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Norcantharin ameliorates the development of murine lupus via limiting STAT3-mediated IL-17-producing cell accumulation

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Abstract

Background: Systemic lupus erythematosus (SLE), a devastating autoimmune disorder, results from the aberrant T cell and B cell activation. Norcantharin (NCTD), a derivative of Cantharidin, is an efficacious anti-cancer drug, whereas the role of NCTD in SLE remains unknown. As a result, we aimed to investigate the therapeutic effect of NCTD on the development of murine lupus.

Methods: MRL/MpJ and MRL/*lpr* female mice were randomly divided into three groups and administered with vehicle control or NCTD at 1 mg/kg or 2 mg/kg. The mortality rate, auto-antibodies, kidney damage, immune complex deposition and lupus-related inflammation level were tested. Furthermore, the proportion of T/B cells or T cell subsets in splenocytes from each group was monitored using flow cytometry. Finally, double-negative (CD3+CD4-CD8-, DN) T cell proliferation and T helper 17 (Th17) cell differentiation *in vitro* as well as regulatory pathways were analyzed for NCTD treatment.

Results: NCTD decreased mortality, the accumulation of anti-dsDNA, splenomegaly and inflammation level of lupus mice. In addition, NCTD-treated MRL/*lpr* mice showed notably ameliorated renal involvement. Moreover, we found that NCTD reduced DN T cell proliferation and Th17 differentiation via mediating the activation of signal transducer and activator of transcription 3 (STAT3).

Conclusions: NCTD effectively suppressed DN T cell proliferation and Th17 cell polarization, thus ultimately contributing to the attenuated the SLE development. Our results revealed that NCTD may be a promising therapeutic agent for SLE.

Keywords: Norcantharin, Systemic lupus erythematosus, STAT3, IL-17-producing cell accumulation

Background

Systemic lupus erythematosus (SLE) is a complicated autoimmune disease, manifested by autoantibody accumulation, systemic inflammation and immune complex deposits in multi-organs [1], which are resulted from abnormal B cell and T cell responses [2, 3]. Recent studies have showed that T cells serve as the commander in SLE pathogenesis with orchestrating not only B cell activation for autoantibody production but also the modulation and differentiation of T helper (Th) cells and inflammation cytokine infiltration in target organs such as kidney, finally resulting in systemic damage [4, 5]. At present, patients suffered from SLE are primarily treated with corticosteroids accompanied by immunosuppressant agents, which lead to numerous side effects. Thus, there is an urgent need to look for more effective and safer therapeutic agents for SLE.

Interleukin-17(IL-17, a.k.a. IL-17A) has been reported to play a central pathogenic role in the development of SLE [6, 7]. Patients with SLE show higher levels of IL-17 in serum accompanied with increased number of IL-17 producing T cells in peripheral blood [8-10]. During SLE, IL-17 may mediate local tissue damage by inducing other inflammatory chemokines and cytokines to promote the recruitment of immune cells such as monocytes and neutrophils [11, 12]. In addition, IL-17 has been demonstrated to have a synergy with B-cell activating factor (BAFF) to promote B cell proliferation and auto-antibody production[13].

It has been shown that double-negative (CD3+CD4-CD8-, DN) T cells as well as Th17 cells mainly produce the increased amounts of IL-17 in SLE progress [10]. It has been demonstrated that DN T cells are improved in SLE patients as well as in lupus-prone mice [14, 15] and the number of DN T cells is positively related to SLE activity [16]. DN T cells invade into multi-organs of SLE patients, where they activate other T cells, induce autoantibody production as well as promote the produce of pro-inflammtory cytokines, such as IFN-γand IL-17 [17]. Th17 cells, a T- cell subset derived from CD4+ T cells, also secret IL-17, IL-21 and IL-22 to regulate the inflammatory process of SLE. Some studies have shown that both circulating Th17 cell percentage and serum IL-17 concentration is positively corrected with SLE Disease Activity Index (SLEDAI)

[18, 19].Thus, it is meaningful to seek novel therapeutic agents for SLE targeting IL-17 producing cells. Norcantharin (NCTD), the demethylated form of Cantharidin isolated from blister beetles, is an anti-cancer drug routinely used in China with less nephrotoxic and phologenic side effects [20]. Previous studies have proved that NCTD showed therapeutic effect in CIA-induced Rheumatoid arthritis (RA) model by regulating the balance of Th17/Treg cells [21]. Moreover, NCTD has also been demonstrated to protect renal function in different nephropathy models [22-24]. However, the role of NCTD in SLE remains unclear. Here, we examined the effect of NCTD in SLE progress and found that NCTD treatment effectively ameliorated lupus syndromes by inhibiting IL-17-producing cell accumulation including DN T cells and Th17 cells in a dose-dependent manner. Further research suggested that NCTD impaired DN T cell proliferation and Th17 cell differentiation both via blocking the activation of signal transducer and activator of transcription 3 (STAT3). Our research reveals NCTD may be as a promising therapeutic drug for SLE treatment.

Materials and methods

Mice

MRL/*lpr* and MRL/MpJ female mice (4-5 weeks) were purchased from SLRC Experimental Animals Co. Ltd. (Shanghai, China). From 12 weeks, NCTD (Sigma-Aldrich, MO, USA), dissolved in PBS, was administered by intraperitoneal injection into MRL/*lpr* or MRL/MpJ mice at 1 mg/kg or 2 mg/kg every day for 9 weeks. Control groups were given PBS as a vehicle control. The deaths were recorded every day. At 20 weeks, the mice were sacrificed, and then the spleens and kidneys were collected. The weight of spleens was measured as well as total splenocyte number was assessed by the Cedex XS cell analysis system (Roche, Mannheim, Germany). All animal experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University.

Renal Histology and Immunofluorescence (IF)

Kidneys were obtained from exsanguinated mice, immediately fixed with 4% formalin and then embedded in paraffin according to standard procedures. Sections (5 μm) were mounted on slides for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining to evaluate the morphology changes and inflammation level in kidneys. Renal pathology was scored by an experienced pathologist blinded to the treatments according to previously described methods [25]. Besides, immune complex (IgG) deposition was assessed with IgG-FITC antibody (Abcam Ltd., MA, USA) in accordance with a previous protocol [26] and the fluorescence intensity was quantified by Image J.

Urinalysis

Urine was manually harvested from 19 weeks. Fresh urine samples were centrifuged at 1500 rpm for 10 minutes at 4°C and pooled for each mouse followed by storing at −80°C until use. Levels of total protein, albumin and creatine in urine were detected with commercially available kits (Dia Sys Diagnostic Systems GmbH, Holzheim, Germany).

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected from whole blood without anticoagulants at 3000 rpm for 10 minutes at 4°C. The concentrations of anti-dsDNA (SHIBAYAGI CoLtd, Shibukawa, Japan), IFN-γ, IL-17A, IL-22, IL-23, IL-6, TNF-α, IL-4 and IL-10 (Thermo Fisher Scientific, MA, USA) in serum were determined with corresponding ELISA kits according to the manufacturer's instructions. In addition, CD4 T cell culture supernatants were also collected and the cytokine production (IL-17A, IFN-γ, IL-4 and IL-10) was detected as described above.

Flow cytometry

To measure T/B cell percentage, single-cell suspensions of spleens were prepared and stained with PE-anti-CD3- and FITC-anti-CD19 antibodies (Thermo Fisher Scientific) followed by FACS analysis using an FC 500 MC system (Beckman Coulter, Fullerton, USA). To analyze the T cell subsets in spleens, single-cell suspensions were re-stimulated with 50 ng/ml PMA (Sigma-Aldrich), 1μg/ml ionomycin (Sigma-Aldrich) and 10 μg/ml Brefeldin A (Sigma-Aldrich) for 5 h. Surface markers were stained with the indicated antibodies: FITC-anti-CD3, PE-anti-CD4, APC-anti-CD8, APC-anti-CD25 and APC-anti-CD4 (Thermo Fisher Scientific). Then cells were fixed with Fixation/ permeabilization Buffer (BD Biosciences, CA, USA), permeabilized with Perm/Wash buffer (BD Biosciences) and stained with the following antibodies: PE-anti-IFN-γ, FITC-anti-IL-17A, APC-anti-IL-4 (Thermo Fisher Scientific) according to the manufacturer's instructions. For Foxp3 intracellular staining, cells were treated with Foxp3 buffer (BD Biosciences), followed with FITC-anti-Foxp3 staining. Stained cells were evaluated by FACS analysis and data were analyzed by FlowJo software (Tree Star).

