Regulation of neutrophil immune responses by Foxp3+ Treg cells in Atopic dermatitis-like inflammation

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Regulation of neutrophil immune responses by Foxp3^{+} Treg cells in Atopic dermatitis-like inflammation

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ABSTRACT

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Regulatory T cells (Treg) are central immune cells that restrict autoimmunity and inflammation in the body. Atopic dermatitis (AD), a chronic inflammatory skin disease with T cell activation as a key feature, in which Th2 cell–mediated responses play a pivotal role. By using Treg depeltion and atopic dematitis-like inflammation mouse model, we demonstrate that the depletion of Foxp3$^+$ Treg led to significantly exacerbated AD-like skin inflammation, including increased recruitment of neutrophils and expression of Th1 cytokine IFN-γ. Neutrophil infiltrating in skin of Treg-depleted mice released more NETs than wild type. Neutralization of IFN-γ abolished neutrophil infiltration and NETosis in Treg-depleted mice. Neutrophils stimulated with IFN-γ were more prone to release NETs in vitro. Finally, Foxp3$^+$ Treg control cutaneous allergic inflammation by regulating IFN-γ-driven neutrophilic infiltration and NETosis.

Our study lights the previously underestimated Treg-IFN-γ-neutrophil inflammatory axis in atopic dematitis-like inflammation.

Key words :Atopic dermatitis, Regulatory T cells, NETosis, IFN-γ

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory condition characterized by skin barrier dysfunction, inflammation, and chronic pruritus[1]. Cellular infiltrates comprising predominantly T helper 2 (Th2) cells play a pivotal role in AD skin lesions, supporting the view that these cells play a role in disease pathogenesis and regression [2]. $CD4^+$ regulatory T cells (Treg) are indispensable constituents of the normal immune system that maintain immunological self-tolerance and homeostasis [3]. Treg express a variety of skin-homing addressins and actually reside in the skin , which demonstrate they may play a key role in skin immune surveillance.[4] There is general consensus that Treg cells play an important role in controlling or suppressing T-cell responses to allergens. Treg cells can exhibit plasticity and have the capacity to adapt in an inflammatory milieu[5]. The expansion of Treg inducing by AD-like inflammation exhibits a Th2-like phenotype in mice during AD-like inflammation[6].The identification of forkhead box P3 (Foxp3), a key transcription factor, is required for their development, maintenance and function[7, 8]. In humans, loss-of-function mutations affecting Foxp3 result in the development of immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome, which are characterized by autoimmunity and severe allergic inflammation, including AD [9]. Foxp3 mutant mice spontaneously exhibit allergic airway inflammation, AD–like skin disease, and increased serum IgE levels, independent of their genetic background [10]. Although these data support the notion that Treg cells can regulate AD skin pathology, the precise mechanism by which Treg cells control AD skin inflammation remains unclear.

AD has been recognized as a Th2 disease for the abundant production of Th2 cytokines, interleukin (IL)-4, IL-5 and IL-13 associated with eosinophilia and elevated serum IgE level. However, Th1 cells have also been involved. Th1 cytokines such as IFN-γ and IL-12 has also been found to be increased in chronic AD lesions[11].The blood signature of patients with AD is characterized by increasing IFN-γ[12]. Which demonstrate that fine-tuning of the delicate balance among Th1, Th2 cells is required for the control of AD.

Treg were shown to inhibit IFN-γ production [13]. Inhibition of IFN-γ production by $CD8^+$ T cells, $CD4^+$ T cells, and $CD56^+$ natural killer (NK) cells associated with expansion of Treg [14]. Although allergic dermatitis is thought to be mediated by Type 2 cytokine, these data also suggest that IFN may play an important role in allergic dermatitis, providing hypotheses for elucidating the mechanism by which Treg control allergic dermatitis. However, these studies make it difficult to interpret how IFN-γ regulates allergic dermatitis when Treg are depleted. Therefore, mechanisms underlying the pathology of Treg depletion during AD-like inflammation remain poorly understood.

In AD patients, Th2 cells, eosinophils, mast cells and dendritic cells are markedly increased in the skin lesions. However, transcriptional studies in patients with AD have revealed an overabundance of neutrophil signatures reflecting tissue neutrophilia [15]. In humans, the neutrophil-to-lymphocyte ratio in patients with AD was significantly higher in patients with AD than in healthy individuals [16]. In mice, neutrophils are the most abundant immune cell subtype in the early stage of AD-like inflammation mouse model [17]. However, the mechanism of

neutrophil-mediated pathology in AD-like skin inflammation remains unclear.

To investigate the mechanisms underlying the pathology and disease exacerbation resulting from Treg depletion, we utilized Foxp3-DTR mice to deplete $Foxp3^+$ Treg specifically, in which the human diphtheria toxin receptor (DTR) is inserted into the Foxp3 locus. This allowed us to eliminate $Foxp3^+$ Treg by the injection of diphtheria toxin (DT) [18].We also examined neutrophilic infiltration and IFN-γ gene expression in MC903-induced allergic skin inflammation in mice. The Treg-IFN-γ-neutrophil inflammatory axis were revealed which may provide new therapeutic strategies for the treatment of allergic dermatitis.

2. Materials and methods

2.1. Animals

Eight-week-old female C57BL/6J mice were purchased from The Jackson Laboratory(Bar Harbor, ME, USA). Foxp3-DTR mice were bred and maintained under specific pathogen-free conditions in accredited animal facilities at Yonsei University. All Foxp3-DTR mice were ≥7 weeks of age when used in experiments described.

2.2. AD-like skin inflammation and in vivo treatments

AD-like skin inflammation was induced by applying 0.5 nmol calcipotriol (MC903; Leo Pharma, Ballerup, Denmark) on mouse ears for eight consecutive days. To deplete $Foxp3^+$ Treg in Foxp3-DTR mice, DT(List Biological Laboratories, California, USA) 500 ng per mouse was injected intraperitoneally (i.p.) on days four and six after the start of MC903 treatment. For IFN-γ neutralization, mice were injected i.p. with 200μg of either anti-mouse-IFN-γ monoclonal antibody (αIFN-γ mAb) (clone XMG1.2; BioXCell, Lebanon, USA) or isotype control antibody (Ctrl Ab) (clone TNP6A7; BioXCell) on days 2, 4, and 6 of MC903 treatment.

2.3. Immunohistochemical staining of myeloperoxidase (MPO)

After deparaffinized, the paraffin sections were blocked using 3% H2O2 for 10 min at 37℃. Antigen retrieval was accomplished in 95℃ boiling water for 20 min in citrate buffer at pH 6.0, which was rinsed later with Tris-buffered saline with 0.01% TWEEN20. Sections were incubated with goat antibodies against mouse MPO(1:500; R&D Systems, Minneapolis, MN, USA) at 4℃ overnight, then incubated with biotinylated horseradish peroxidase-conjugated rabbit anti-goat antibodies (1:200; Vector Laboratories, Burlingame, CA, USA) for 30 minutes. The colored product was developed by incubation with 3,30-diamino-benzidine tetrahydrochloride dihydrate. Stained samples were examined under a light microscope.

2.4. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

The TUNEL assay was performed on mouse skin sections using the In-Situ Cell Death Detection Kit (Roche, USA) according to the manufacturer's recommendations. Nuclei were stained with DAPI and the images taken with fluorescence microscope (Olympus, Tokyo, Japan) and analyzed by ImageJ software.

2.5. Visualization of NETosis by immunofluorescence (IF)

Skin sections were incubated with primary anti-citrullinated histone H3 (CitH3; 1:200; Abcam; Cambridge, MA, USA) and anti-MPO (1:40; R&D systems) overnight at 4℃. After washing, the

sections were incubated with secondary Alexa Fluor 555-labeled anti-IgG antibodies (1:1000; Invitrogen, Carlsbad, CA, USA), Alexa Fluor 488-labeled anti-IgG antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and DAPI (1:1000;Invitrogen) for 2 h in the dark at 37℃. Images of the samples were subsequently acquired using a fluorescence microscope and analyzed using CellSens Standard imaging software (Olympus).

2.6. Flow cytometry

The whole ear skin samples were minced and digested in 1.6 mg/mL collagenase A (Roche Diagnostic, GmbH, Mannheim, Germany) and 0.1mg/mL DNase I (Roche) for 2 h at 37 ℃. For analysis of intracellular cytokine production, the ear tissues were incubated with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) in the presence of 10 μg/mL Brefeldin A (Sigma, St. Louis, MO, USA) for 5 h, and resultant cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA). Cells were stained with following fluorochrome-conjugated antibodies: anti-mouse BV421-labeled CD45, BV786-labeled CD90.2, Percp-cy5.5-labeled CD11b, APC-Cy7-labeled LyG, PE-Cy7-labeled LyC, PE-Cy5-labeled F4/80, and PE-Cy7-labeled IFN-γ antibodies from BioLegend (San Diego, CA, USA). APC-labeled interleukin (IL)-4 and IL-13 were purchased from eBioscience (San Diego, CA, USA). Stained cells were analyzed using an LSRFortessa flow cytometer (BD Biosciences). All flow cytometric data were analyzed using FlowJo software (Treestar, Ashland, OR, USA).

2.7. Quantitative real-time polymerase chain reaction

The Qiagen RNeasy Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used to extract total RNA from the ear skin of mice, which was then reverse transcribed into cDNAs using the Prime Script RT master MIX (Takara Bio, Shiga, Japan) following the manufacturer's instructions. The mRNA expression levels of IL-4, IL-5, IL-13, IFN-γ, Cleaved caspase-3, RIRK3, MLKL, Cxcl1, IL-33, IL-17a, IL-1β, and IL-10 were analyzed using qRT-PCR, with HPRT as an internal reference. The primer sequences used are shown in Table 1.

Table 1. Primer sequences used for real-time qPCR.

