Role of regulatory T cells in imiquimod-induced psoriasiform dermatitis model

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Role of regulatory T cells in imiquimod-induced psoriasiform dermatitis model

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ABSTRACT

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Psoriasis is a common immune-mediated, chronic inflammatory skin disease characterized by widespread erythematous plaque with typical silvery white scale. Among various immune cells, T cells play a pivotal role in development of these characteristic skin changes. In psoriasis, dysregulation of the regulatory T (Treg) cell compartment has been reported. In human psoriasis, defects in the function of Treg cells, as well as a resistance of effector T cells to Treg cell-mediated suppression could contribute to failed T cell regulation. From this background, I hypothesized modulation of Treg cells function can be a feasible therapeutic strategy for the control of chronic inflammation of psoriasis. This study aims to observe the phenotype of Treg cells in imiquimod (IMQ)-induced animal model for psoriasis and to elucidate the functional role of Treg cells using Treg-depletion mice model.

Firstly, repetitive application of IMQ on the shaved back skin and one side of ear of mice successfully induced psoriasiform dermatitis model. Interestingly, splenic $CD4^{+}Foxp3^{+}$ Treg cells were significantly increased in active group, and their expression of markers related with suppressive function, PD-1, CTLA-4, and GITR, are increased on Treg cells in active group. However, prolonged

application of IMQ more than one week did not exacerbate IMQ-induced skin inflammation and did not changed splenic and lymph node Treg cells and their suppressive markers.

To understand the direct role of Treg cells in IMQ-induced mice model, Treg cell depletion model using Foxp3-DTR-eGFP mice was adopted. Unexpectedly, the transient Treg depletion before the induction of skin inflammation did not worsen IMQ-induced inflammatory change. Moreover, the main source of IL-17 in this mice model, and IL-17-producing γδ T cells were not changed after Treg cell depletion before the induction of skin inflammation. Contrary to early response, transient Treg cell depletion before the starting of late period, skin inflammation was exacerbated. Then, to compare relative importance of IL-17 and Treg depletion, transient Treg-depleted mice were treated with anti-IL-17A monoclonal antibody. The treatment with anti-IL-17A monoclonal antibody partially rescued mice from the exacerbation of disease associated with Treg cell depletion.

Taken together, these findings show that Tregs are important for the controlling of on-going inflammation in IMQ-induced skin inflammation. And transient Treg cell depletion acts, at least partly, through increasing IL-17 producing γδ T cells and inflammatory cytokines such as IFN-γ. These results suggest possibility that Treg cells therapy may be of benefit in psoriasis as a supportive therapeutics to current anti-psoriatic treatments based on anti-IL-17 strategies. Further investigations to reveal the detailed dynamic change of Treg cells and their functional phenotypes on different time points in IMQ-induced skin change will be needed for the comprehensive understanding of psoriasis.

Key words : Animal model, diphtheria toxin, imiquimod, interleukin-17, psoriasis, regulatory T cell

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I. INTRODUCTION

Psoriasis is a common immune-mediated, chronic inflammatory skin disease characterized by hyperproliferative keratinocytes and extensive infiltration of various immune cells, including different types of T cells, dendritic cells (DCs), macrophages, and neutrophils. Among these cells, T cells play a pivotal role in development of these characteristic skin changes with inflamed scaly plaques. In particular, activated T helper (Th)1 and Th17 responses are frequently observed in the patients and have been considered to be responsible for typical skin change.[1,2] Th17 cells were initially seen as primary IL-17 producers in psoriasis. However recent analyses from psoriasis patients as well as mouse models have shown that neutrophils, mast cells, γδ T cells, natural killer (NK) cells, and innate lymphoid cells (ILC) also release substantial amounts of IL-17.[3] During inflammatory process, these

IL-17-producing immune cells collaborate with Th1 cells, and macrophages which secrete tumor necrosis factor (TNF)-α, another important cytokine in psoriasis.[2]