Purification of T cells and DN T cells

Total CD3 T cells isolated from spleens of MRL/*lpr* mice were obtained with negative selection using a Mouse T Cell Isolation Kit (Stem Cell Technologies Inc, Vancouver, Canada) according to the manufacturer's instructions. On the basis, DN T cells were purified from these CD3 T cells by Fluorescent cell sorting with anti-CD3, anti-CD4 and anti-CD8 antibodies using BD FACSAria (BD

Biosciences).

DN T cell proliferation assay

Purified DN T cells were re-suspended in RPMI 1640 with 10% heat-inactivated FBS, 50 U/ml of penicillin and 50 U/ml of streptomycin. Then 1×10^5 DN T cells, in a volume of 100 μl per well, were cultured in a 96-well plate, with 1 μg/ml anti-CD3 (Biolegend, CA, USA) and 0.5 μg/ml anti-CD28 (Biolegend) to stimulate naïve T cells. These cells were cultured for 48 hours in the presence of control or NCTD (5 or 10 μg/ml) and the cell number was monitored as above by the Cedex XS cell analysis system (Roche).

Purification of CD4 T cells and in vitro Th17 cell differentiation

Total CD4 T cells from spleens of MRL/*lpr* mice were purified with negative selection using a Mouse T Cell Isolation Kit (Stem Cell Technologies Inc) according to the manufacturer's instructions. The purity was determined by FACS analysis (>98% CD4+). For T cell differentiation, the above purified naïve CD4+ T cell were cultured in RPMI 1640 with 10% heat-inactivated FBS and 2 mM L-glutamine in the presence of 1 μg/ml anti-CD3 (Biolegend) and 0.5 μg/ml anti-CD28 (Biolegend), followed by treatment with or without NCTD. Then, 2 ng/ml transforming growth factor-1(TGF-β1) (Peprotech, Rocky Hill, NJ, USA), 20 ng/ml IL-6 (Peprotech), 10 μg/ml anti-IL-4 antibody (Biolegend) and 10 μg/ml anti- IFN-γ antibody (Biolegend) were added to drive Th17 cell polarization. After 4 days, cells were collected for FACS analysis and cell culture supernatants were also collected to detect the IL-17A production as described above.

Immunoblotting analysis

DN T cells purified from MRL/*lpr* mice administrated with NCTD or control, as well as DN T cells treated with or without NCTD were homogenized in RIPA buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease and phosphatase inhibitors (Beyotime Biotechnology). Cell lysates (40 μg) were separated on SDS-PAGE and immunoblotted using antibodies against the following proteins: phospho-STAT3 (Y705) (Cell Signaling Technology, MA, USA), STAT3 (Cell Signaling Technology) and β-actin (Sigma-Aldrich). CD4+ T Cells treated with or without NCTD (10 μg/ml) were stimulated with IL-6

or TGF-β1 in time gradient and immunoblotted using antibodies against the following proteins: phospho-STAT3 (Y705) (Cell Signaling Technology), STAT3 (Cell Signaling Technology), p-Smad2 (Cell Signaling Technology), p-Smad3 (Abcam Ltd), Smad2 (Cell Signaling Technology), Smad3 (Abcam Ltd) and β-actin (Sigma-Aldrich).

Statistical analysis

All results were expressed as the mean ± SE and were determined by t-test or two-way ANOVA with Graphpad Prism 8, as appropriate. If ANOVA was significant, individual differences were determined with Tukey post-test. In our studies, P values < 0.05 were considered statistically significant.

Results

NCTD treatment significantly prevents SLE development of MRL/ lpr mice

To well define the role of NCTD in SLE, lupus prone female MRL/*lpr* mice were used. According to previous research, MRL/*lpr* mice spontaneously develop symptoms of SLE similar to human SLE from 12 weeks [27]. As a result, MRL/MpJ and MRL/*lpr* female mice were treated with vehicle or NCTD (1 mg/kg and 2 mg/kg) everyday from 12 weeks of age to 20 weeks (Fig. 1a). At the stage, the death of mice was recorded and survival curves displayed that there was a raised tendency of survival rate in the 1 mg/kg NCTD treatment group and a notably improvement in 2 mg/kg NCTD treatment group compared with the control group (Fig. 1b). Besides, the level of serum anti-dsDNA antibodies, a diagnostic marker for SLE[28], was significantly decreased in a dose and time-dependent manner for NCTD treatment (Fig. 1c). Moreover, the weight of spleens isolated from NCTD-treated MRL/*lpr* mice was substantially reduced (Fig. 1d). Altogether, our data suggest that NCTD is able to alleviate lupus symptoms and improves survival rate of MRL/*lpr* mice dose-dependently.

Restored renal structures and functions in NCTD-administrated MRL/lpr mice

Next, we analyzed the renal structure and function of MRL/*lpr* mice. Histologically, lupus mice spontaneously developed progressive crescent glomerulonephritis and substantial inflammatory cell infiltration in kidneys [25], which were assessed by histologic examination of H&E and PAS-stained kidney sections (Fig. 2a-b). According to the composite score integrating glomerular deposition, glomerular crescent formation, immune cell infiltration and endocapillary proliferation of PAS-stained sections, we found that NCTD prevented renal damage of MRL/*lpr* mice and the inhibitory effect of 2 mg/kg/d NCTD was much better than that of 1 mg/kg/d (Fig. 2b-c). Moreover, to monitor the effects of NCTD on renal function, urine samples were collected to measure the total protein and albumin: creatinine ratio. As expected, we observed that NCTD-treated MRL/*lpr* mice showed a notable decrease in total protein (Fig. 2d), as well as a reduction in albumin: creatinine ratio (Fig. 2e) in urine, indicating a prominent restoration

in renal function for the use of NCTD. Considering the immune complex deposition as an important pathological mechanism in lupus nephritis (LN), IgG deposit in kidneys was evaluated via IgG staining. As shown in Fig.2f-g, IgG deposit in the glomerulus was remarkably inhibited for NCTD administration in MRL/*lpr* mice. Collectively, these results clearly define that NCTD prevents and restores renal damage in MRL/*lpr* mice in a dose-dependent way.

NCTD limits inflammatory responses of MRL/lpr mice

Previous studies have proved that lots of cytokines are closely linked to the severity of SLE, including IFN-γ, IL-17A, IL-22, IL-23, IL-6, TNF-α and so on [29, 30]. During SLE pathogenesis, IFN-γ is secreted by Th1 and DN T cells; IL-17A is mainly produced by Th17 and DN T cells; IL-22, IL-6 and TNF-α are also expressed by Th17 cells; Meanwhile, IL-23 is indispensable for the later stabilization of Th17 cells and is able to induce the production of IL-17, IL-22 , IL-6 and TNF-α [31-33]. Besides, IL-4 and IL-10 were respectively secreted by Th2 and Treg cells. To further characterize the effect of NCTD in the development of SLE, we measured the levels of representative cytokines in serum of MRL/*lpr* mice at 20 weeks. There was a significant reduction in levels of IFN-γ, IL-17A, IL-22, IL-23, IL-6 and TNF-α for NCTD treatment (Fig. 3a-f), while no significant differences were observed in IL-4 and IL-10 levels between NCTD-administrated and vehicle control groups (Fig. 3g-h). These data prove that CTS treatment inhibits the inflammatory response, particularly IL-17-related cytokine dominant inflammation in MRL/*lpr* mice.

Decreased lymphoproliferation and T cell accumulation in MRL/lpr mice for NCTD treatment

MRL/*lpr* mice develop massive lymphoproliferation and finally result in splenomegaly [27, 34]. The spleen cells from vehicle-treated mice were more than that of NCTD-treated mice (Fig. 4a) consistent with the results of spleen weight (Fig. 1d). According to our results about inflammatory cytokines (Fig.4), we speculate that NCTD regulated T cell functions. Therefore, we further explored the effect of NCTD on lymphoproliferation in spleens by analyzing the T and B populations using flow cytometry. Results showed that no significant differences in B cell percentage and number between NCTD-administrated and

vehicle-treated mice (Fig. 4b-c), while the proportion and total number of T cells were reduced in NCTD groups compared to control group (Fig. 4d-e). On this basis, further analyses found that the percentage and number of DN T cells were remarkably decreased for NCTD treatment (Fig. 4f-g). Besides, despite the improvement of CD4+ and CD8+ T cell proportion, the number of CD4+ T cells was reduced in NCTD-treated group, while the number of CD8+ T cells was not been effected (Fig.4f-g). These results suggest that NCTD suppresses T cell hyperproliferation and reduces the generation of pathogenic T cells in MRL/*lpr* mice.