Gene name	Sequences
HPRT	Forward: 5′-TCAGTCAACGGGGGACATAAA-3′
	Reverse: 5′-GGGGCTGTACTGCTTAACCAG-3′
Ifng	Forward: 5′-GATGCATTCATGAGTATTGCCAAGT-3′
	Reverse: 5′-GAGGACCACTCGGATGAGCTC-3′

Il4	Forward: 5′-AGATCATCGGCATTTTGAAC-3′
	Reverse: 5′-TTTGGCACATCCATCTCCG-3′
Il5	Forward: 5′-CGCTCACCGAGCTCTGTTG-3′
	Reverse: 5′-CCAATGCATAGCTGGTGATTTTT-3′
Il13	Forward: 5′-AACAGCAACTCCCACTCTTC-3′
	Reverse: 5′-CCTGTTGCTGTAGCCGTATT-3′
Il33	Forward: 5′-TGA GGA GCT GAG CAA CAT CA-3′
	Reverse: 5′-ATC CAG GGC TAC ACA GAA CC-3′
Il17a	Forward: 5′-CAGCAGCGATCATCCCTCAAAG-3′
	Reverse: 5′- CAGGACCAGGATCTCTTGCTG -3′
Il1β	Forward: 5′-TGACGGACCCCAAAAGATGA-3′
	Reverse: 5′-GCTCTTGTTCATGTGCTGCT-3′
Il10	Forward: 5′-CCCTTTGCTATGGTGTCCTT-3′
	Reverse: 5′-TGGTTTCTCTTCCCAAGACC-3′
Cxcl1	Forward: 5′-CCGAAGTCATAGCCACACTCAA-3′
	Reverse: 5′-GCAGTCTGTCTTCTTTCTCCGTTA-3′
Cxcl2	Forward: 5′-GAAGTCATAGCCACTCTCAAGG-3′
	Reverse: 5′-CCTCCTTTCCAGGTCAGTTAGC-3′
Cleaved-caspase3	Forward: 5′-TGTCATCTCGCTCTGGTACG-3′
	Reverse: 5′-AAATGACCCCTTCATCACCA-3′
Bax	Forward: 5′-GGAGCAGCTTGGGAGCG-3′
	Reverse: 5′-AAAAGGCCCCTGTCTTCATGA-3′
RIRK3	Forward: 5′-CAGTGGGACTTCGTGTCCG-3′
	Reverse: 5′-CAAGCTGTGTAGGTAGCACATC-3′
MLKL	Forward: 5′-TTAGGCCAGCTCATCTATGAACA-3′
	Reverse: 5′-TGCACACGGTTTCCTAGACG-3′

2.8. Isolation of primary neutrophils from the mouse bone marrow and in vitro treatments

Wild type (WT) mice were euthanized and their tibias, fibula, and femurs were extracted and

collected. Muscle and connective tissues were removed from the bones, and the bone marrow from the cleaned bones was collected in clean tubes by centrifugation. Red blood cells from the bone marrow were removed using a Red Blood Cell Lysis buffer (eBioscience, no. 00-4300-54). Neutrophils were isolated from other bone marrow cells using a STEMCELL (StemCell Technologies, Vancouver, Canada). The purity of the cells was determined by labeling with lineage positive antibody using LSRFortessa FCM (BD Biosciences). These cells were incubated with 100 ng/mL IFN-γ (Peprotect, Rocky Hill, NJ, USA) or untreated for 4 h at 37°C.

2.9. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde in PBS for 15 min. After fixation, cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked for 1 h with 10% horse serum in PBS. The cells were incubated with primary anti-CitH3 (1:200; Abcam) and anti-MPO (1:500; R&D Systems) at 4℃ overnight. After washing with PBS, the cells were stained with the respective secondary antibodies for 2 h at 37℃ in a blocking buffer (PBS + 2% horse serum). Nuclei were counterstained with 1 μg/mL of DAPI for 4 min. Images of the cells mounted on slides were acquired using a fluorescence microscope and analyzed with CellSens Standard imaging software.

2.10.Statistics

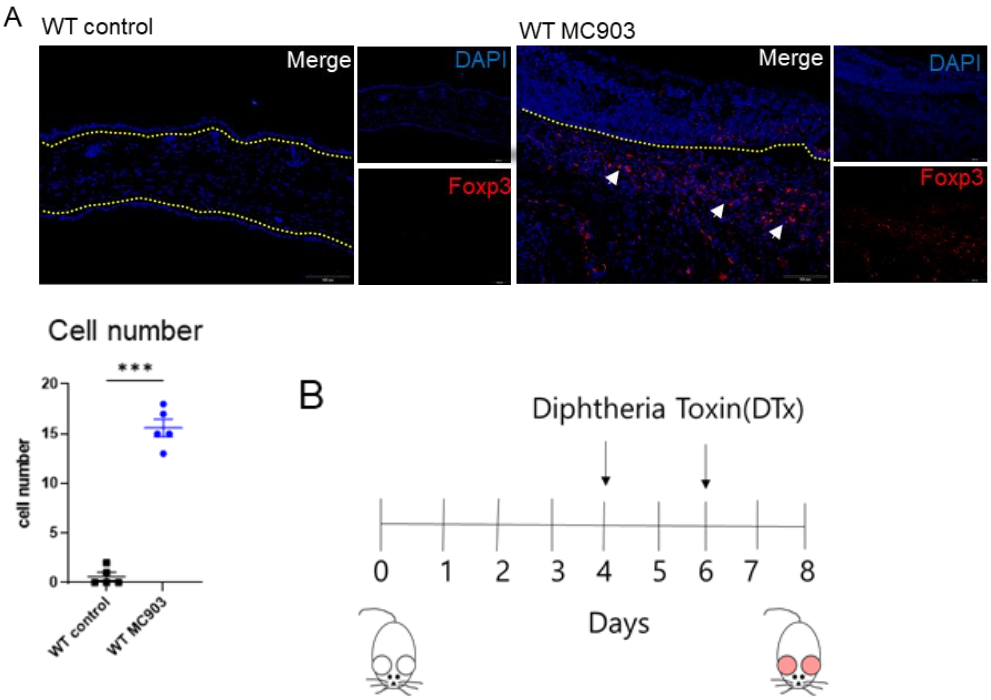
Data were analyzed with unpaired Student's two-tailed t-test, one-way ANOVA, unless otherwise stated, using the Prism software (GraphPad software Inc., San Diego, CA, USA). $P<0.05$ was considered to be significant.

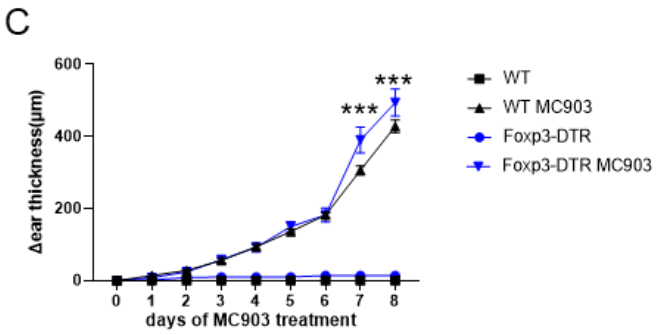
3. Results

3.1. Foxp3$^+$ Treg cells accumulate in AD-like skin inflammation and control the MC903-induced cutaneous inflammation severity

To explore the role of Treg in the MC903-induced AD-like skin inflammation model, we first used IF to detect Foxp3$^+$ Treg in untreated and MC903-treated ear skin of WT mice. The results revealed a notable increase in the number of Treg in the dermis and epidermis following MC903 treatment (Fig. 1A). To determine the role of Treg in the immunopathology of AD-like inflammation, we treated Foxp3- DTR mice and WT mice with MC903 for eight consecutive days, injecting 500 ng of DTx to Foxp3-DTR mice as same as WT mice on days four and six (Fig. 1B). FCM analysis demonstrated that Treg cell depletion was highly efficient after DT injection at day6 and 8(Fig. 2). The skin inflammation in Treg cell-depleted mice was dramatically exacerbated, resulting in steadily increasing thickening of the ear skin (Fig. 1C). Histological analysis of Treg cell–depleted skin revealed an increase in dermal thickness and an accumulation of inflammatory cells in the dermal and epidermal skin layers (Fig. 1D and E). These findings suggest that Foxp3$^+$ Treg accumulate during AD-like skin inflammation and that the depletion of Treg exacerbates MC903-induced AD-like inflammation in mice.

We found that IL-5 and IL-13 were significantly increased in Treg-depleted mice, However, depletion of Treg did not induce higher levels of IL-4 and IL-10 expression significantly than those in WT mice. Meanwhile, IFN-γ unexpectedly increased significantly in Treg-depleted mice. (Fig. 1F). We hypothesized that a novel inflammatory mechanism might be involved in Treg cell–depleted allergic skin inflammation.





