF-β is positively correlated with the expression of Mal in Tregs, implying that Mal may be required in the maintenance of TGF-β expression in Tregs. Since the Toll/IL-1R domain-containing adapter-inducing interferon-β (TRIF) and MyD88 are also involved in the TLR signal transduction pathway [15], we examined the expression of TRIF and MyD88 in Tregs by Western blotting. The results showed that TRIF and MyD88 were detected in Tregs, the levels of which were comparable between the HC group and the AR group as well as between the NR group and AR group of nasal Tregs (Fig. 2E–F).

3.3. TGF-β mRNA spontaneously decay in Tregs that can be counteracted by Mal activation

Next, we isolated Tregs from HC subjects. The Tregs were cultured in medium alone and harvested at time points of 0, 20, 40, 60, 80 and 100 min, respectively. Expression of TGF-β by the Tregs was assessed by RT-qPCR. The results showed that TGF-β mRNA decayed spontaneously in the culture in a time-dependent manner (Fig. 3A). Since exposure to TLR agonists can induce Tregs [13,14], exposure to TLR agonists may counteract the spontaneous TGF-β mRNA decay in Tregs. To test the inference, we added LPS, or SEB, into the culture. Indeed, the spontaneous TGF-β mRNA decay in Tregs was efficiently counteracted; such an effect was abolished by depleting either Mal or MyD88 expression in Tregs by RNAi (Fig. 3B, Fig. S4). The results demonstrate that TGF-β mRNA decays spontaneously in Tregs that can be counteracted by the presence of agonists of TLR2 or TLR4, suggesting that TLR2/4 signals may be required in stabilizing the expression of TGF-β in Tregs.