NCTD treatment blocks STAT3-mediated DN T cell proliferation

According to our previous data, DN T cell accumulation was inhibited for NCTD treatment. STAT3 pathway has been demonstrated to directly regulate T cell survival and proliferation [35]. As a consequence, we firstly detected the STAT3 activation in DN T cells purified from NCTD and vehicle-treated MRL/*lpr* mice and results showed that NCTD notably suppressed STAT3 activation in a dose-dependent manner (Fig. 5a-b). To further confirm the role of NCTD in mediating DN T cell proliferation, we purified DN T cells from spleens of MRL/*lpr* mice. The DN T cells were cultured with or without NCTD for 48 hours. We found that the proliferation of DN T cell was blocked for NCTD treatment with the stimulation of anti-CD3 and anti-CD28 antibodies (Fig. 5c). Consistently, the phosphorylation of STAT3 was also reduced in NCTD-treated DN T cells (Fig. 5d-e). Taken together, these data identify that NCTD treatment impairs DN T cell proliferation by inhibiting STAT3 activation and contributes to the attenuated SLE development.

Impaired Th17 polarization of NCTD-treated MRL/lpr mice

Our analysis of cytokines in serum has revealed that the level of Th17-associated cytokines (IL-17A, IL-22, IL-6 and IL-23) in serum was diminished for NCTD treatment (Fig.3b-e). These indicated the potential role of NCTD on Th17 cell differentiation. To verify our supposition, we monitored the proportion of T cell subsets by flow cytometry and found that the percentage of Th17 cells (CD4+IL-17A+) dramatically decreased in the NCTD-treated MRL/*lpr* mice compared to the vehicle control groups (Fig.6a-b). However,

no obvious diminution of Th1, Th2 or Treg cells was observed for the use of NCTD (Fig.6a, c-g). To further confirm our conclusion, total CD4+ T cells isolated of splenocytes from individual MRL/*lpr* mice, treated with or without NCTD for 9 weeks, were stimulated with anti-CD3 and anti-CD28 for 24 hours. Then the culture supernatants were collected and cytokine secretion was examined. The results exhibited that the production of IL-17A was blocked in CD4+ T cells isolated from NCTD-treated MRL/*lpr* mice (Fig.6h), while the level of and IFN-γ, IL-10 and IL-4 remained unchanged (Fig.6i-k). Together, our findings indicate that NCTD administration disturbs Th17 polarization *in vivo*.

NCTD suppresses Th17 cell differentiation via blocking IL-6-STAT3 pathway

To further clarify the direct role of NCTD in Th17 cell polarization, we subsequently analyzed the percentage of Th17 cell under the Th17 cell differentiation condition with or without NCTD treatment. Results showed that NCTD notably inhibited Th17 cell generated from CD4+ naïve T cells in a dose-dependent manner (Fig.7a-b). We subsequently analyzed the production of IL-17A and found that the IL-17A expression was decreased (Fig.7c) consistent with our above results (Fig.6h).

Th17 cell differentiation is dependent on the activation of STAT3 induced by IL-6, which promotes the expression of retinoic acid-related orphan receptor γt (RORγt), the key transcription factor of Th17 cells [36]. Besides, in conjunction with IL-6, TGF-β1 also promotes CD4+ T cell differentiation into Th17 cells [37, 38]. We therefore detected the activation of IL-6-STAT3 and TGF-β1-Smad2/3 pathways. As shown in Fig.7d-e, STAT3 phosphorylation was dramatically suppressed for NCTD administration in response to IL-6 stimulation time-dependently, while the level of STAT3 remains unchanged. At the same time, we analyzed the phosphorylation of Smad2 and Samd3 under TGF-β1 stimulation. Results showed that NCTD treatment had no influence on the phosphorylation and expression of Smad2/3 (Fig.7f-h). These observations clearly define that NCTD effectively disrupts IL-6-STAT3 pathway rather than TGF-β1-Smad2/3 pathway to regulate Th17 cell polarization.

Discussion

NCTD, isolated from Mylabris, has been used against various human cancers by inhibiting cell proliferation, inducing apoptosis or anti angiogenesis [39-41]. Previous study has proved that NCTD is able to protect renal functions via blocking renal inflammation and proteinuria [42], while the role of NCTD in SLE remains unknown. SLE is associated with lots of clinical and immunologic manifestations, in which LN is the most important predictor for morbidity and mortality [43]. Nephritis development in SLE patients are indicated by proteinuria, diffuse inflammation and decreased renal function for multiple pathogenic factors including aberrant cell apoptosis, autoantibody accumulation and immune complex deposition [44]. Our results unveiled that NCTD remarkably ameliorated SLE symptom, especially LN via blocking STAT3-dependent DN T cell accumulation and Th17 cell differentiation.

We found that NCTD suppressed the progress of SLE dose-dependently, as illustrated by improved survival rate, suppressed autoantibody production (anti-dsDNA), alleviative systemic inflammatory response and relieved LN as well as restored renal structure and functions (Fig.1-3). Then, analysis on spleen cells showed that NCTD significantly attenuated splenomegaly accompanied with the reduced percentage and number of T cells. Further research proved the number of DN T cells and CD4+ T cells decreased in response to NCTD (Fig.4). Moreover, *in vivo* and *in vitro* results identified that phosphorylation of STAT3 was prominently impaired for NCTD treatment, resulting in repressed DN T cell proliferation (Fig.5). In addition, analysis of CD4+ T cell subsets exhibited that NCTD specifically inhibited Th17 cell polarization while had no affect on Th1, Th2 and Treg cell differentiation (Fig.6). Consistent with *in vivo* results, NCTD blockaded Th17 cell polarization and IL-17A secretion *in vitro* via positively mediating IL-6-STAT3 signaling (Fig.7). Our findings indicate that NCTD may be an attractive therapeutic drug for SLE.

IL-17, a pro-inflammatory cytokine, is involved in the pathogenesis of SLE. The elevated level of IL-17 is positively corrected with the SLE disease activity. The deficiency of IL-17 protected mice from SLE associated with decreased auto-antibodies and LN [45, 46]. During SLE development, DN T cells and Th17

cells produce the main amounts of IL-17. Therefore, IL-17-producing cells would be a potential target for SLE treatment. Our results showed that NCTD significantly blocked DN T cell accumulation and limited Th17 cell differentiation, while the percentage of Th1, Th2 and Treg cells remained unchanged. Consistently, Th17-related cytokines including IL-17A, IL-22 and IL-23 in serum were decreased for NCTD treatment. On the other hand, the level of IL-4 (Th2 cytokine) and IL-10 (Treg cytokine) had not been affected. However, Th1 cytokine IFN-γ in serum was also reduced though Th1 cell percentage was not inhibited for NCTD treatment because DN T cells were also an important source for IFN-γ except Th1 cells during SLE pathological course. Moreover , IL-6 , required to activate STAT3, was produced by Th17 cells and also reduced in response to NCTD, which resulted in a continual circle of attenuated Th17 differentiation.

STAT3, an essential transcription factor in the pathogenesis of SLE [47], orchestrates multiple aspects of T cell function including regulating T cell activation, proliferation and Th17 cell differentiation[4, 35]. In our previous study, we have demonstrated that STAT3 signaling positively regulated DN T cell proliferation in MRL/*lpr* mice [48]. Here, we found that NCTD notably decreased STAT3 phosphorylation of DN T cells and resulted in disrupted DN T cell proliferation. Moreover, Th17 cell polarization was also inhibited responded to NCTD by impairing IL-6-stimualted STAT3 activation rather than TGF-β1-dependent Smad2/3 pathway. These results were consistent with the conclusion that NCTD has no influence on Treg cell differentiation because TGF-β1-Smad2/3 signaling is also responsible for Treg cell generation [49]. Further studies focusing on the mechanism of NCTD regulated STAT3 activation may contribute to the development and application of NCTD in SLE progression.