Regulatory T (Treg) cells, which are characterized by expression of the transcription factor Foxp3, play an indispensable role in establishing and maintaining immune homeostasis.[4] In psoriasis, dysregulation of the Treg compartment has been reported.[5-7] Theoretically, several mechanisms including defects in the number and function of Treg cells, as well as a resistance of effector T cells to Treg cell-mediated suppression, could each contribute to failed T cell regulation.[8] First, the lower proportion of epidermal or dermal $Foxp3^+$ cells in lesional skin was reported from human studies.[9,10] However there are also conflicting results on the number of lesional Treg cells despite of different analyzing methods.[5,11] Instead, psoriatic $CD4^+CD25^{hi}$ Treg cells are functionally deficient in suppressing effector T cell responses, and higher numbers of psoriatic Treg cells are required to provide suppression similar to that of Treg cells for healthy controls.[5] Moreover, $Foxp3^+$ Treg cells are critical in the balance of proinflammatory and anti-inflammatory signals, because they are sensitive to the cytokine environment.[6] Treg cells from psoriasis patients show a predominant STAT3 phosphorylation by exposure to pro-inflammatory cytokines, leading to their impaired functions in suppressing T cell activation.[12] Especially high IL-6 from lesional endothelial cells, and dendritic cells enable responder T cells

escape from Treg cell suppression.[6] Furthermore, under predominance of IL-23, conversion of $Foxp3^+$ cells to IL-17-producing cells may occur.[7] $Foxp3^+$ Treg cells from patients with severe psoriasis have an enhanced propensity to lose Foxp3 expression, while maintaining a high level of RORγt expression.[7] Interestingly, $CD4^+CD25^{hi}$ $Foxp3^+$ Tregs from healthy individuals can convert into inflammation-associated Th17 cells, and this Treg cell change was further promoted by the proinflammatory cytokines, such as IL-1ß, IL-21, and IL-23.[13,14]

Clinically, therapeutic responses to anti-psoriasis treatments such as photochemotherapy or narrow-band UVB are associated with restoration of Treg cells count or their suppressive function.[15-17] From this background, I hypothesized modulation of Treg cells function can be a good therapeutic strategy for the control of chronic inflammation of psoriasis. Although convincing evidences have proven the involvement of dysfunctional Treg cells in the pathogenesis of psoriasis, the pivotal role of these Treg cells in psoriasis is not clarified. This study aims to observe the phenotype of Treg cells in imiquimod (IMQ)-induced animal model for psoriasis and to elucidate the functional role of Treg cells using Treg-depletion animal model.

II. MATERIALS AND METHODS

1. Mice

Normal C57BL/6 and BALB/C mice were purchased from Orient Bio Inc. (Sungnam, Korea). Foxp3-DTR-eGFP transgenic mice on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained by breading at animal facility of Avison Biomedical Research Center, Yonsei Medical Center, Seoul, Korea. Animals were kept under strictly monitored specific and opportunistic pathogen-free conditions. All experimental protocols involving animal studies have been approved by the Animal Care and Use Committee of Yonsei Medical Center, Seoul, Korea.

2. Induction of IMQ-induced psoriasiform skin inflammation in mice

To examine the changes in regulatory T cells, an IMQ-induced mouse model of psoriasis originally developed by Van der Fits *et al*. was used.[18] The backs of mice were shaved, and the remaining hairs were completely removed with depilatory cream (Veet™, Reckitt Benckiser, Cedex, France). The next day, commercially available 30 mg of 5% IMQ cream (Aldara™, 3M Pharmaceuticals, Loughborough, Leicestershire, UK) was applied to the dorsal skin for 6 to 14 consecutive days. To measure the ear thickness change, 20 mg of IMQ cream was also applied on one side of ear.