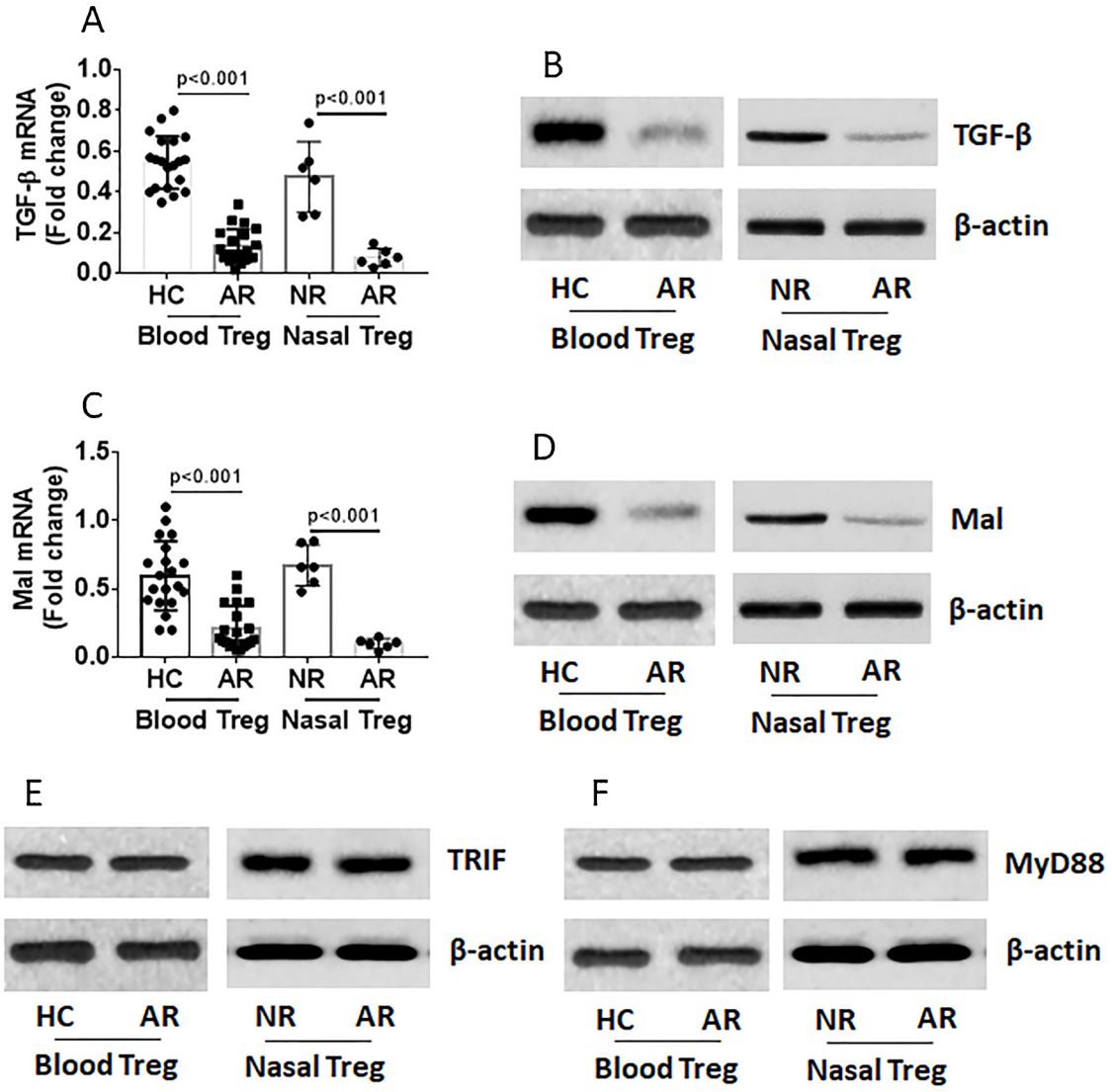

Fig. 2. AR Tregs express lower levels of TGF-β and Mal. Blood samples were collected from AR patients (n = 20) and HC subjects (n = 20). Nasal mucosa samples were collected from 6 patients with non-allergic rhinitis/nasal polyps (NR) and 6 patients with allergic rhinitis/nasal polyps (AR). Tregs were isolated from the blood samples (Blood Treg) and nasal mucosal samples (Nasal Treg) and analyzed by RT-qPCR and Western blotting. A, bars indicate TGF-β mRNA levels in Tregs. B, immunoblots indicate TGF-β protein levels in Tregs. C, bars indicate Mal mRNA levels in Tregs. B, immunoblots indicate Mal protein levels in Tregs. E–F, immunoblots indicate protein levels of TRIF (F) and MyD88 (G). Data of bars are presented as mean ± SEM. Each dot inside bars presents data of an independent experiment. The immunoblots were results of one experiment representing 3 independent experiments (proteins from each sample were pooled within each group).

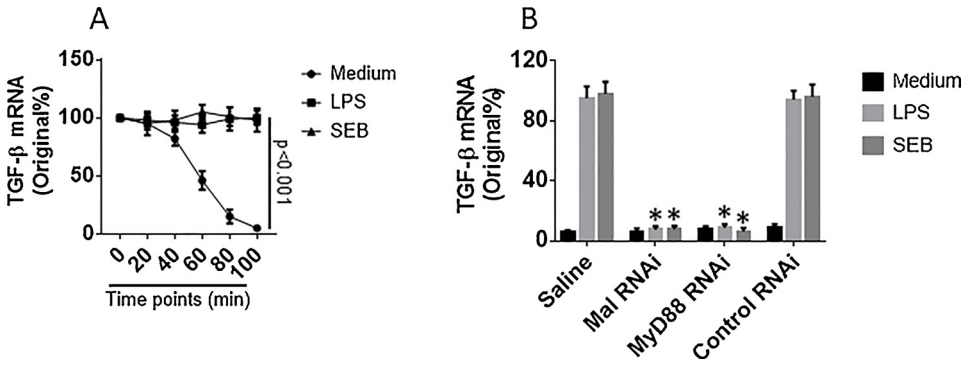

Fig. 3. TLR agonists counteract TGF-β mRNA decay in Tregs. Tregs were isolated from blood samples collected from HC subjects. A, Tregs were cultured with or without the presence of LPS (100 ng/ml), or SEB (100 ng/ml), respectively. Tregs were harvested at the indicated time points and analyzed by RT-qPCR. Curves indicate TGF-β mRNA levels in Tregs at indicated time points (presented as percentage of value at time point 0). B, Mal-deficient or MyD88-deficient or wild type Tregs were cultured with or without the presence of LPS (100 ng/ml), or SEB (100 ng/ml), respectively. The cells were harvested 100 min later and analyzed by RT-qPCR. Bars indicate TGF-β mRNA levels in Tregs. Data of bars are presented as mean ± SEM. *p < 0.01, compared with the saline group. The data represent results obtained from 3 independent experiments.