Conclusions

As illustrated in Fig. 8, our findings clearly indicated that NCTD significantly alleviates the lupus symptoms of MRL/*lpr* mice by blocking IL-17-producing cell accumulation including DN T cells and Th17 cells dose-dependently. Furthermore, we found NCTD impaired DN T cell proliferation and inhibited Th17 cell

differentiation both via disrupting STAT3 activation. This study suggests that NCTD may have potential clinical values in treating SLE or other IL-17-telated diseases.

Abbreviations

BAFF: B-cell activating factor; DN T cell: Double-negative T cell; ELISA: Enzyme-linked immunosorbent assay; H&E: Hematoxylin and eosin; IF: Immunofluorescence; IL-17: Interleukin-17; LN: Lupus nephritis; NCTD: Norcantharin; PAS: Periodic acid-Schiff; RORγt: Retinoic acid-related orphan receptor γt; RA: Rheumatoid arthritis; SLEDAI: SLE Disease Activity Index; SLE: Systemic lupus erythematosus; STAT3: Signal transducer and activator of transcription 3; TGF-β1: Transforming growth factor-1; Th cell: T helper cell; Th17 cell: T helper 17 cell

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Authors' contributions

Y.Z., L.J. and C.P. designed the research, Y.Z., L.J., Y.X., Z.X., L.H. and H.C. performed the experiments, collected and analyzed the data. C.P. and Y.Z. wrote the paper.

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests.

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Figure legends

Fig.1. NCTD treatment significantly prevents SLE development of MRL/*lpr* mice

(a) MRL/*lpr* and MRL/MpJ female mice were randomly divided into three groups and administered with vehicle control, NCTD 1 mg/kg or 2 mg/kg via i.p. every day for 9 weeks as the indicated scheme.

(b) Survival curve of 12- to 20-week-old MRL/*lpr* mice subjected to either control or NCTD treatment. n= 10 mice/group. * $p < 0.05$.

(c) The serum was collected from 12 week and level of anti-dsDNA antibody was monitored, n=4 /group. ** $p < 0.01$, *** $p < 0.001$.

(d) Spleen weight to tibia length ratios of MRL/*lpr* and MRL/MpJ mice subjected to either vehicle or NCTD treatment (1 mg/kg and 2 mg/kg). n=4, 5, 7, or 9 mice/group. * $p < 0.05$, ** $p < 0.01$.

Fig.2. NCTD administration attenuated renal involvement in MRL/*lpr* mice

(a,b) Representative images of H&E and PAS staining from kidneys of 20-week-old MRL/*lpr* and MRL/MpJ mice treated with vehicle control or NCTD. Scale bar = 50 μm, n=4, 7, or 9 mice/group.

(c) Histologic damage of kidneys was evaluated with pathological scores. The score of each mouse was calculated from the total scores of observed five glomeruli. n=4, 7, or 9 mice/group. * $p < 0.05$, *** $p < 0.001$.

(d) Decreased urine protein level was found in NCTD-treated MRL/*lpr* mice. n=4, 7, or 9 mice/group. * $p < 0.05$, ** $p < 0.01$.

(e) Albumin and creatinine in urine were analyzed and the ratio of albumin to creatinine was declined for NCTD treatment in MRL/*lpr* mice. n=4, 7, or 9 mice/group. * $p < 0.05$, ** $p < 0.01$.

(f) IgG deposition in the glomeruli was revealed by immunofluorescence staining of IgG (green). White arrows indicate glomeruli. Representative images were shown. Scale bar = 50 μm, n= 4, 7, or 9 mice/group.

(g) The mean fluorescence intensity of IgG accumulation was assessed by Image J. n=4, 7, or 9 mice/group. * $p < 0.05$,** $p < 0.01$.

Fig.3. Decreased inflammation level in NCTD-treated MRL/*lpr* mice

(a-h) The levels of cytokines (IFN-γ, IL-17A, IL-22, IL-23, IL-6, TNF-α, IL-4 and IL-10) in serum were determined with ELISA. n=4 mice/group. * $p < 0.05$, *** $p < 0.001$.

Fig.4. Reduced lymphoproliferation and T cell accumulation for NCTD administration

(a) Total splenocyte number was counted in MRL/*lpr* mice treated with vehicle control or NCTD. n=4, 7, or 9 mice/group. * $p < 0.05$.

(b,c) Percentage and number of total B cells in spleens from MRL/*lpr* mice were analyzed via flow cytometry. n=4 mice/group.

(d,e) Percentage and number of total T cells in spleens from MRL/*lpr* mice were analyzed via flow cytometry. n=4 mice/group. * $p < 0.05$,** $p < 0.01$.

(f,g) Flow cytometric analysis of CD3 gated cells to identify T cell subsets including DN (CD4-CD8-), CD4 and CD8 cells from spleens of 20-week-old MRL/*lpr* mice treated with vehicle control or NCTD. Then the percentage and total number of T cell subsets were quantified according to the results of flow cytometry. n= 4 mice/group. * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$.

Fig.5. NCTD treatment blocked DN T cell proliferation by inhibiting STAT3 activation

(a,b) Western blot assay and densitometry analysis indicating the phosphorylation of STAT3 (Y705) was blocked in DN T cells isolated from the spleens of NCTD-treated MRL/*lpr* mice. β-actin was used as loading control. Data were representive of three independent experiments. * $p < 0.05$, *** $p < 0.001$.

(c) DN T cells purified from spleens of MRL/*lpr* mice were cultured (1×10^5 cells/well) in 96-well plates for 48 hours in the presence of vehicle control or NCTD (5 or 10 μg/ml) as well as incubated with anti-CD3 and anti-CD28 antibodies to determine DN T cell proliferation. n=3 /group. * $p < 0.05$, ** $p < 0.01$.

(d,e) DN T cells purified from spleens of MRL/*lpr* mice were cultured (1×10^5cells/well) in 96-well plates for 48 hours in the presence of control or NCTD (5 or 10 μg/ml) as well as incubated with anti-CD3 and anti-CD28 antibodies. Cells were then obtained and immuneblotted with anti-p-STAT3 and anti-STAT3

antibodies. Results showed that NCTD suppressed STAT3 activation in DN T cells. β-actin was used as loading control. Data were representive of three independent experiments. *** $p < 0.001$.

Fig.6. Impaired Th17 cell differentiation *in vivo* for NCTD treatment

(a-c) Cells isolated from spleens of vehicle control and NCTD-treated MRL/*lpr* mice were gated on CD4 and stained with anti-IL-17A and anti-IFN-γ antibodies. Representative flow cytometry plots (a) and statistical data (b,c) showing a significant decline of Th17 cell proportion and unchanged Th1 cells for NCTD treatment. n=4mice/group. * $p < 0.05$,** $p < 0.01$.

(d,e) Representative flow cytometry plots (d) and statistical data (e) showing no significant difference of Treg cell (CD4+CD25+Foxp3+) proportion for NCTD treatment. n=4mice/group.

(f,g) Representative flow cytometry plots (f) and statistical data (g) showing no significant difference of Th2 cell (CD4+IL-4+) proportion for NCTD treatment. n=4mice/group.

(h-k) Total CD4+ T cells isolated from spleens of MRL/*lpr* mice treated with control or NCTD were stimulated with anti-CD3 antibody for 24 hours. Then, the culture supernatants were collected and the cytokine production (IL-17A, IFN-γ, IL-10 and IL-4) was detected via ELISA. n=4 mice/group. * $p < 0.05$,** $p < 0.01$.

Fig.7. NCTD inhibited IL-6-STAT3-dependent Th17 differentiation *in vitro*

(a,b) Naïve CD4 T cells from MRL/*lpr* mice were collected and activated with anti-CD3 and anti-CD28, as well as with or without NCTD, then ploarized into Th17 cells for 4 days. Representative flow cytometry plots (a) and statistical data (b) displayed an observably decreased Th17 cell (CD4+IL-17A+) percentage for NCTD treatment. * $p < 0.05$, *** $p < 0.001$.

(c) The culture supernatants were collected and IL-17A level was monitored via ELISA. ** $p < 0.01$.

(d,e) Western blot assay and densitometry analysis indicating NCTD blocked the phosphorylation of STAT3 (Y705) upon IL-6 stimulation in T cells. β-actin was used as loading control. Data were representive of three independent experiments. * $p < 0.05$.

(f-h) Western blot assay and densitometry analysis indicated NCTD had no effect on the phosphorylation of Smad2/3 upon TGF-β1 stimulation in T cells. β-actin was used as loading control. Data were representive of three independent experiments.