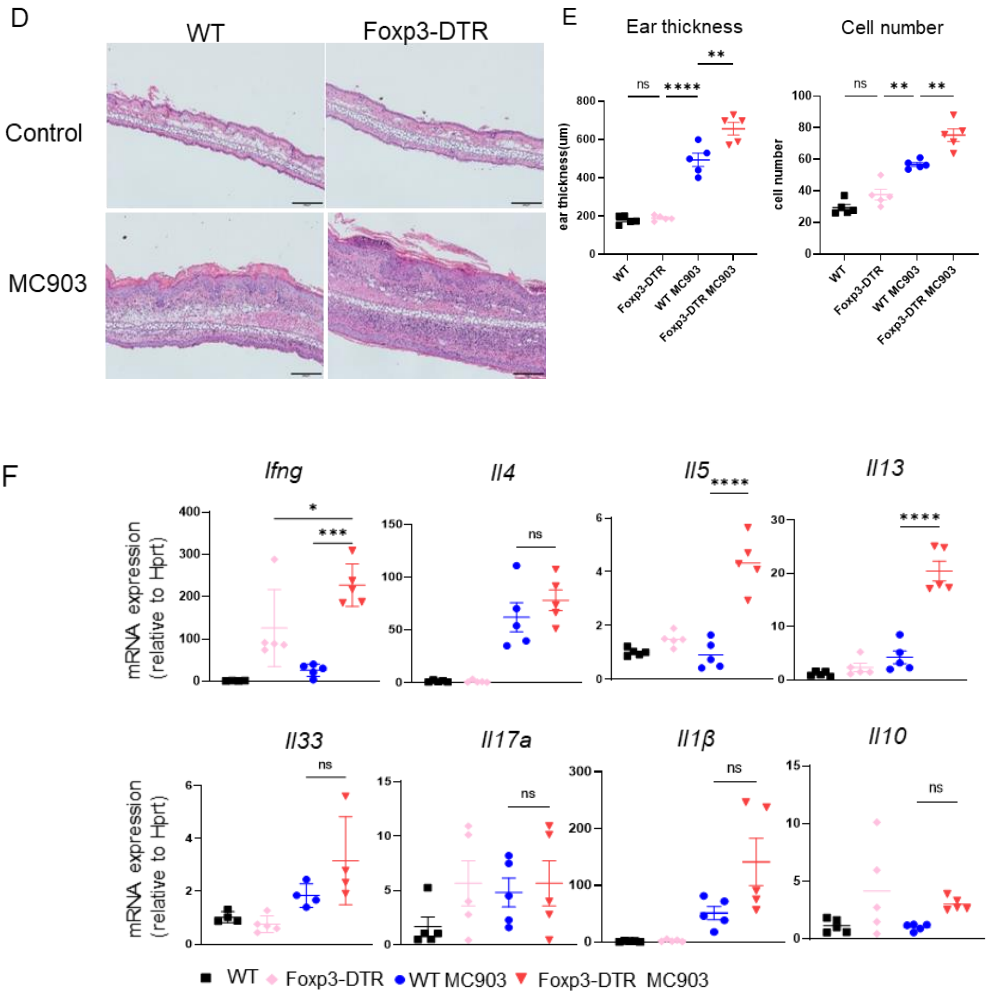
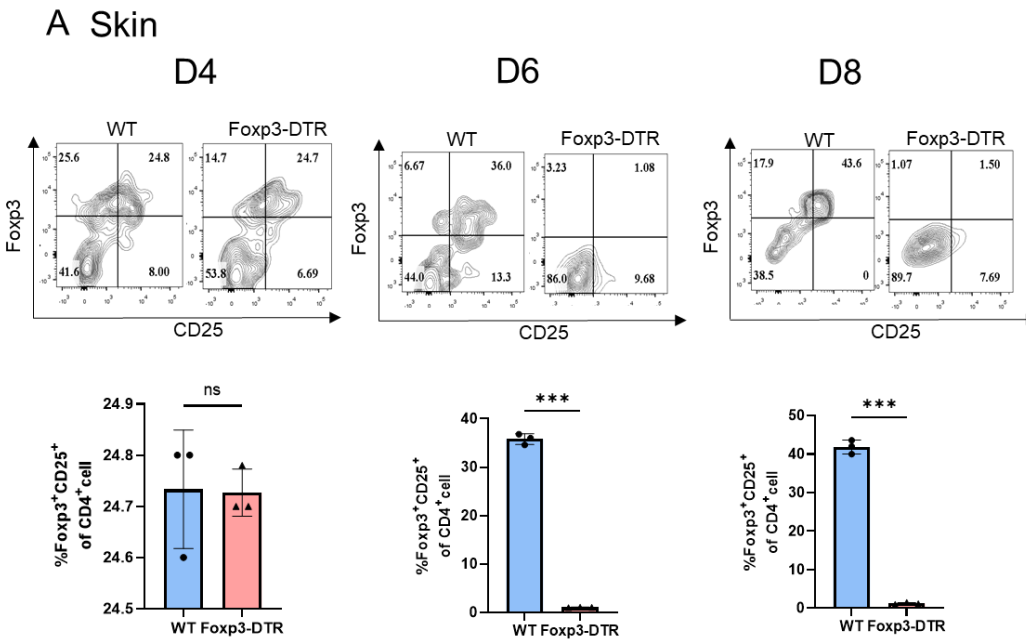


Figure 1. Foxp3$^+$ Treg cells accumulate in AD-like skin inflammation and control the MC903-induced cutaneous inflammation severity. (A) Representative immunofluorescence staining of Foxp3$^+$ cell (examples indicated by arrows) in Ethanol and MC903-treated ear skin of B6 wild type mice. Scale bars, 100 μm. Right, FOXP3$^+$ cell counting of immunofluorescence staining. (B) Schematic illustration of the experimental setup. (C) Δear thickness over the course of the treatment with Ethanol or MC903. (D) Representative H&E staining of ear tissue sections. Scale bars, 200 μm. (E) Epidermal thickness and infiltrated whole cells were quantified based on the results of H&E staining. (F) After 8 days of Ethanol or MC903 application, quantitative RT-PCR were

performed for the indicated inflammatory genes. Five samples were examined from each group. Data were showed as mean ± standard error of the mean (SEM). ****P<0.0001, ***P<0.001, **P<0.01, ns, not signification.



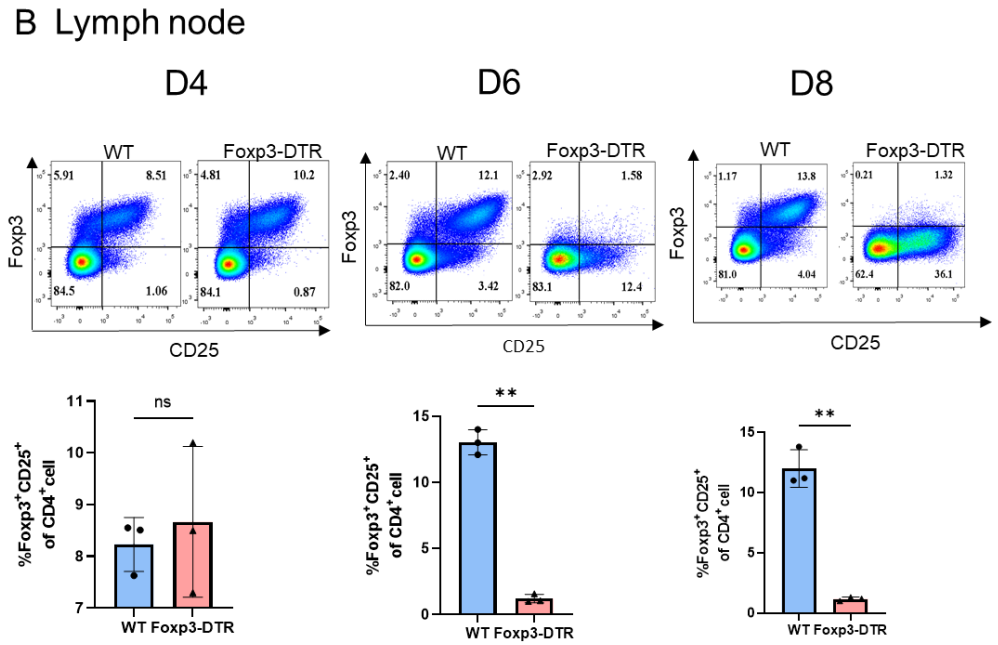


Figure 2. Treg cell was highly efficient depleted by DT injection. WT and Foxp3-DTR mice were treated 500 ng diphtheria toxin (DTx) during MC903 treatment, after injection of DTx at day4, 6 and 8, ear skin (A) and lymph nodes (B) were collected for flow cytometry. Three samples were examined from each group. Data were showed as mean ± standard error of the mean (SEM). ***P<0.001, **P<0.01, ns, not signification.

3.2. Upregulation of T-bet and GATA-3 by Treg cells during AD-like skin inflammation

As our data showed that both type 1 and type 2 inflammation were increased in MC903-treated skin by depletion of Treg compared to WT skin (Fig. 1F), which indicated the Treg induced in the MC903 topical application are specifically associated with Th1 inflammation. It has been reported that the transcription factor T-bet in Treg plays a crucial role in regulating Th1-type responses[19]. To further understand the molecular characteristics underlying Treg in MC903 model, we performed an experiment to clarify the expression profiles of T-bet and GATA-3 from Treg in skin and skin-draining lymph nodes (SDLNs) with or without MC903 treatment. We found that both T-bet and Gata-3 expressions from Treg were significantly increased by MC903 treatment (Fig. 3), indicating that Treg could actively suppress both type 1 and type 2 immune response elicited in MC903 model.