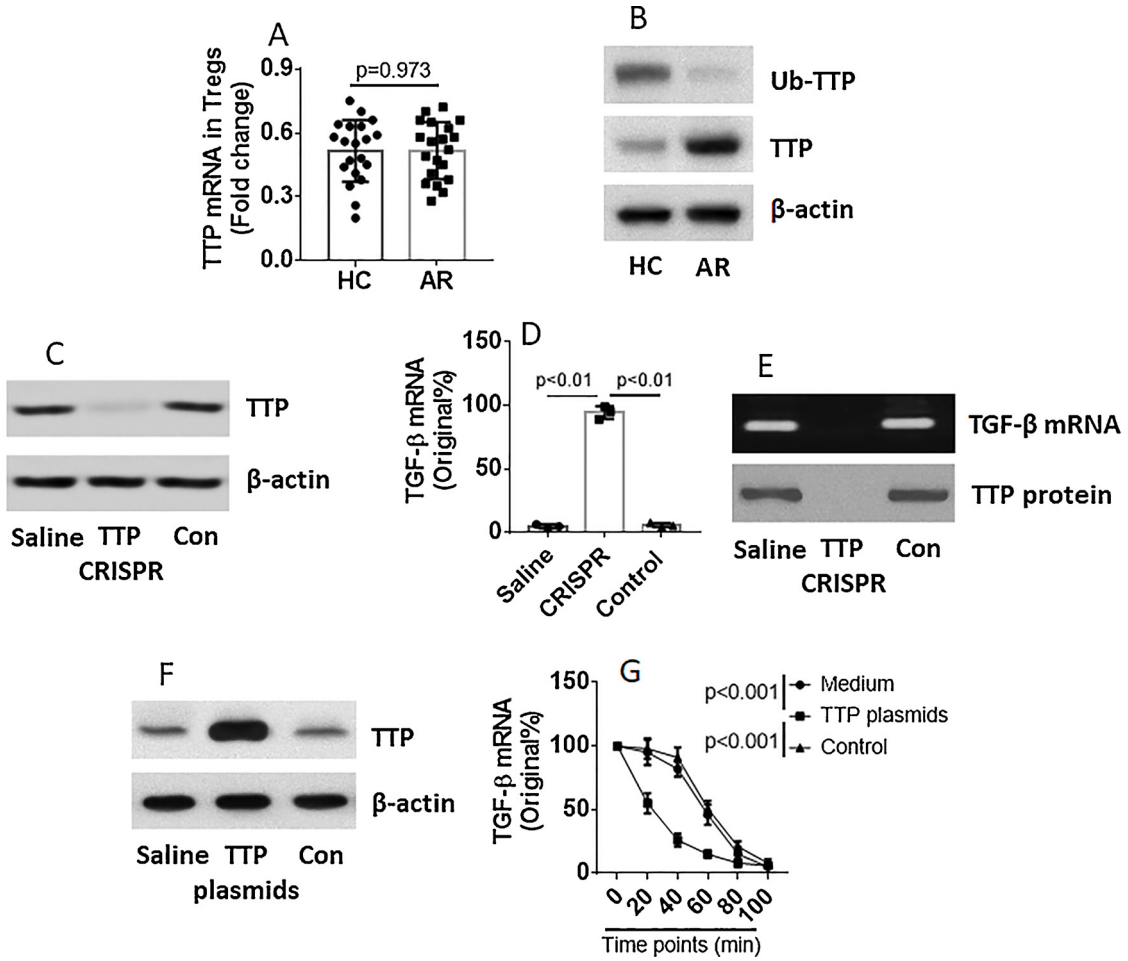

Fig. 4. TTP speeds up TGF-β mRNA to decay in Tregs. A–B, Tregs were prepared from HC subjects (n = 20) and AR patients (n-20). Bars show TTP mRNA levels in Tregs. Immunoblots show TTP proteins and ubiquitinated TTP proteins in Tregs. C, TTP expression was depleted in Tregs by CRISPR. D, bars show depletion of TTP counteracted TGF-β mRNA decay. E, a complex of TTP/TGF-β mRNA was detected in Tregs with or without TTP depletion. F, HC Tregs were over expressed TTP by transfecting with TTP-expressing plasmids. G, curves show TTP-over expression speeds up TGF-β mRNA decay in HC Tregs. Data of bars are presented as mean ± SEM. Each dot inside bars present data from one sample (tested in triplicate). Protein samples for immunoblots were pooled per group. The data of immunoblots are from one experiment representing 3 independent experiments. Data of D and G represent 3 independent experiments.