Fig.8. A summarization about the protective effect and underlying mechanisms of NCTD in SLE development

NCTD treatment significantly impaired DN T cell accumulation and Th17 cell differentiation via inhibiting STAT3 pathway, finally resulting in alleviated lupus symptoms in lupus mice.

Fig.1

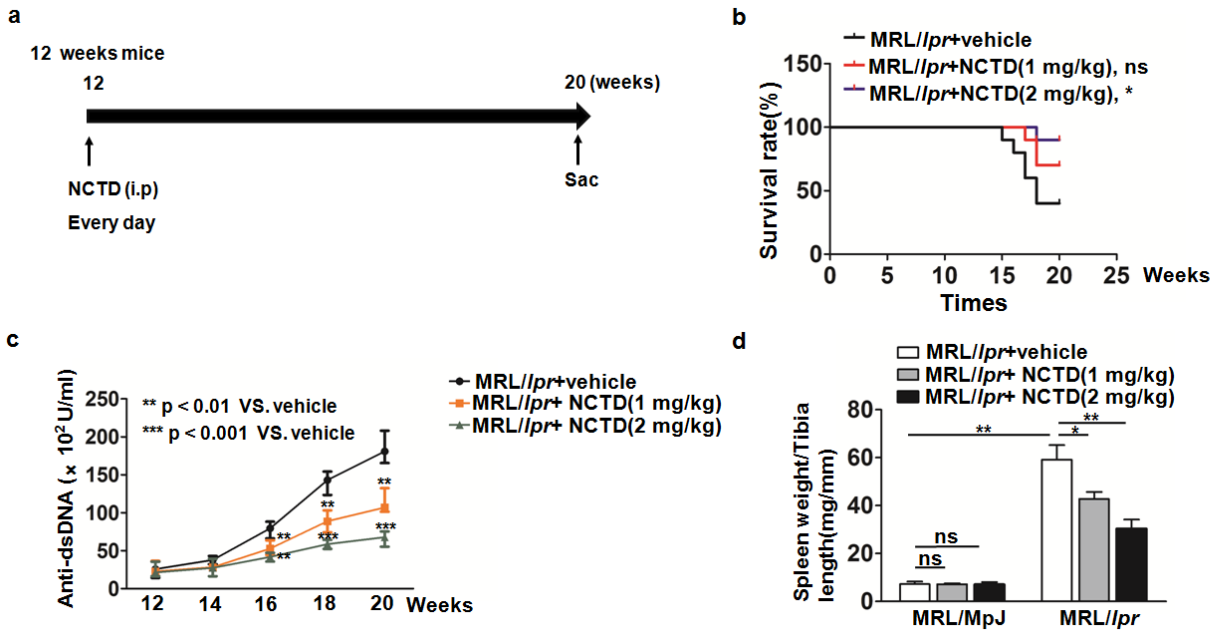

a
12 weeks mice
12
20 (weeks)
NCTD (i.p)
Every day
Sac
b
MRL/lpr+vehicle
MRL/lpr+NCTD(1 mg/kg), ns
MRL/lpr+NCTD(2 mg/kg), *
Survival rate(%)
Times
Weeks
c
** p < 0.01 VS. vehicle
*** p < 0.001 VS. vehicle
MRL/lpr+vehicle
MRL/lpr+ NCTD(1 mg/kg)
MRL/lpr+ NCTD(2 mg/kg)
Anti-dsDNA (× 10² U/ml)
Weeks
d
MRL/lpr+vehicle
MRL/lpr+ NCTD(1 mg/kg)
MRL/lpr+ NCTD(2 mg/kg)
Spleen weight/Tibia length(mg/mm)
ns
MRL/MpJ
MRL/lpr

Fig.2

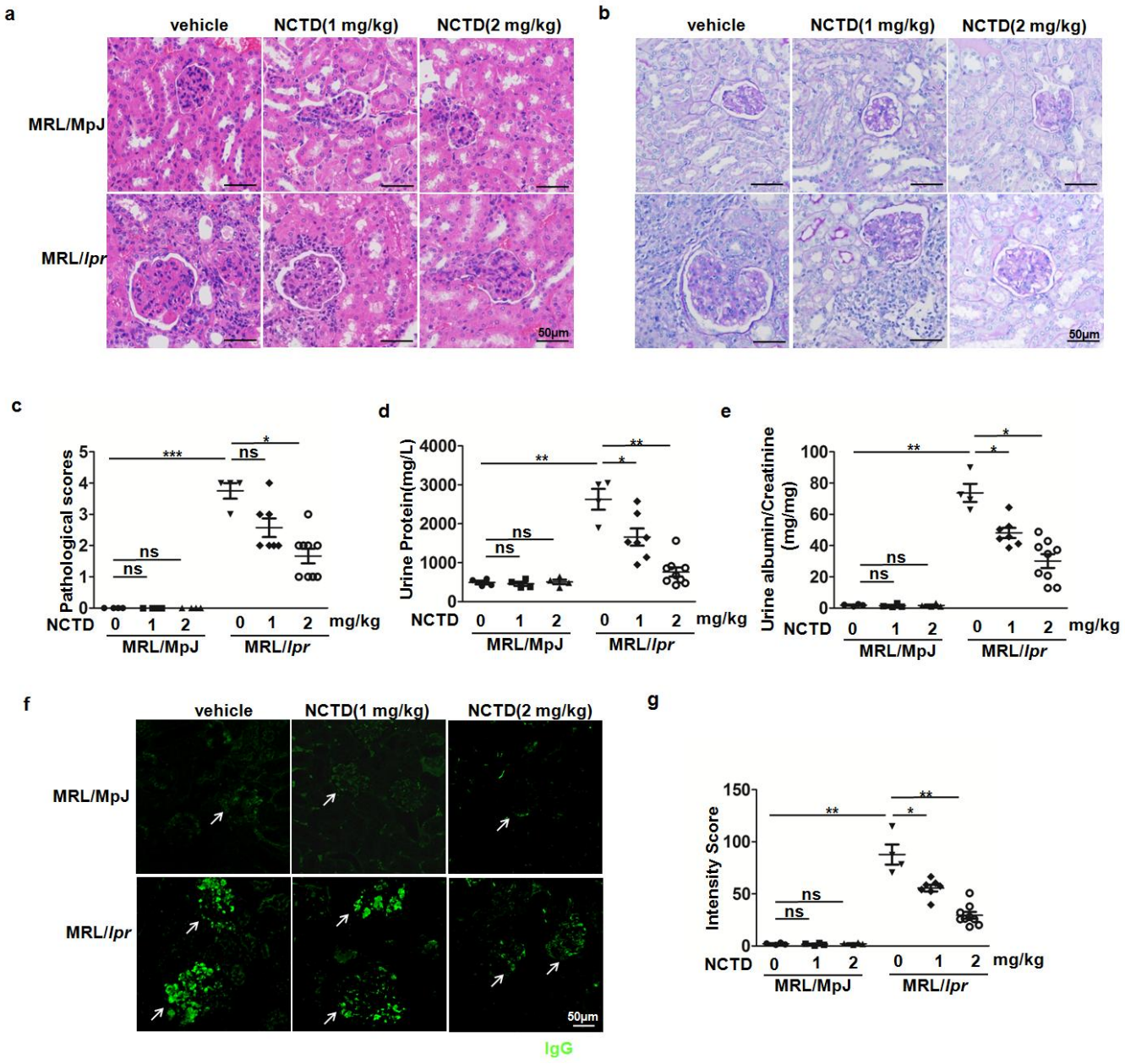

a
vehicle
NCTD(1 mg/kg)
NCTD(2 mg/kg)
MRL/MpJ
MRL/lpr
50μm
b
vehicle
NCTD(1 mg/kg)
NCTD(2 mg/kg)
50μm
c
Pathological scores
***
ns
ns
*
ns
NCTD 0 1 2 0 1 2 mg/kg
MRL/MpJ
MRL/lpr
d
Urine Protein(mg/L)
**
ns
ns
*
**
NCTD 0 1 2 0 1 2 mg/kg
MRL/MpJ
MRL/lpr
e
Urine albumin/Creatinine (mg/mg)
**
ns
ns
*
*
NCTD 0 1 2 0 1 2 mg/kg
MRL/MpJ
MRL/lpr
f
vehicle
NCTD(1 mg/kg)
NCTD(2 mg/kg)
MRL/MpJ
MRL/lpr
50μm
IgG
g
Intensity Score
**
ns
ns
*
**
NCTD 0 1 2 0 1 2 mg/kg
MRL/MpJ
MRL/lpr