3. Depletion of Foxp3+ cell

Foxp3-DTR-eGFP mice were injected intraperitoneally with 1 μg diphtheria toxin (DT, Sigma-Aldrich, St. Louis, MO, USA) in 100 μl PBS 48 hr before or during IMQ-induced inflammation induction. Depletion of Foxp3+ Treg cells was confirmed by flow cytometry on lymph node and spleen 48 hr after DT injection. For sustained depletion of Treg cells, DT was repetitively injected every other day.

4. Scoring severity of skin inflammation

To score the severity of skin inflammation, a modified scoring system based on the clinical psoriasis area and severity index (PASI) was adopted.[19] Erythema, scaling, and thickness (induration) were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The level of erythema and scale were scored by careful observation. The ear thickness was serially measured with a vernier caliper (Ozaki MGF. CO. LTD, Tokyo, Japan) at the IMQ-applied ear. The cumulative score (sum of erythema, scaling, and thickening scores) served as a measure of the severity of inflammation similarly to clinical PASI scoring (range 0–12).

5. Flow cytometric analysis of mouse skin, lymph node and spleen cells.

Dermal single-cell suspensions were prepared as follows. Skin samples were incubated for 1.5 hr at 37℃ in PBS containing 2mg/ml Dispase-II (Roche

Diagnostics GmbH, Mannheim, Germany). Epidermal and dermal sheets were cut into small pieces, incubated in 0.1% collagenase D and 0.1 mg/ml DNase I (Roche) in RPMI 1640 (Gibco Life technologies, Carlsbad, CA) supplemented with 2% FBS, 1% penicillin streptomycin and 1% 1 M HEPES buffer at 37℃ for 1.5 hr. Enzyme activities were neutralized with 5 mM EDTA-PBS and filtered through a cell strainer with a 70 μm porous nylon strainers (BD Falcon, San Jose. CA. USA) to obtain single cells for flow cytometry.

To prepare total skin sample, the step of epidermal separation using dispase-II (Roche) was skipped. Then the samples were transferred to 15 ml conical tubes and incubated in RPMI 1640 containing 1 mg/ml collagenase A (Roche) and 0.1 mg/ml DNase I at 37℃ for 1 hr in a shaker. Single cells were obtained by filtering using 70 μm porous nylon cell strainers.

Harvested spleens and skin-draining lymph nodes (axillary and brachial) were minced on a 70 μm porous nylon cell strainers with the plunger of a syringe. Erythrocytes from spleen were removed using RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). Single cells obtained from skin, spleen, and skin-draining lymph nodes were washed with PBS and stained with the fluorescence conjugated antibodies for each FACS analysis. Intracellular staining was performed as described in intracellular fixation buffer (BD Biosciences, San Jose, CA, USA), after stimulation with phorbol 12-myristate 13-acetate (PMA, 200 ng/ml)/ionomycin (500 ng/ml), and brefeldin A (5 μg/ml, Sigma-Aldrich) for 2 hr.

To perform surface staining, cells were stained for 30 min at 4℃ in PBS containing 1% FBS and 2 mM EDTA (FACS buffer) with following anti-mouse antibodies; Fc blocker (BD Pharmingen, San Jose, CS, USA), CD45, CD3, CD4, CD8, γδ TCR, TCRβ, CD25, programmed death-1 (PD-1), glucocorticoid induced TNFR-related protein (GITR), and CTLA-4 (eBioscience, San Diego, CA, USA). Intracellular staining was performed with APC-conjugated anti-IL-17A and PE-conjugated anti-IL-22 PE (eBioscience). Samples were measured on a FACS LSR Fortessa or LSR Ⅱ (BD Biosciences, San Jose, CA, USA) and analyzed using Flow Jo software (Tree Star, Ashland, Jackson, OR, USA).