3.4. Tristetraprolin (TTP) speeds up TGF-β mRNA decay in Tregs

Published data indicate that tristetraprolin (TTP) can bind mRNA to induce mRNA decay [16]. We wondered if TTP was also associated with TGF-β mRNA decay in Tregs. To this end, HC Tregs and AR Tregs were prepared and analyzed by RT-qPCR and Western blotting. The results showed that TTP was detected in both HC and AR Tregs. The mRNA levels of TTP were comparable between HC and AR Tregs (Fig. 4A), while the protein levels of TTP were higher in AR Tregs (Fig. 4B). The results implicate that the degradation of TTP is impaired in AR Tregs. We then assessed the ubiquitination of TTP in Tregs. The results showed less ubiquitinated TTP in AR Tregs than that in HC Tregs (Fig. 4B). The results suggest that the degradation of TTP is impaired in AR Tregs. To test the role of TTP in TGF-β mRNA degradation in Tregs, HC Tregs were prepared; the expression of TTP was depleted in Tregs by CRISPR (Fig. 4C). The TTP-deficient and TTP-sufficient Tregs were cultured and harvested 100 min later. As analyzed by RT-qPCR, TGF-β mRNA decay was observed in TTP-sufficient Tregs that was abrogated by depletion of TTP (Fig. 4D). The results confirm that TTP does play a critical role in TGF-β decay in Tregs. Further, a complex of TTP/TGF-β mRNA was detected in Tregs (Fig. 4E), which were treated with the procedures of Fig. 4D. Furthermore, by overexpressing TTP (Fig. 4F), the TGF-β mRNA decayed more quickly in HC Tregs (Fig. 4G).

3.5. Absence of Mal signals impairs Treg's immune suppressive function

By employing Mal-deficient (Mal-d) mice, we obtained an in vivo mouse model of absence of Mal signals. Spleen cells were prepared from Mal-d mice and wild type (WT) mice; the cells were examined by flow cytometry. The results showed that the frequency of Tregs in Mal-d mice was comparable with that of WT mice (Fig. 5A–B), while the protein levels of TGF-β were significantly lower in Mal-d mice as compared with WT mice (Fig. 5C). To test the immune suppressive function of Tregs, $CD4^+$ effector T cells (Teffs) and Tregs were isolated from spleen cells. The Tregs and Teffs were cocultured for 3 days in the presence of anti-CD3/CD28 Abs and analyzed by flow cytometry. Tregs isolated from Mal-d mice showed much weaker immune suppressive effect on Teff proliferation (Fig. 5D–E). Tregs isolated from Mal-d mice showed higher TTP expression than that of WT mice (Fig. 5F). The results suggest that the Mal signals are required in the maintenance of Tregs' immune suppressive function.

3.6. Absence of Mal signal worsens AR response in mice

Following published data [17], Mal-d mice and WT mice were treated with the AR-induction procedures. As shown by Fig. 6, the AR response was induced in WT mice, including increases in nasal itch, sneezing, levels of IL-4, IL-5, specific IgE and mouse mast cell protease-