Fig.3

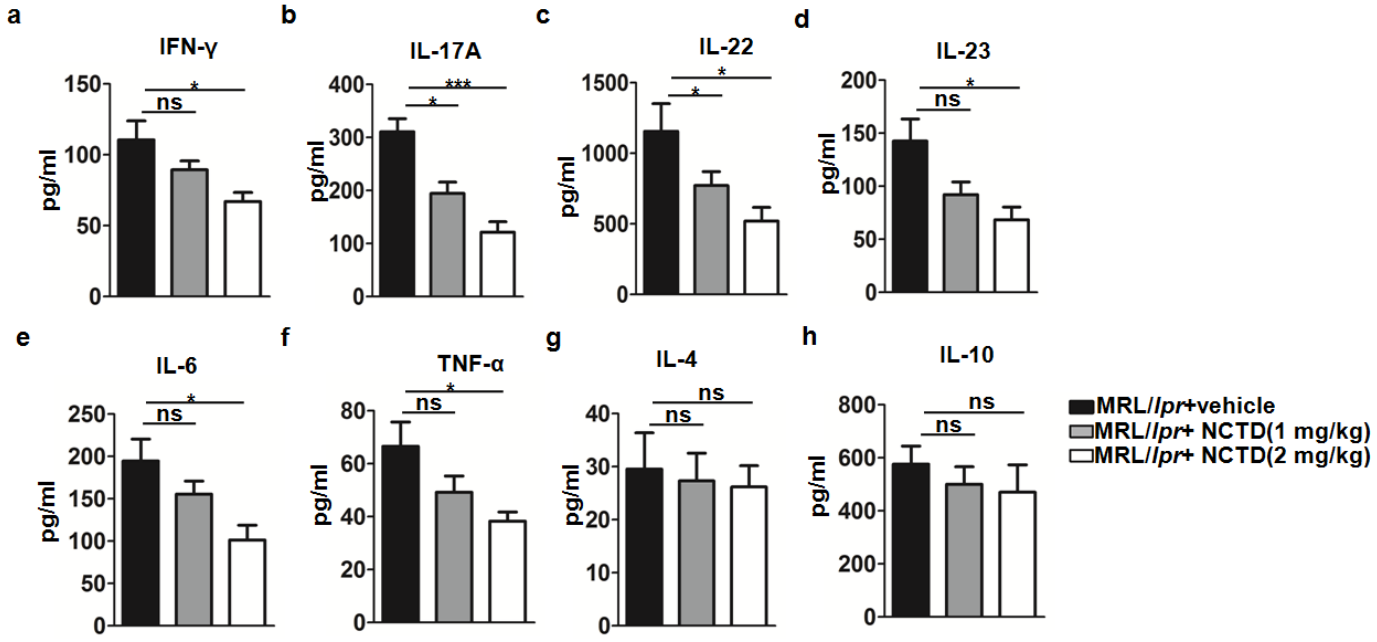

a
IFN-γ
pg/ml
150
100
50
0
*
ns
b
IL-17A
pg/ml
400
300
200
100
0
***
*
c
IL-22
pg/ml
1500
1000
500
0
*
*
d
IL-23
pg/ml
200
150
100
50
0
*
ns
e
IL-6
pg/ml
250
200
150
100
50
0
*
ns
f
TNF-α
pg/ml
80
60
40
20
0
*
ns
g
IL-4
pg/ml
40
30
20
10
0
ns
ns
h
IL-10
pg/ml
800
600
400
200
0
ns
ns
MRL/lpr+vehicle
MRL/lpr+ NCTD(1 mg/kg)
MRL/lpr+ NCTD(2 mg/kg)

Fig.4

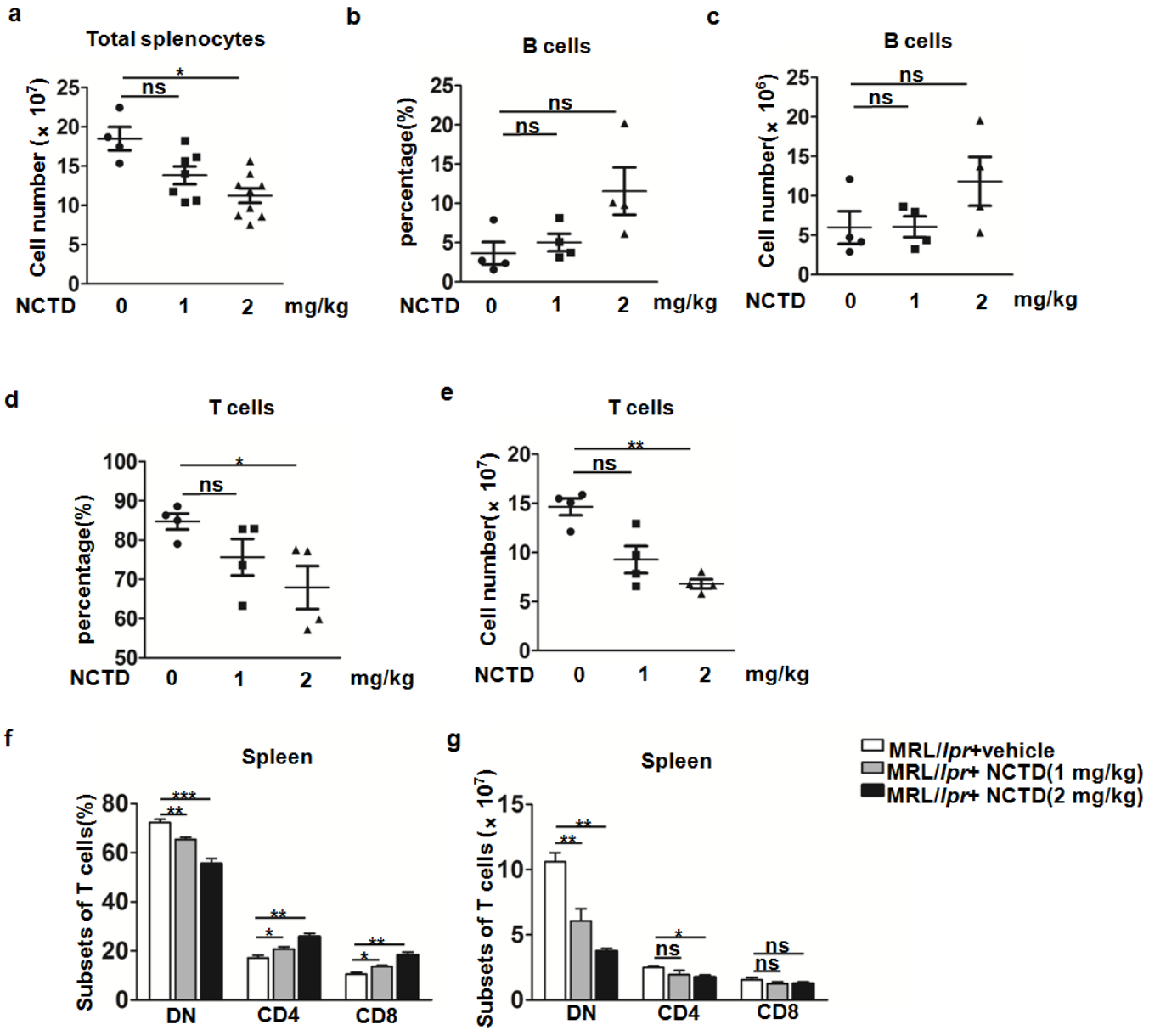

a
Total splenocytes
Cell number (× 10^7)
ns
*
NCTD 0 1 2 mg/kg
b
B cells
percentage(%)
ns
ns
NCTD 0 1 2 mg/kg
c
B cells
Cell number(× 10^6)
ns
ns
NCTD 0 1 2 mg/kg
d
T cells
percentage(%)
ns
*
NCTD 0 1 2 mg/kg
e
T cells
Cell number(× 10^7)
ns
**
NCTD 0 1 2 mg/kg
f
Spleen
Subsets of T cells(%)
DN CD4 CD8
g
Spleen
Subsets of T cells (× 10^7)
DN CD4 CD8
MRL/lpr+vehicle
MRL/lpr+ NCTD(1 mg/kg)
MRL/lpr+ NCTD(2 mg/kg)