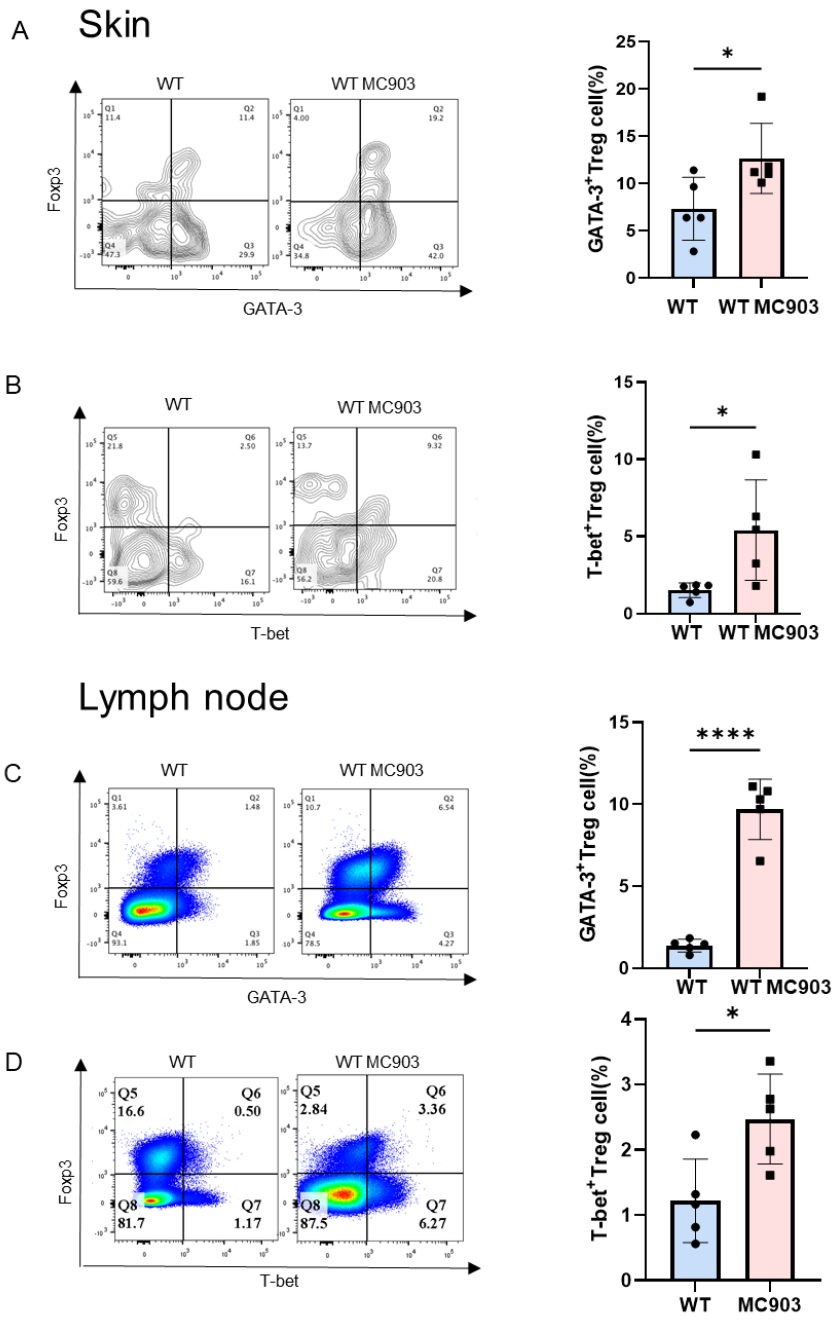


Figure 3. Upregulation of T-bet and GATA-3 by Treg cells during AD-like skin inflammation.

Following 8 days of MC903 application, T-bet and GATA-3 expression were analyzed in the skin (A, B) and skin draining lymph node (C, D) cells of WT mice by flow cytometry (n= 5). Data were presented as the mean ± SD. ****P<0.0001, **P<0.01, *P<0.05.

3.3. Depletion of Treg cells enhances IFN-γ production by $CD4^+$ T cells

IFN-γ is predominantly produced by NK cells during the antiviral innate immune response[20]. To clarify whether the aggravated AD-like inflammation in Treg cell-depleted mice was dependent on which type of IFN-γ^+ T cell responses, we next analyzed lymphocytes from the draining lymph nodes and the ear skin after MC903 application using flow cytometry. We found that total T cell, CD4, CD8, NK cell tend to expand more in Treg depleted mice than WT mice, during MC903 treatment the significant differences of total T cell, CD4, CD8 were show between Treg depleted mice and WT mice. However, NK cells didn't show difference between Treg depleted and WT mice (Fig. 4). Meanwhile, the percentage of IFN-γ^+ $CD4^+$T cells was significantly higher in in both skin and lymph nodes of Treg cell–depleted mice. No significant differences were observed between $CD8^+$ T cells and NK T cells (Fig. 5). These data demonstrate that IFN-γ was significantly enriched in Treg cell–depleted mice during MC903-induced allergic skin inflammation.

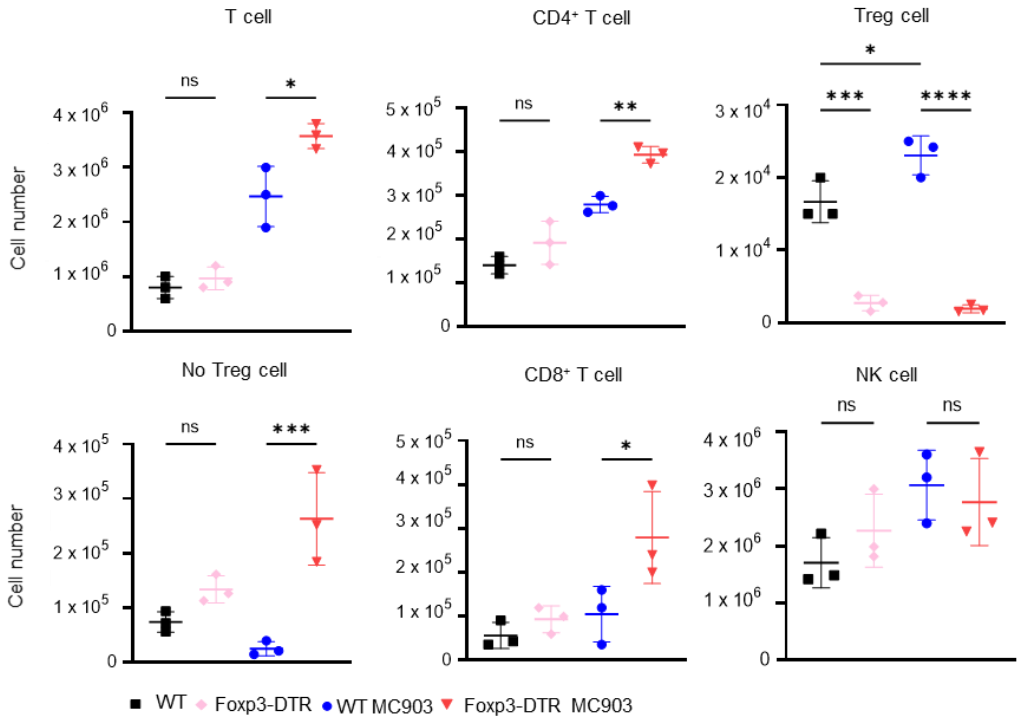
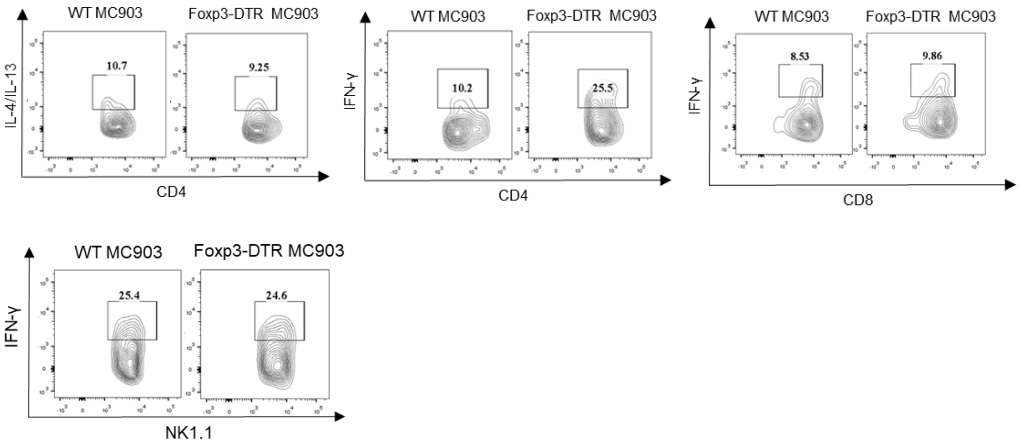
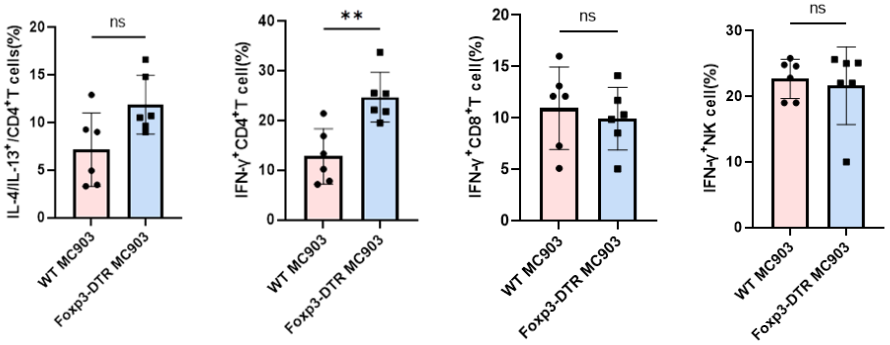


Figure 4. Increased cell numbers of CD3$^+$ T cells after depletion Treg under steady state and MC903 treatment. Following 8 days of MC903 application, T cell, CD4$^+$ (Treg/non-Treg), CD8$^+$, and NK$^+$ cell counts were analyzed in the skin.

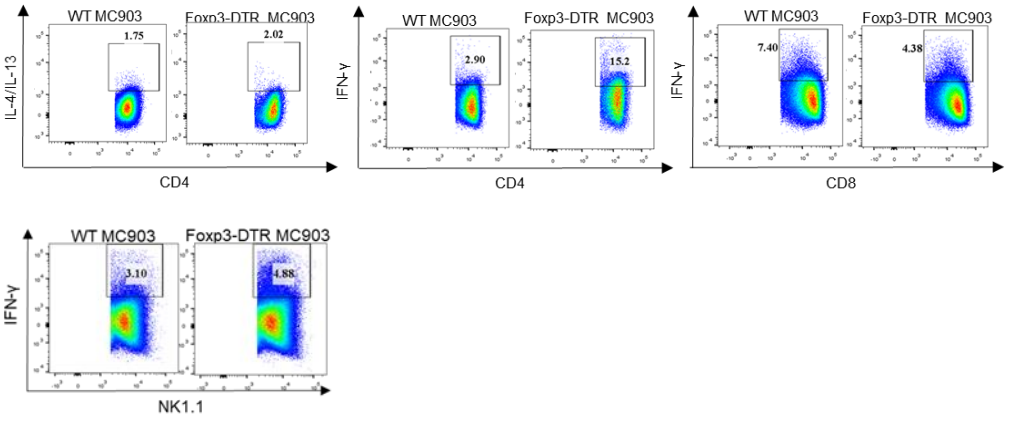
A Skin



B



C Lymph node



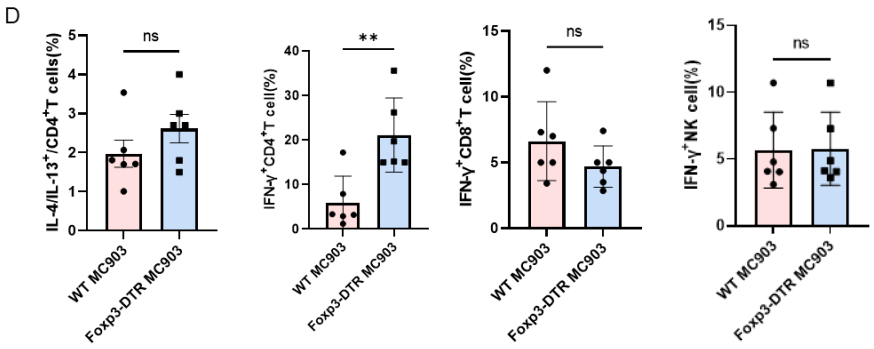


Figure 5. Depletion of Treg cells enhanced IFN-γ$^+$ expression by CD4$^+$ T cells. Following 8 days of MC903 application, IL-4/IL-13 and IFN-γ-producing T cell responses were analyzed in the skin (A, B) and skin draining lymph node cells (C, D) of WT and Foxp3-DTR mice by flow cytometry (n= 6). Data were presented as the mean ± SD. ***P*<0.01, ns, no significance.