6. RNA preparation and real-time PCR

Total RNA was isolated from the whole skin using the Hybrid-R™ (GeneAll Biotechnology, Seoul, Korea) after controlled crushing of the tissue according to the manufacturer's recommended protocol. Complementary DNA (cDNA) was synthesized using reverse-transcription kit (Takara, Shiga, Japan). One microgram of complementary cDNA was used for a quantitative real-time PCR reaction using the SYBR Green mixture (Takara) according to the manufacturer's recommendation (Applied Biosystems, Foster City, CA, USA). Samples were first heated for 30 sec at 95 ℃, before amplification as follows; 42 cycles of two-step PCR program at 95 ℃ for 5 sec and 60 ℃ for 30 sec. For SYBR Green PCR, melting point analysis was carried out by

heating the amplicon from 60 ℃ to 95 ℃. The expression levels of gene transcript was normalized to GAPDH and relative quantitation of gene expression was calculated by ΔΔCt method. Sequences of PCR primers were as shown in Table 1.

Table 1. Primer sequences for RT-PCR

IL-17A	Forward	GGACTTCCTCCAGAATGTG
	Reverse	ATCTATCAGGGTCTTCATTGC
FOXP3	Forward	TTGGCCAGCGCCATCTT
	Reverse	TGCCTCCTCCAGAGAGAAGT
IL-23p19	Forward	ACTAAGAGAAGAAGAGGATGAAGA
	Reverse	GCAGAACTGGCTGTTGTC
IFN-γ	Forward	CTGTCATAATAATATTCAGAC
	Reverse	CGAGCTTTAAAAGATAGTTCC
GAPDH	Forward	CGTGCCGCCTGGAGAAACC
	Reverse	TGGAAGAGTGGGAGTTGCTGTTG

7. Histology

Mice tissues were harvested, transferred for 24 hr at 4 ℃ in a fresh solution of 4% paraformaldehyde. Paraffin-embedded tissues were stained with H&E according to manufacturer's protocol.

8. Anti-IL-17A monoclonal antibody treatment

For the *in vivo* neutralization of IL-17A, wild type (WT) and Foxp3-depletion mice were injected intraperitoneally with 400 μg of anti-mouse IL-17A mAb (clone 17F3, BioXcell,West Lebanon, NH) in 100 μl PBS twice or thrice during the course of the IMQ-induced skin inflammation. Total skin, spleen, and skin-draining lymph nodes were harvested and analyzed by real-time PCR and flow cytometry.

9. Statistical analysis

The significance of differences between groups was determined by Kruskal-Wallis Test with Dunn's *post-hoc* test, one-way analyses of variance (ANOVA) with Tukey's *post-hoc* test, or Mann-Whitney U tests. The significance of differences among repetitive, observational results from groups was determined by Wilcoxon matched-pairs signed rank test. All analyses were performed using the Statistical Package for the Social Sciences version 22.0 (SPSS Inc., Chicago, IL, USA) or GraphPad statistical software

(GraphPad Software, Inc. La Jolla, CA, USA). Differences were considered statistically significant when the P value was less than 0.05.

III. RESULTS

1. Development of IMQ-induced mouse model

To develop IMQ application-induced skin inflammation, IMQ cream was applied on the shaved back skin and one side of ear of mice for 7 consecutive days. As previously described, 2 or 3 days after the start of IMQ application, the back skin of the mice started to shows inflammatory signs of erythema, scaling, and induration (thickening).[18] Daily measured ear thickness changes in mice are shown in Figure 1.

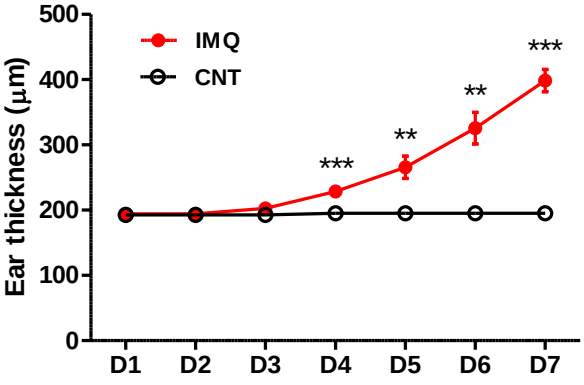


Figure 1. Change of ear thickness after IMQ application in C57BL/6 mice. Commercially available 5% IMQ cream was applied to one side of ear of mice for 7 consecutive days. Ear thickness was measured daily. Overall, thickness of IMQ-applied ear in mice (n=4) was significantly increased compared with untreated controls (CNT, n=4). Significance is shown in comparison with control mice on each observed day using unpaired t test, *P< 0.05, **P< 0.01, ***P< 0.001). Data are mean ± SD.