Fig.5

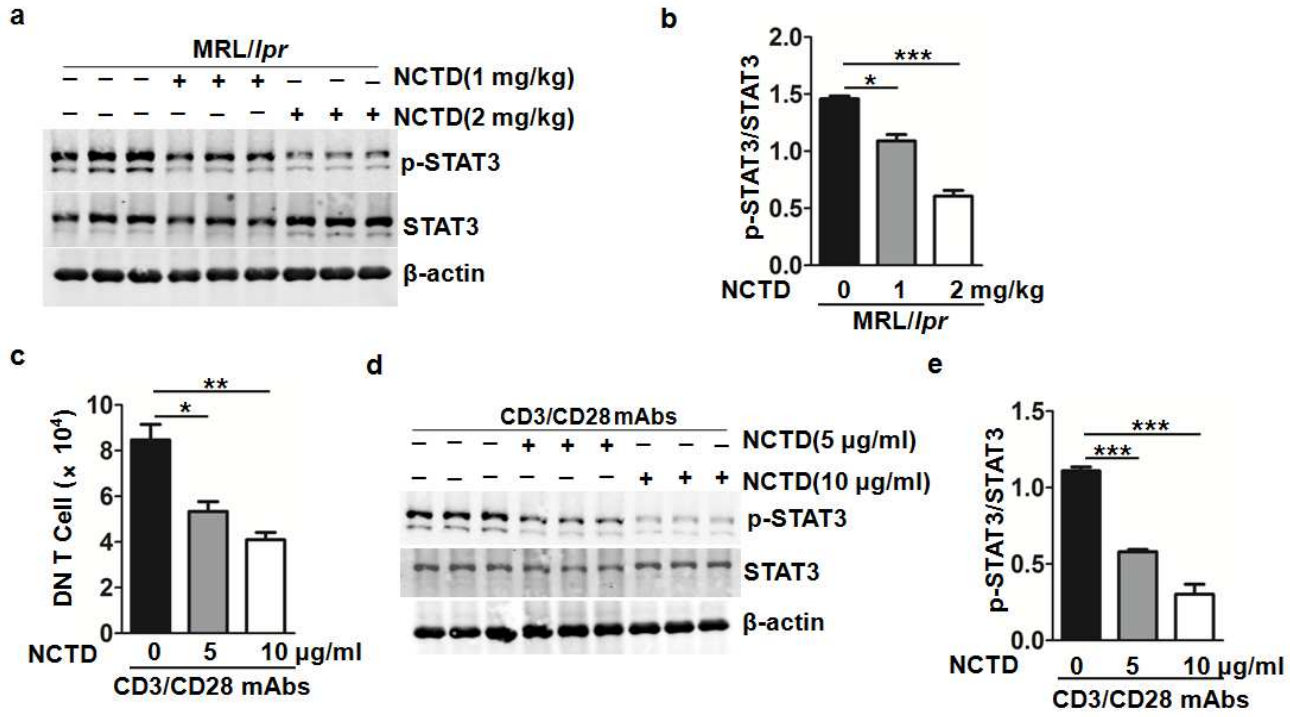

a
MRL/lpr
NCTD(1 mg/kg)
NCTD(2 mg/kg)
p-STAT3
STAT3
β-actin
b
p-STAT3/STAT3
NCTD
0 1 2 mg/kg
MRL/lpr
c
DN T Cell (× 10⁴)
NCTD
0 5 10 μg/ml
CD3/CD28 mAbs
d
CD3/CD28 mAbs
NCTD(5 μg/ml)
NCTD(10 μg/ml)
p-STAT3
STAT3
β-actin
e
p-STAT3/STAT3
NCTD
0 5 10 μg/ml
CD3/CD28 mAbs

Fig.6

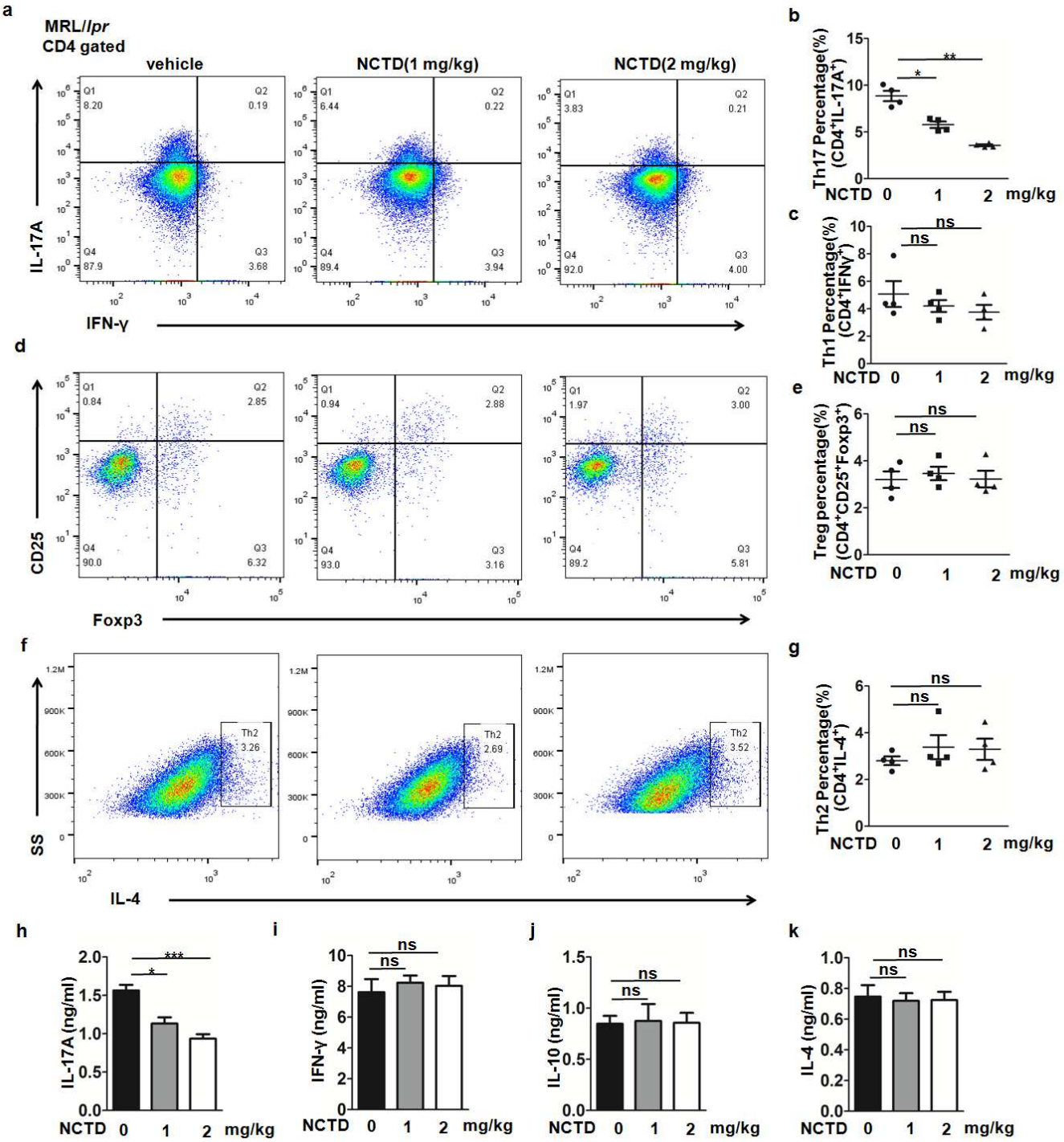

a
MRL/lpr
CD4 gated
vehicle
NCTD(1 mg/kg)
NCTD(2 mg/kg)
IL-17A
IFN-γ
Q1 8.20
Q2 0.19
Q4 87.9
Q3 3.68
Q1 6.44
Q2 0.22
Q4 89.4
Q3 3.94
Q1 3.83
Q2 0.21
Q4 92.0
Q3 4.00
b
Th17 Percentage(%) (CD4+IL-17A+)
NCTD 0 1 2 mg/kg
c
Th1 Percentage(%) (CD4+IFNγ+)
d
CD25
Foxp3
Q1 0.84
Q2 2.85
Q4 90.0
Q3 6.32
Q1 0.94
Q2 2.88
Q4 93.0
Q3 3.16
Q1 1.97
Q2 3.00
Q4 89.2
Q3 5.81
e
Treg percentage(%) (CD4+CD25+Foxp3+)
f
SS
IL-4
Th2 3.26
Th2 2.69
Th2 3.52
g
Th2 Percentage(%) (CD4+IL-4+)
h
IL-17A (ng/ml)
i
IFN-γ (ng/ml)
j
IL-10 (ng/ml)
k
IL-4 (ng/ml)
ns
*
**
***