3.4. Neutrophilic infiltration in Treg cell–depleted MC903-induced allergic skin inflammation

Previous studies had shown an overabundance of neutrophil signatures in patients with AD [17]. Broggi et al. demonstrated that neutrophils derived from blood, bone marrow, and peritoneal cavity all express IFNLR1 [21], which may indicate that neutrophils can respond to treatment with IFN-γ. To investigate the aggravated allergic skin inflammation in Treg cell-depleted mice was dependent on which type of inflammatory cells, we analyzed the ear skin by FCM. The results showed myeloid cells were significantly higher in the Treg depleted group than controls, while there was not significant difference in lymphoid cells. We gated on $CD45^+$ cells, it was founded neutrophil and monocyte all significantly increased (Fig. 6A and B). We analyzed ear skin using immunohistochemistry (IHC) of MPO. A substantial number of MPO–positive cells were detected in the epidermis and dermis of the skin of Treg cell–depleted mice after MC903-treatment (Fig. 6C). Neutrophils accumulated in the epidermis due to mass erosion, and their area was dramatically increased in Treg cell–depleted mice than in WT mice. The number of dermal neutrophils was also increased in Treg cell–depleted mice (Fig. 6D). Cxcl1 and Cxcl2 are important chemokines that mediate neutrophil recruitment [22, 23]. Expression of Cxcl1 and Cxcl2 was significantly enhanced in Treg cell–depleted mice. Previous research has shown that activated neutrophils can lead to monocytes recruitment, which supports the simultaneous occurrence of neutrophil and monocyte recruitment. Overall, these results suggest that Treg depletion augments neutrophil accumulation, which plays a vital role in the development of MC903-induced allergic skin inflammation.

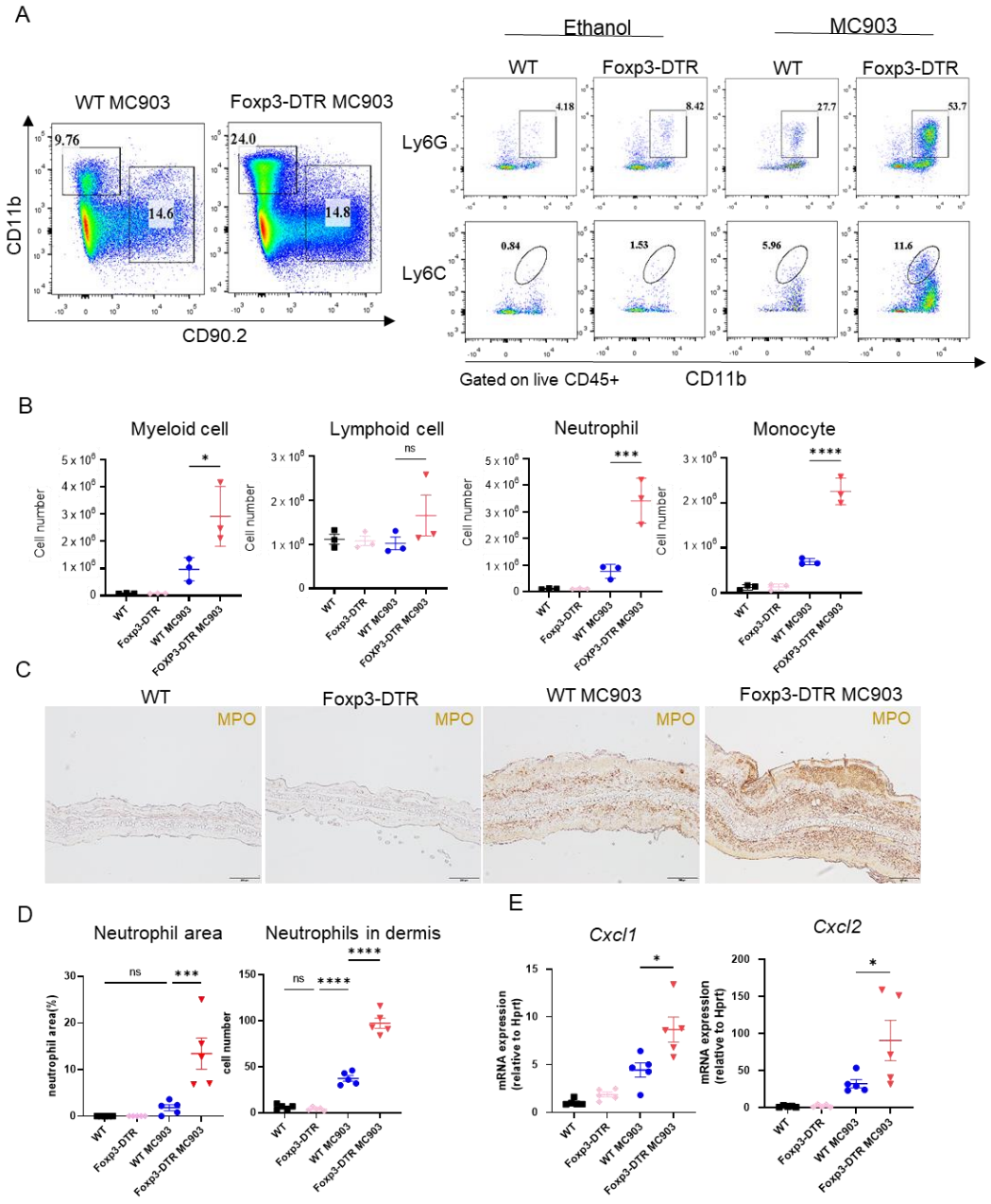


Figure 6. Depletion of Treg cells in MC903 model enhanced cutaneous neutrophils infiltration.

(A, B) Ethanol or MC903-treated ear skin cell suspensions were analyzed by flow cytometry. The flow plots and frequencies of $CD11b^+$, $CD90.2^+$, $CD11b^+Ly6G^+$, $CD11b^+Ly6C^+$ cells were shown. (C) Representative immunohistochemical (IHC) staining of MPO in Ethanol or MC903-treated ear skin of B6 wild type mice and Foxp3-DTR mouse. Scale bars, 200 μm. (D) Percent of neutrophil area in epidermis and cell number of neutrophils in dermis were counted by IHC staining. (E) Gene expression of Cxcl1 and Cxcl2 are shown though quantitative RT-PCR. Data were presented as the mean ± SEM. ****P <0.0001, ***P <0.001, *P <0.05, ns, no significance.

3.5. Depletion of Treg cells enhances MC903-induced skin apoptosis and necroptosis

Increased IFN-γ induces apoptosis and necroptosis of keratinocytes in patients with AD [24, 25]. First, to investigate whether IFN-γ can induce cell death in Treg cell–depleted mice, we monitored the gene expression of apoptosis and necroptosis maker. Cleaved caspase-3 and Bax were highly expressed in Treg cell–depleted mice after MC903 treatment, indicating increased apoptosis. RIRK3 and MLKL, which are involved in necroptosis, were more frequently detected in Treg cell–depleted mice (Fig. 7A). We then performed TUNEL staining, a feature of both apoptotic and necrotic cells. As shown, the proportion of both TUNEL-positive epidermal and dermal cells in the Treg cell–depleted group was significantly higher than that in the WT group after MC903-treatment (Fig. 7B and C). Consistently, we revealed enhanced apoptosis and necroptosis by using IHC of cleaved caspase-3, RIRK3, and MLKL, all of which showed more positive cells in the Treg cell–depleted group (Fig. 7D and E). Therefore, we conclude that Treg reduced apoptosis and necroptosis in MC903-induced allergic skin inflammation in mice.