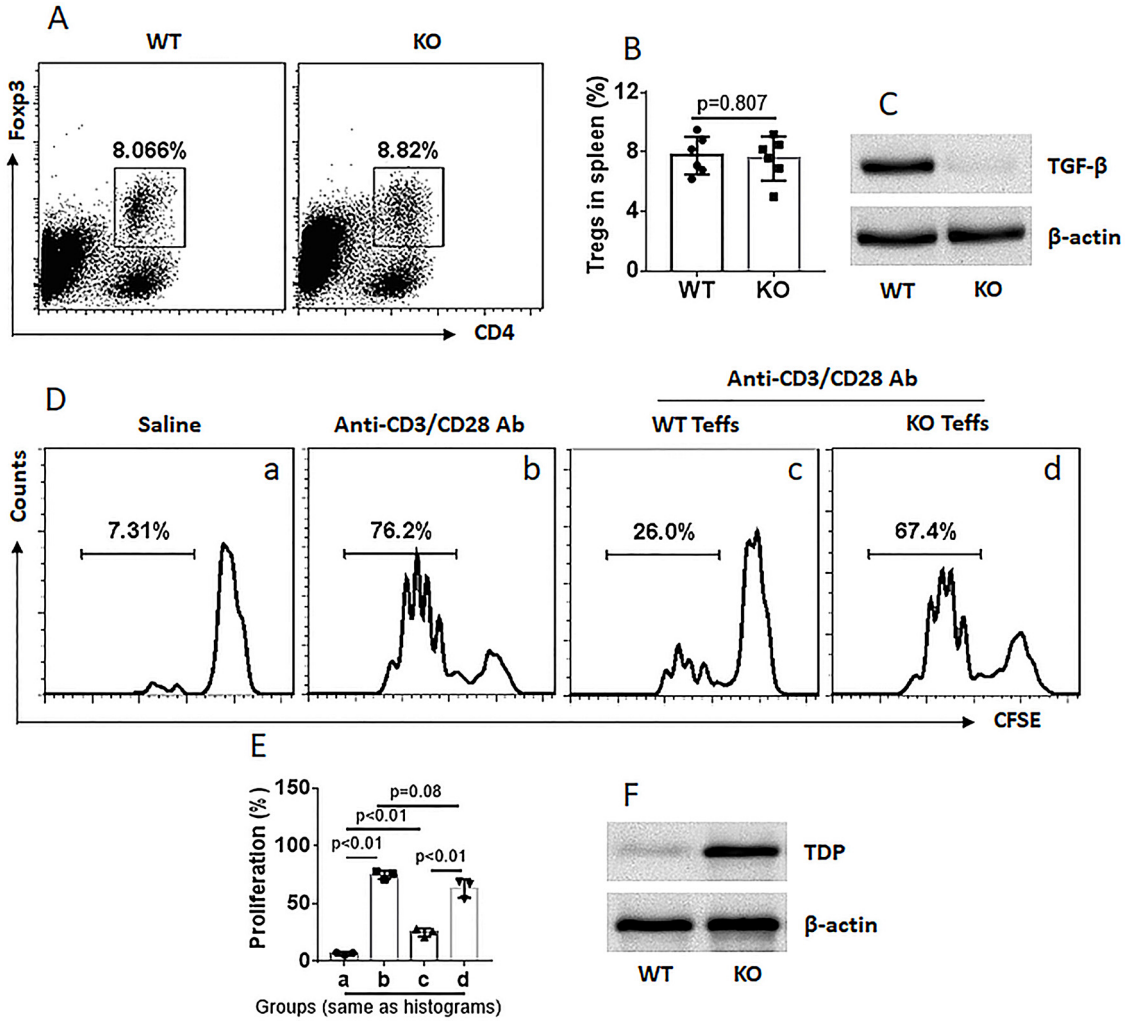

Fig. 5. Absence of TLR signal compromises Treg immune suppressive function. Spleen cells were prepared from Mal-knockout (KO) mice and wild type (WT) mice. A–B, spleen cells were analyzed by flow cytometry. The gated dot plots indicate $CD4^+$ $Foxp3^+$ Tregs (A); the bars indicate summarized Treg frequency (B). C–F, Tregs and CD4+ $CD25^-$ effector cells (Teffs) were isolated from the spleen. C, the immunoblots show TGF-β protein levels in Tregs. D–E, Tregs and Teffs were cocultured for 3 days with the treatment denoted above each subpanel. The gated histograms indicate proliferating Teffs (D). Bars indicate summarized frequency of proliferating Teffs (E). F, immunoblots indicate TTP protein levels of isolated Tregs. Data of bars are presented as mean ± SEM. Each dot inside bars present data obtained from individual mouse (B) or an independent experiment.

1 in nasal mucosal extracts, while the AR response was much worse in Mal-d AR mice. Passively transplantation of Mal-sufficient Tregs to Mal-d mice efficiently inhibited the AR response while transplantation with Mal-deficient Tregs did not. The results demonstrate that absence of Mal signals worsens AR response.

4. Discussion

The present data reveal an important phenomenon that the Mal signals are required in the maintenance of Treg's immune suppressive function. Although the relevant number of Tregs in AR patients is comparable with that of healthy control subjects, the immune suppressive function is impaired in AR Tregs. Importantly, TGF-β mRNA decays in AR Tregs spontaneously. TTP speeds up TGF-β mRNA to decay. Mal prevents TTP from inducing TGF-β mRNA decay. Therefore, insufficient Mal signal induces Treg dysfunction. Indeed, deficiency of Mal worsens experimental AR as observed in the present study.