Fig.7

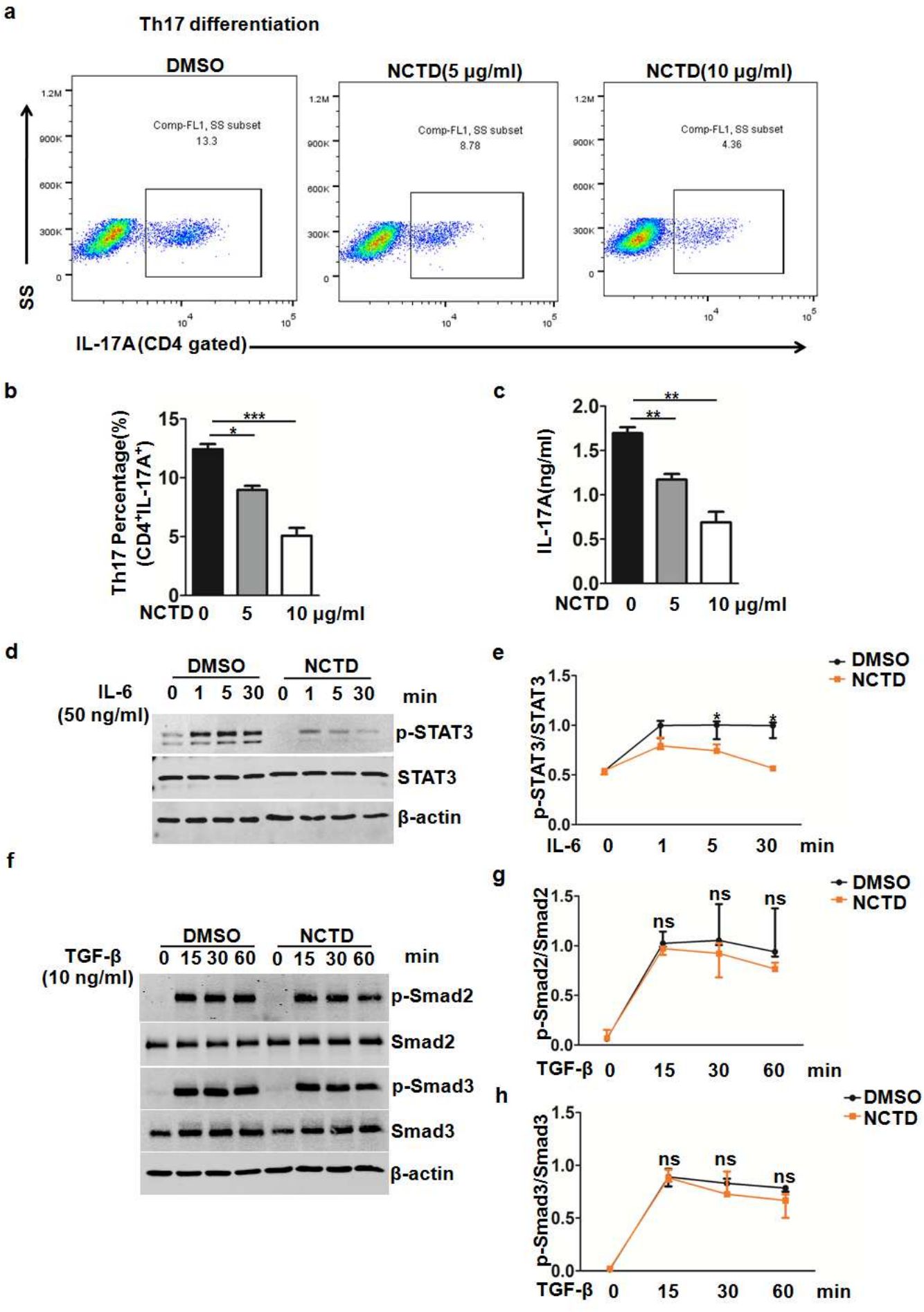
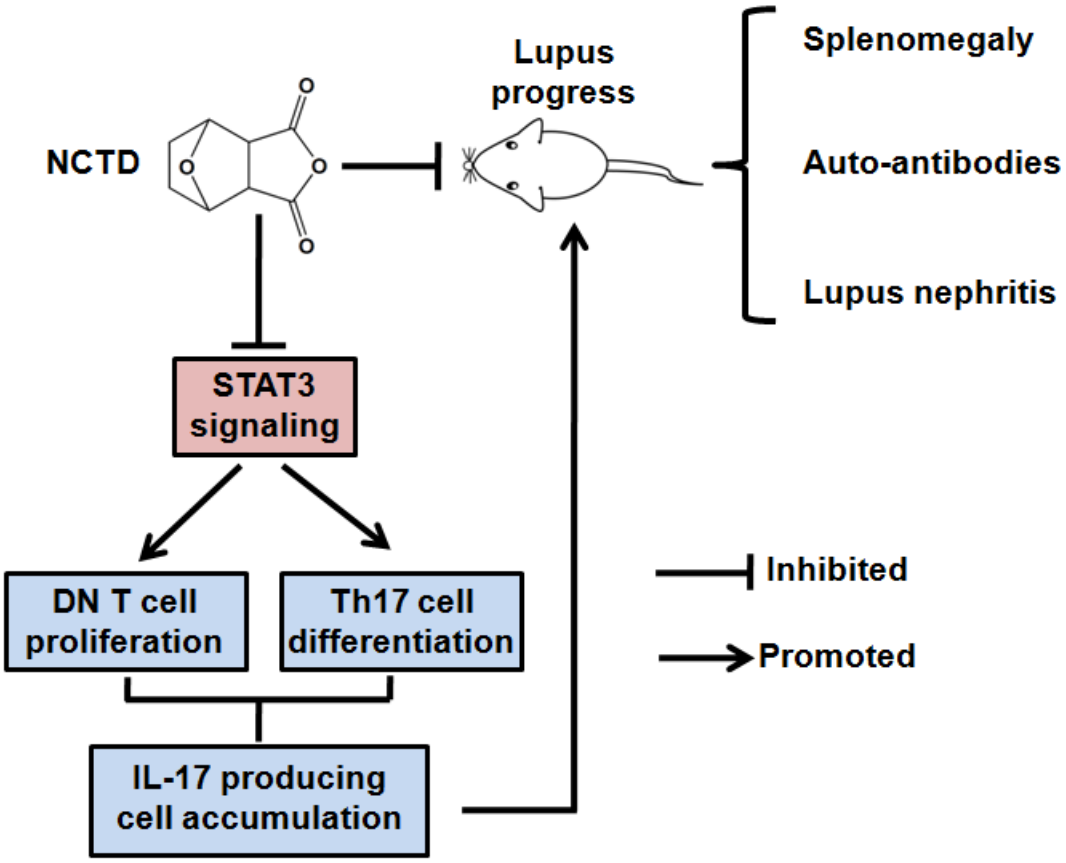

a
Th17 differentiation
DMSO
NCTD(5 μg/ml)
NCTD(10 μg/ml)
Comp-FL1, SS subset
13.3
8.78
4.36
SS
IL-17A(CD4 gated)
b
Th17 Percentage(%)
(CD4+IL-17A+)
NCTD 0 5 10 μg/ml
c
IL-17A(ng/ml)
NCTD 0 5 10 μg/ml
d
DMSO
NCTD
IL-6
(50 ng/ml)
0 1 5 30 0 1 5 30 min
p-STAT3
STAT3
β-actin
e
DMSO
NCTD
p-STAT3/STAT3
IL-6 0 1 5 30 min
f
DMSO
NCTD
TGF-β
(10 ng/ml)
0 15 30 60 0 15 30 60 min
p-Smad2
Smad2
p-Smad3
Smad3
β-actin
g
DMSO
NCTD
p-Smad2/Smad2
ns
TGF-β 0 15 30 60 min
h
DMSO
NCTD
p-Smad3/Smad3
ns
TGF-β 0 15 30 60 min

Fig.8


Lupus progress
Splenomegaly
NCTD
Auto-antibodies
Lupus nephritis
STAT3 signaling
DN T cell proliferation
Th17 cell differentiation
Inhibited
Promoted
IL-17 producing cell accumulation