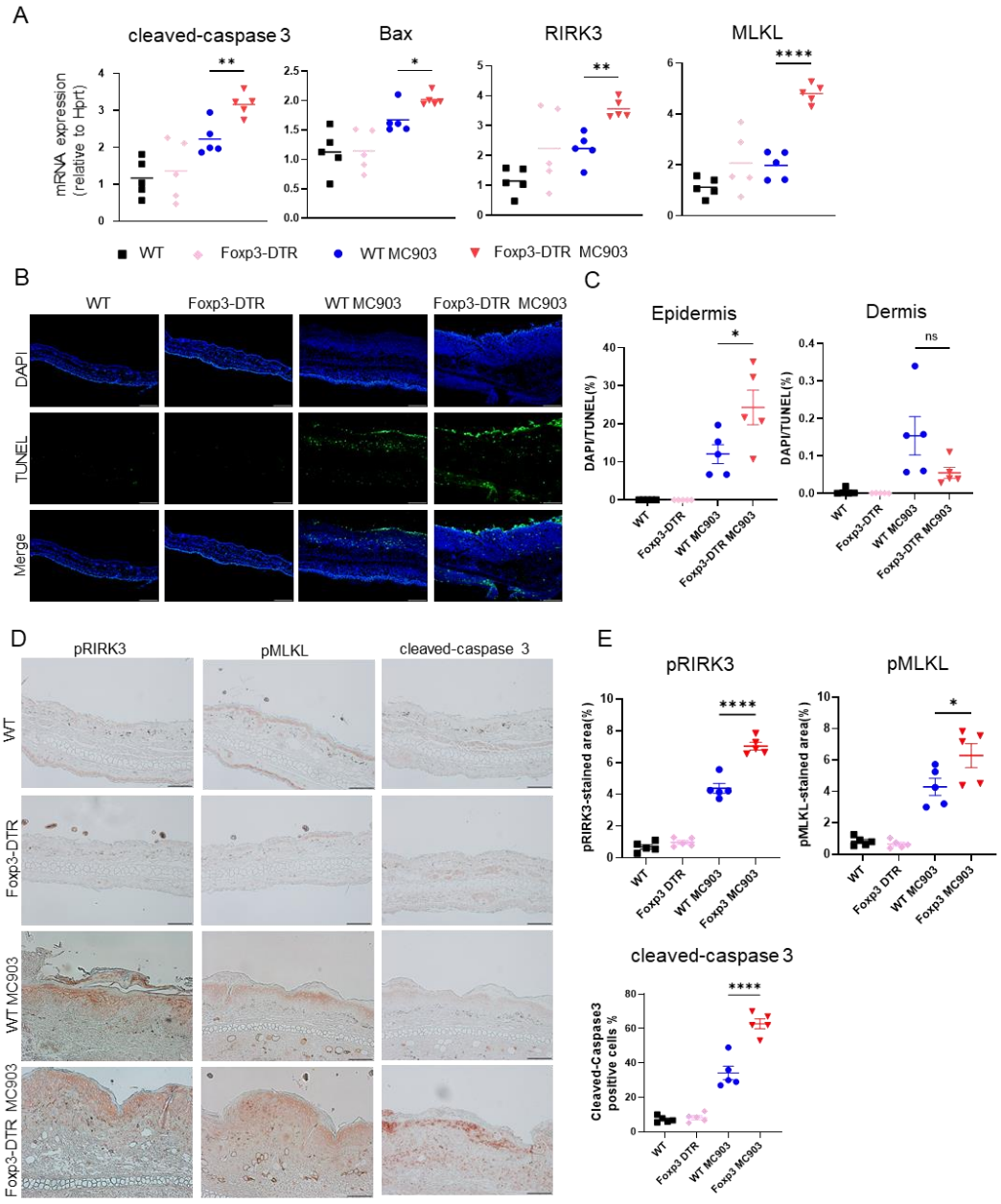


Figure 7. Depletion of Treg cells enhanced MC903-induced skin apoptosis and necroptosis. (A) mRNA gene expression of cleaved-caspase 3, Bax, RIRK3, MLKL.
(B) TUNEL-stained (green fluorescence) cells in the ear skin from mice, co-stained with DAPI (blue fluorescence) to detect the nucleus. Scale bars, 200 μm.(C) The percent of microscopic

quantification of TUNEL-positive cells in four groups. (D) Representative immunohistochemical staining of pRIPK3, pMLKL, and cleaved-caspase 3. Scale bars,100 μm. (E) The ratio of microscopic quantification of pRLRK3, pMLKL-stained area, cleaved-caspase 3-positive cells in ear skin of four groups. Results are representative of five independent experiments. Data were presented as the mean ± SEM. ****P <0.0001, **P <0.01, *P <0.05, ns, no significance.

3.6. An increased NETosis in AD-like skin inflammation was observed in Treg-depleted mice

Neutrophils displaying a marked loss of characteristic morphology are prone to undergoing NETosis [26]. NETosis is a unique form of neutrophil death that differs from apoptosis and necrosis; however, NETosis and apoptosis can occur simultaneously [27]. We found that neutrophils that infiltrated the epidermis of Treg cell–depleted mice also lost their morphology (Fig. 6C), and increased apoptosis and necroptosis were detected by using TUNEL and IHC staining (Fig. 7B and D). Therefore, we hypothesized that neutrophils infiltrating the epidermis also undergo NETosis. To identify the role of NETosis in Treg cell–depleted mice, we performed IF microscopy of the ear skin stained with DAPI (DNA), MPO, and CitH3, the specific markers of neutrophil extracellular traps (NETs) formation [28, 29]. NETs were detected in surprisingly high amounts in AD-like inflammation in Treg cell–depleted mice but were scarcely found in MC903-treated WT mice, as shown quantitatively by measuring the percentage of CitH3 stained areas within the MPO-positive areas of the skin lesion (Fig. 8). These results unexpectedly indicated that neutrophil infiltration in cutaneous lesions occurred simultaneously with NETosis, which plays an important role in exacerbating AD-like inflammation.

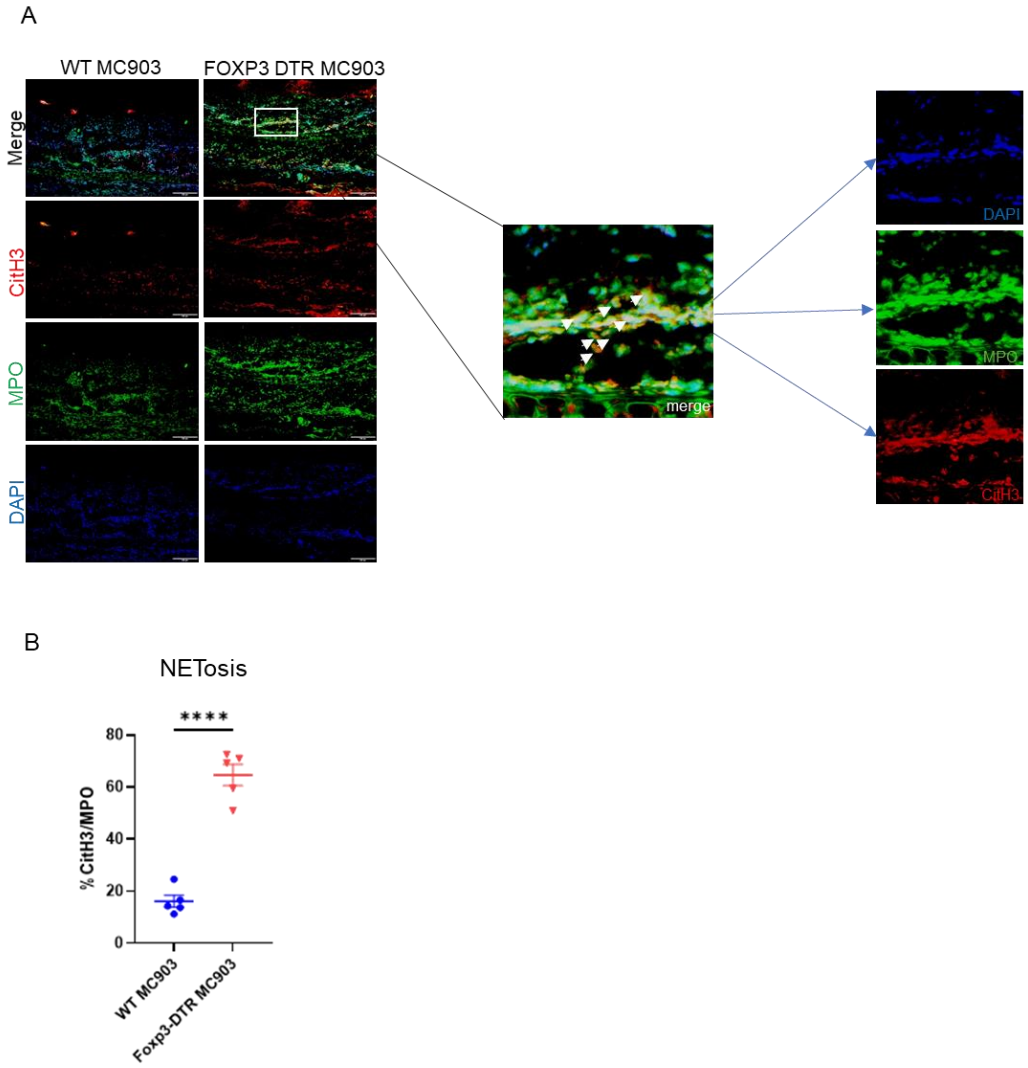
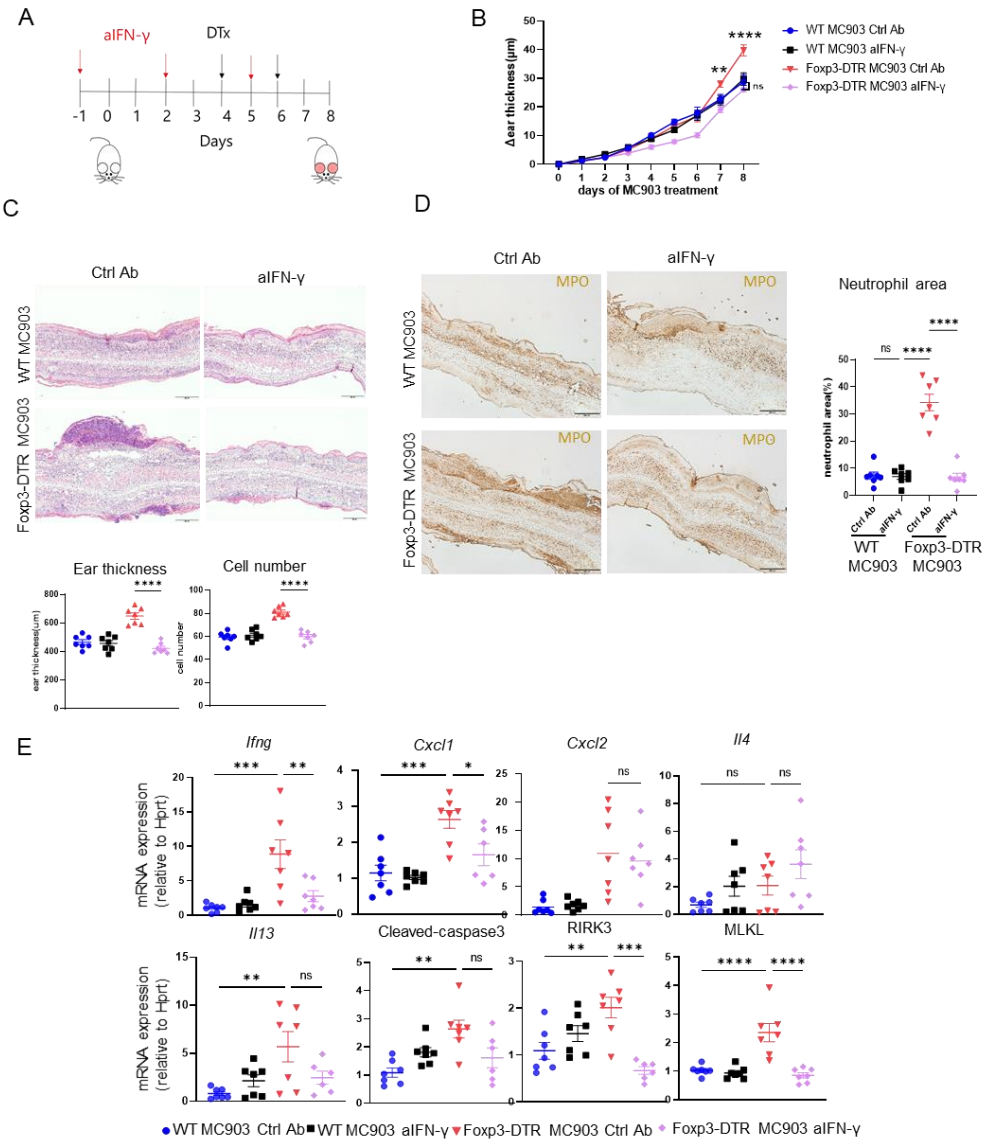