We found that the relevant number of peripheral Tregs of AR patients was comparable with that in healthy control subjects. This is concordant with previous observations. For example, Han et al observed the peripheral Treg number was equivalent to that in healthy controls [18]. Some researchers found that the number of Tregs was much less in patients with AR [19] or both asthma and AR [20]. Malmhall et al even found that the number of Treg was higher in patients with allergic diseases that was down regulatable after treatment with steroids [21]. The mixed results about Tregs in allergic diseases suggest dynamic changes in Tregs in the course of allergic diseases or the number of Treg can be affected by multiple factors.

However, we observed that the immune suppressive function of AR Tregs was impaired, although the number of AR Treg was maintained at similar levels for the HC group. This result suggests that the immune regulatory system may be dysfunctional in AR patients. The inference is supported by our clinical observation. Higher levels of Th2 cytokines, specific IgE and tryptase were detected in the serum as well as in the nasal secretions. Tregs are one of the major immune regulatory cells [4]. The main function of Treg is to suppress other immune cell's activities [4]. The aberrant higher levels of Th2 cytokines and specific IgE in AR patients mirrors the dysfunctional status of the immune regulatory system; this is in accordance with the impairment of Treg suppressive function observed in the present study. Recent reports also found that Tregs could not suppress basophil activities [22], which may be partially because basophils lack the contact-dependent receptors (CTLA-4 and LAG-3) [22]. On the other hand, Tregs also induce activation of basophils via activating the IL-3/STAT5 signal pathway [22].