Figure 8. Treg deletion showed an increased level of NETosis in atopic dermatitis-like skin inflammation. (A) After 8 days treatment of MC903, ear skin section were stained with DAPI (blue), MPO (green) and citrullinated histone H3 (citH3; red). NETosis are visualized by colocalization of DAPI CitH3 and MPO staining (merged images; n=5 mice/group). Scale bars, 100 μm. (B) Percentage of NET area in the skin normalized to MPO positive signal. Data were presented as the mean ± SEM. ****P<0.0001, each dot represents an individual mouse; n=5 mice/group.

3.7. Neutralization of IFN-γ abolished neutrophil infiltration and NETosis

Previous studies showed that IFN-γ induces NETosis in the salivary gland and lung tissue [30, 31]. However, whether IFN-γ can induce NETosis in the skin remains unknown. To investigate the role of IFN-γ in NETosis in Treg cell–depleted mice during MC903-treatment, WT, and Treg cell–depleted groups were treated with αIFN-γ mAbs to neutralize IFN-γ during MC903-treatment (Fig. 9A). Treg cell–depleted mice treated with αIFN-γ mAbs exhibited reduced skin ear thickness and infiltrating cell numbers compared to Ctrl mAbs treated Treg cell–depleted mice (Fig. 9B and C). IHC staining revealed less neutrophil infiltration in Treg cell–depleted mice treated with αIFN-γ than in the Ctrl mAbs group (Fig. 9D). Consistently gene expression for neutrophil migration (Cxcl1), apoptosis (cleaved-caspase 3), and necroptosis (RIRK3, MLKL) all significantly decreased in Treg cell–depleted mice treated with αIFN-γ mAbs. There were no significant differences in IL-4 or IL-13 gene expression between these groups (Fig. 9E). NETs were detected in abundance in the AD-like inflammation from Treg cell–depleted mice but were practically non-existent in the AD-like inflammation from mice treated with αIFN-γ, as shown quantitatively (Fig. 9F). These findings demonstrate that elevated levels of IFN-γ are required for neutrophil infiltration and increased NETosis in exacerbated AD-like inflammation triggered by depletion of Treg.

To further investigate whether IFN-γ is sufficient to induce NETosis in neutrophils, we performed IF microscopy of mouse neutrophils stained with DAPI, MPO, and CitH3. Cells were incubated with IFN-γ or untreated, and statically imaged at the end of the 4 h stimulation period. The NETs release of group stimulated by IFN-γ performed increasingly compared to untreated control (Fig. 9G). Our results revealed that neutrophil infiltration and exacerbated NETosis are dependent on overexpression of IFN-γ in Treg cell–depleted mice during AD-like inflammation, showing an unexpected mechanism underlying acerbated AD-like inflammation pathogenesis induced by Treg depletion.



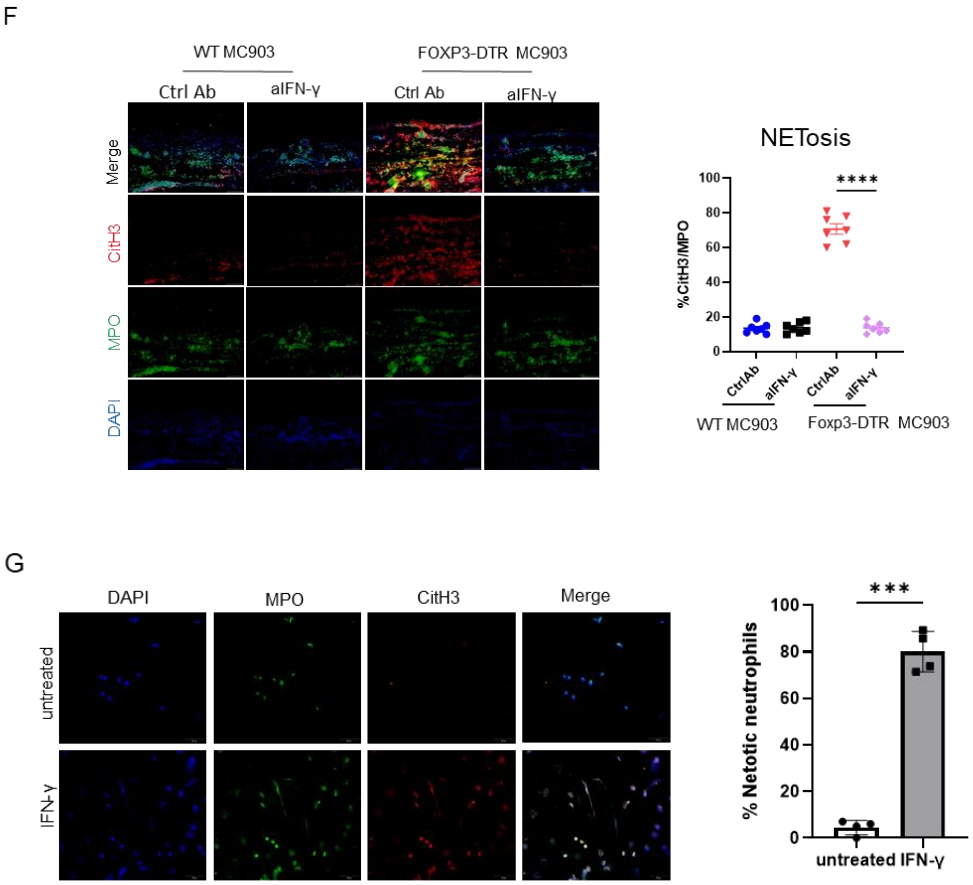


Figure 9. Neutralization of IFN-γ abolished neutrophil infiltration and NETosis.
(A) Schematic illustration of the experimental setup. (B) Δear thickness of ear skin over the course of MC903 treatment and (C) representative H&E staining, Scale bars, 200 μm. Epidermal thickness and cell number of H&E staining. (D) Gene expression of IFN-γ, Cxcl1, Cxcl2, IL-4, IL-13, cleaved-caspase 3, RIRK3 and MLKL were showed after the treatment. (E) Representative immunohistochemical staining of MPO in MC903-treated ear skin with treated anti-IFN-γ or CtrlAb. Neutrophil infiltration area of immunohistochemical staining of MPO. (F) Representative immunofluorescence staining with citrullinated histone H3 (citH3; red), MPO (green), and DAPI (blue). NETosis are visualized by colocalization of DAPI, CitH3 and MPO staining (merged images; n=7 mice/group). Scale bars, 100 μm. Percentage of NET area in the skin normalized to MPO positive signal. (G) Representative immunocytofluorescence pictures from untreated and IFN-γ stimulation group. Scale bars, 40 μm. The percentage of cells that had undergone NETosis (defined by typically NET morphology and costaining of $DAPI^{+}$ Cith3) was quantified in a blinded manner from five fields in four independent experiments. All Data are presented as the mean ± SEM.

****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, ns, no significance.

4. DISCUSSION

In this study, we showed that allergic skin inflammation was exacerbated under conditions depleting Treg and that over-expression of IFN-γ led to the aggravation of allergic skin inflammation through enhanced neutrophilic infiltration and NETosis. To the best of our knowledge, no previous studies have reported the involvement of Treg in allergic skin inflammation through the modulation of neutrophilic infiltration with accelerated NETosis.

Although observations suggest that Treg control the severity of psoriasiform inflammation [32-34], no studies have been conducted on Treg and AD skin inflammation. Several studies have indicated that Treg are increased in the blood and skin of patients with AD compared with healthy controls. In addition, elevated Treg cell numbers are significantly correlated with AD severity [35-41]. In the present study, Treg depletion exacerbated AD-like inflammation. Thus, Treg play an important role in preventing inflammatory skin diseases.

Foxp3-expressing Treg cells suppress autoimmunity [42], Treg inhibit proinflammatory Th1 cell responses by reducing the expression of IFN-γ [43]. Although previous studies showed that IFN-γ/Th1 immune response is increased in chronic AD patients[44], it has not been well-understood the pathogenic role of IFN-γ/Th1 in AD as AD is considered as a type 2 inflammation-mediated disease. Our data that depletion of Treg induced exacerbated inflammation in the MC903 topical application are specifically associated with Th1 specific IFN-γ expression. It has been reported that the transcription factor T-bet in Treg plays a crucial role in regulating Th1-type responses. In contrast, GATA-3 is canonical Th2 transcription factor controls $Foxp3^+$ regulatory T cell fate during inflammation[45].However, the role of T-bet in $Foxp3^+$ Treg during Th2 inflammation remain unclear. Although MC903 model is considered as type 2 immune response-mediated AD-like dermatitis model, we found that both T-bet and GATA-3 expressions from Treg were significantly increased by MC903 treatment, indicating that Treg could actively suppress both type 1 and type 2 immune response elicited in MC903 model. This model comprises a mixture of Th1 and Th2 response accompanied by neutrophilic infiltration as we observed[17, 46], and the role of type 1 immune response has been underestimated so far. Besides, blocking of IFN-γ successfully rescued an exacerbated inflammation in Treg-depleted mice, suggesting that type 1 immune response is responsible for driving an enhanced inflammation. As Treg in the skin and SDLNs are relatively poised to express GATA-3 rather than T-bet to suppress type 2 inflammation and its related pro-fibrotic changes at baseline [45, 47-49], it is likely that an increased expression of T-bet is more sensitively required to suppress an increased type 1 over type 2 inflammation in MC903 model.

It has been reported that $Foxp3^+$ Treg depletion resulted in a significant increase in IFN-γ gene expression[50], which is consistent with our results. An increased IFN-γ level in Treg-depleted mice at steady state would somewhat influence the overall effect of MC903 treatment in this condition. However, interestingly, Treg-depleted control mice did not show a spontaneous neutrophilic

infiltration and related clinical and histological inflammation in the absence of MC903 treatment, indicating that MC903 triggers an IFN-γ-dependent neutrophilic inflammation as shown in our results. Specifically, blocking of IFN-γ successfully rescued an exacerbated inflammation by MC903 application in Treg-depleted mice, suggesting that Treg-IFN-γ-dependent neutrophilic inflammation is largely mediated by MC903 treatment. However, the effect of an induction of type 1 immune response at the steady state needs further evaluation in the future.

IFN-γ^+ CD4 T cell accumulation was significantly increased by Treg depletion during neutrophilic airway inflammation [51]. In this study, we showed that $CD3^+$, $CD4^+$, $CD8^+$ T cell were significantly increased in Treg depleted mice than WT mice. However, NK cells didn't show significant difference between Treg depleted and WT mice. It has been reported that $CD4^+$ T cell and CD8 T^+ cell proliferated in the absence of $Foxp3^+$ Treg, and there was a remarkable influx of $CD3^+$ T cells in Treg depleted mice during Th2 inflammation [52]. Other study showed that ablation of Treg during inflammation does not enhance the number of NK cells in contrast to steady state depletion because Treg depletion fails to favor an outgrowth of NK cell [53]. Simultaneously, the depletion of Treg also exacerbated the expression of IFN-γ^+CD4^+, demonstrating that Treg suppressed $CD4^+$ T cell immune response by reducing the expression of IFN-γ in AD-like inflammation. It has been reported that the mechanisms for Treg-mediated suppression of IFN-γ production from $CD4^+$ T cells through suppressive cytokines[54] (IL-10 or TGF-β) or direct regulation of protein synthesis[55]. The mechanism of how Treg suppresses CD4 T cells expressing IFN-γ needs further clarification.

IFN-γ is known to be capable of inducing apoptosis of tumor cells [56, 57], and has the potential to synergistically induce necroptosis in cancer cells [58, 59], but whether it alone can induce necroptosis has not been established. Our data showed aggravated apoptosis and necroptosis in Treg cell–depleted mice during AD-like inflammation, which were abolished by neutralization of IFN-γ, suggesting that IFN-γ induced exacerbated apoptosis and necroptosis in Treg cell–depleted mice during AD-like inflammation.

Neutrophils are the first immune cells to infiltrate AD skin [17]; however, the mechanism by which neutrophils affect AD remains uncertain. MC903-induced neutrophil recruitment and activation were detectable on day three and reached a maximum on day nine [60]. Treg depletion exacerbates disease severity and neutrophil infiltration in intestinal mucositis [61], allergic airway inflammation [51], and contact hypersensitivity [62]. Simultaneously, $Foxp3^+$ Treg demonstrated the capacity to inhibit neutrophil accumulation in the skin via downregulation of the chemoattractants Cxcl1 and Cxcl2 [63]. We found that exacerbated inflammation with Treg depletion showed neutrophil infiltration in the cutaneous skin, consistent with the increased expression of Cxcl1 and Cxcl2, which are the main chemokines involved in neutrophil migration [64]. In our study, depletion of Treg exacerbated AD-like inflammation and may have affected neutrophil accumulation by upregulating Cxcl1 and Cxcl2.NETosis is a unique cell death pathway that occurs in neutrophils during NET release [65]. NETs are large, extracellular, web-like structures composed of cytosolic and granular proteins assembled on a scaffold of decondensed chromatin [66].

dsDNA released by NETosis promotes type-2 immune responses [67]. Neutrophils undergoing NETosis displayed a marked loss of characteristic morphology [26]. Our results showed that neutrophils infiltrating the epidermis display a loss of morphology and can only be analyzed by the area of mass erosion. Previous studies have shown that NET drives autoimmunity in skin diseases such as psoriasis [68, 69], Stevens-Johnson syndrome toxic epidermal necrolysis [26], and Behçet's Disease [70, 71]. To the best of our knowledge, no studies have previously reported the in modulation of neutrophilic inflammatory response with accelerated NETosis in AD. Our study revealed accelerated NETosis and neutrophil infiltration in Treg cell–depleted mice during AD-like inflammation, which was inhibited by neutralization of IFN-γ. Mature neutrophils strongly express genes that increase their response to type II IFNs. IFN-γ, in combination with complement factor 5a, has been shown to induce healthy neutrophil NETosis [72]. We also proved that IFN-γ induced NETosis in neutrophils independently. Although future mechanistic studies are required to fully understand the contribution of NETosis to disease progression, these observations suggest that inhibiting IFN-γ or NET release may provide new therapeutic strategies for the treatment of allergic dermatitis.

Overall, our results from a mouse model of allergic skin inflammation, where Treg are depleted during inflammation, indicate that excessive NET formation correlated with disease aggravation. Our findings provide insights into how uncontrolled neutrophil infiltration may exert a detrimental effect during allergic skin inflammation. Moreover, we show that this process is controlled by IFN-γ as demonstrated by reduced NETosis and neutrophil infiltration when IFN-γ was neutralized during AD-like inflammation of Treg cell–depleted mice. However, IFN-γ responses counter-balanced in Th2-dominant inflammation in the presence of Treg, consistent with our results that administration of anti-IFN-γ antibody to WT mice with MC903 treatment has no anti-inflammatory effect. It has not been understood yet whether the functionality and number of Treg are different according to the state of AD, which might influence the extent of Th1/IFN-γ-dependent inflammation in AD. Our findings demonstrate an underestimated Treg-IFN-γ-neutrophil inflammatory axis, which explains the mechanism of exacerbated AD-like inflammation induced by Treg depletion, and report the presence of NETosis in allergic skin inflammation for the first time.

5. CONCLUSION

In this study, we elucidate a role of Treg in AD-like skin inflammation. Depletion of Treg exacerbated AD-like skin inflammation through inducing neutrophil infiltration and NETosis. We also demonstrate that neutrophil infiltration and NETosis were controlled by IFN-γ as demonstrated by reduced NETosis and neutrophil infiltration when IFN-γ was neutralized during AD-like inflammation of Treg cell–depleted mice. This study demonstrates an underestimated Treg-IFN-γ-neutrophil inflammatory axis, which explains the mechanism of exacerbated AD-like inflammation induced by Treg depletion, and report the presence of NETosis in allergic skin inflammation for the first time. Future study is required to fully understand the underlying mechanism of Treg in the control of the IFN-γ-producing $CD4^+$ T cell and how IFN-γ regulating neutrophil infiltration and NETosis during AD-like inflammation of Treg cell–depleted mice. We anticipate that our knowledge would be translated to the clinical settings to improve the therapeutic strategies for the treatment of allergic dermatitis through modulating IFN-γ *in vivo*.

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Abstract in Korean

아토피 피부염 유사 환경에서 Foxp3$^+$ Treg 세포에 의한 호중구 면역 반응 조절

조절 T 세포(Treg)는 신체의 자가면역과 염증을 제한하는 중앙 면역 세포입니다. 아토피성 피부염(AD)은 T 세포 활성화가 핵심 특징인 만성 염증성 피부 질환으로, Th2 세포 매개 반응이 중추적인 역할을 합니다. Treg 제거 및 아토피성 피부염 유사 염증 마우스 모델을 사용하여 우리는 Foxp3$^+$ Treg의 고갈이 호중구 모집 증가 및 Th1 사이토카인 IFN-γ 발현을 포함하여 AD 유사 피부 염증을 상당히 악화시켰다는 것을 입증합니다. Treg가 고갈된 생쥐의 피부에 침투한 호중구는 야생형보다 더 많은 NET를 방출했습니다. IFN-γ의 중화는 Treg가 고갈된 마우스에서 호중구 침윤 및 NETosis를 폐지했습니다. IFN-γ로 자극된 호중구는 시험관 내에서 NET를 방출하는 경향이 더 컸습니다. 마지막으로 Foxp3$^+$ Treg는 IFN-γ에 의한 호중구 침윤과 NETosis를 조절하여 피부 알레르기 염증을 조절합니다.

이번 연구는 아토피성 피부염 유사 염증에서 이전에 과소평가되었던 Treg-IFN-γ-호중구 염증 축을 강조합니다.

핵심되는 말: 아토피성 피부염, 조절 T 세포, NETosis, IFN-ɣ