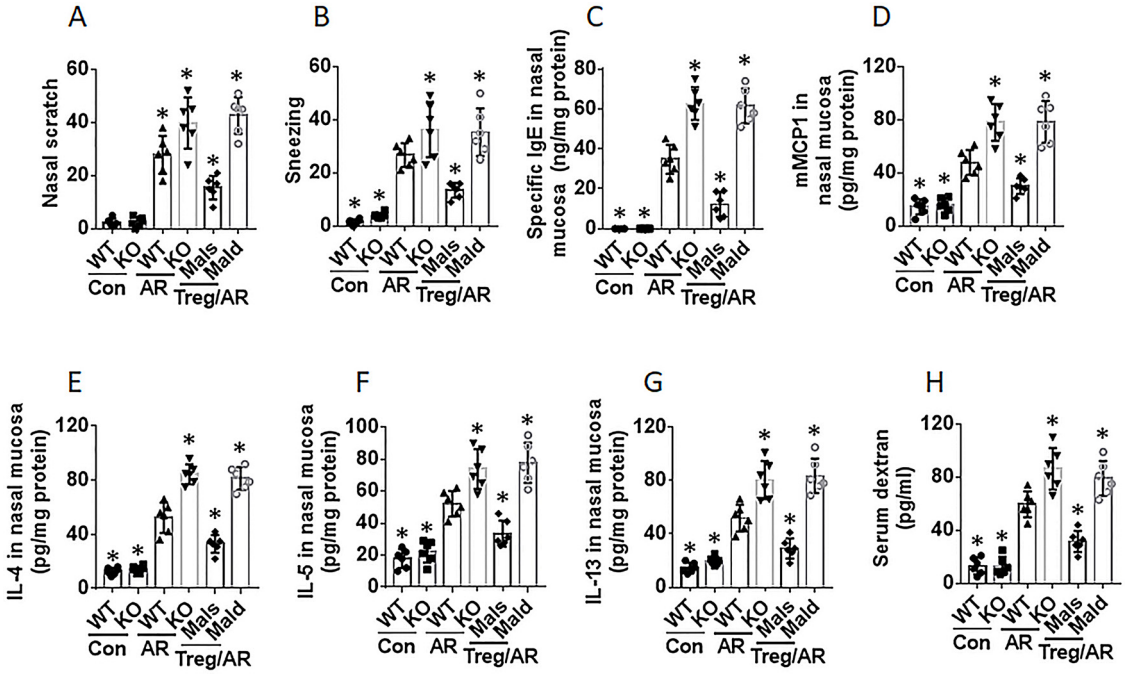

Fig. 6. Absence of Mal signals worsens AR response. An AR mouse model was developed with Mal knockout (KO) mice and the wild type mice (WT; C57BL/c mice). A–B, nasal scratch (nasal itch) and sneezing were recorded 0–30 min after the last exposure to specific antigens. C–D, bars indicate serum levels of specific IgE and mouse mast cell protease-1 (mMCP1). E–G, bars indicate Th2 cytokine levels in protein extracts of nasal mucosa. H, bars indicate serum TRITC-dextran levels that represent nasal epithelial barrier permeability. Data of bars are presented as mean ± SEM. Each dot inside bars presents data obtained from one mouse. *$p < 0.01$, compared with the WT AR group. Mals: KO AR mice received Mal-sufficient Treg transplantation. Mald: KO AR mice received Mal-deficient Treg transplantation. Con: Control mice were treated with saline. Treg: Mice received adoptive transfer with Mal-sufficient Tregs or Mal-deficient Tregs at 2×10^6 Tregs/mouse through tail vein injection on day 0 and day 14, respectively. Each group consists of 6 mice.

Whether this phenomenon is because the Tregs are at a dysfunctional status needs to be further investigated.

The data showed that levels of TGF-β mRNA in Tregs declined spontaneously in the culture, a phenomenon called RNA decay [23]. RNA decay is a natural phenomenon; it is usually triggered by binding with certain proteins or miRNAs [24]. TTP was the protein to form a complex with TGF-β mRNA in Tregs to induce the RNA decay as observed in this study. Previous studies also found that TTP bound tumor necrosis factor (TNF)-α mRNAs to suppress TNF-α production. Deficiency of TTP results in TNF-α-related disorders, such as arthritis [25]. We found that the TGF-β mRNA decay in Tregs could be counteracted by the presence of TLR agonists. Mal is a common checkpoint in the signal transduction pathway of TLRs. Most TLR agonists activate Mal or/and MyD88 first, then activate the downstream components [10]. Our data expand the knowledge at this point by showing that Mal prevents TTP from inducing TGF-β mRNA decay. The present data suggest that TGF-β mRNA decays spontaneously after transcription in Tregs, which can be counteracted by TLR agonists in the micro environment. Since TLR agonists naturally exist in living environment, they can be absorbed into the body through the airway, skin or gastrointestinal tract, and therefore to contribute to stabilizing TGF-β mRNA in Tregs.

Low levels of Mal were observed in AR Tregs in this study; it was positively correlated with the expression of TGF-β in AR Tregs. This implies that the low expression of Mal is associated with lower levels of TGF-β in Tregs. Although the causative factors of the low expression of Mal is not clear, the downstream effects of which were revealed in the present study. Mal prevented TTP from inducing TGF-β mRNA decay in Tregs. The data implicate that the absence or insufficient Mal expression in Tregs may result in a scenario that TTP is out of control in Tregs, and thus, speed up the TGF-β mRNA decay in Tregs and impair the immune suppressive function of Tregs.

Because of Mal is a common checkpoint of TLRs, TLRs recognize microbial stimuli, the absence or insufficient Mal activities can result in a similar situation of shorting of microbial stimuli. The prevalence of allergic disease increases rapidly in the world in the recent decades [1]. The hygiene hypothesis has been proposed to explain this phenomenon that may be a result of the disappearance of chronic infectious diseases and reduced exposure to microbial products [26]. The lack of microbial stimuli may result in Th2 polarization in the body to facilitate initiation of allergic response [27]. The present data add novel information to the hygiene hypothesis and expand the mechanistic scope of the hygiene hypothesis. Insufficient TLR signals may result in TTP out of control and induce or speed up TGF-β mRNA decay in Tregs, and thus, compromise Treg's immune regulatory function, which contribute to the initiation of allergic diseases or/and other immune disorders in the body.

In summary, the present data show that the absence of Mal signal speeds up TGF-β mRNA decay in Tregs and impairs Treg's immune regulatory function, which may contribute to initiation of allergic diseases. Therefore, Mal or/and TTP may be novel therapeutic targets in the treatment of immune diseases.

Conflict of interest

None to declare.

Author contributions

MZ, GY, XRG, YYZ, JQL, FM, ZQL, MZZ, LHM and XQL performed experiments, analyzed data and reviewed the manuscript. XWZ, DBL and PCY organized the study and supervised experiments. PCY designed the project and prepared the manuscript.

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