Overall, thickness of IMQ-applied ear in mice was significantly increased since three day after IMQ application compared with untreated controls. (P =0.0223 in Wilcoxon matched-pairs signed rank test). The clinical score and modified psoriasis area severity index (PASI) score were measured daily (Figure 2).

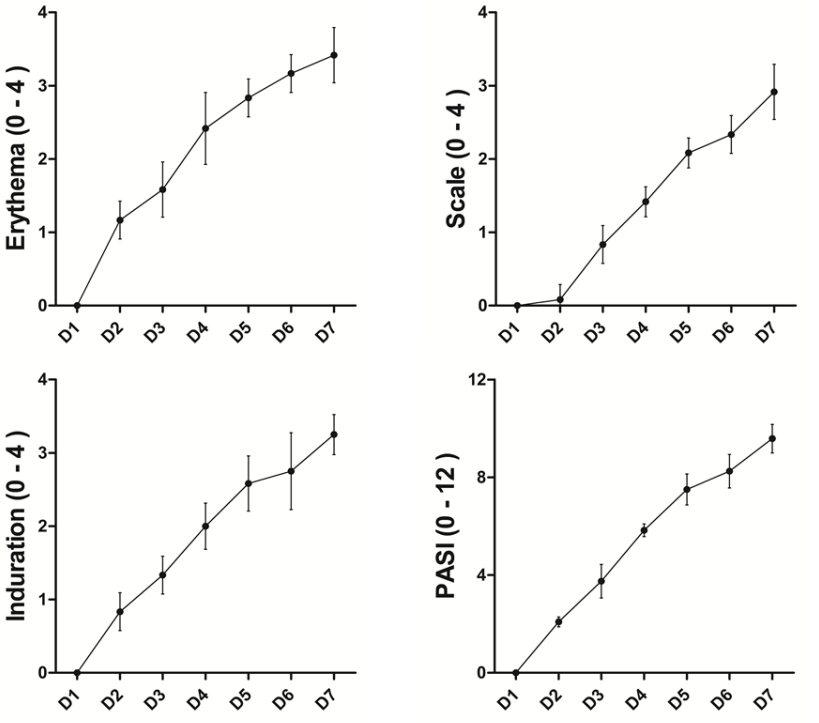


Figure 2. Clinical score and PASI after IMQ application in C57BL/6 mice. Erythema, scaling, and induration of the back skin were scored daily on a scale from 0 to 4. The cumulative score (sum of scores for erythema, scale, induration) is depicted as modified PASI. Data are mean scores ± SD (n=4).

2. The change of regulatory T cells in IMQ-induced skin change

To observe the change of Treg cells in IMQ-induced mice model, the experiment was designed with three groups as follows: (i) IMQ-untreated control group (CNT), (ii) IMQ-applied group for 7 consecutive days (active group), and (iii) spontaneous resolving after 7 day application of IMQ (resolving group) (Figure 3A). Both the proportion of splenic CD4 T cells and CD8 T cell was significantly decreased in active group (Figure 3B, 3C). Interestingly, after gating of splenic CD4 T cell, $Foxp3^+$ Treg cells were significantly increased in active group (Figure 4). To analyze the expression of Treg cell markers which are related with suppressive function such as PD-1, CTLA-4, and GITR, flow cytometric analysis was performed. The PD-1, CTLA-4, and GITR expressing $Foxp3^+$ cell were increased in active group compared with CNT mice (Figure 5).

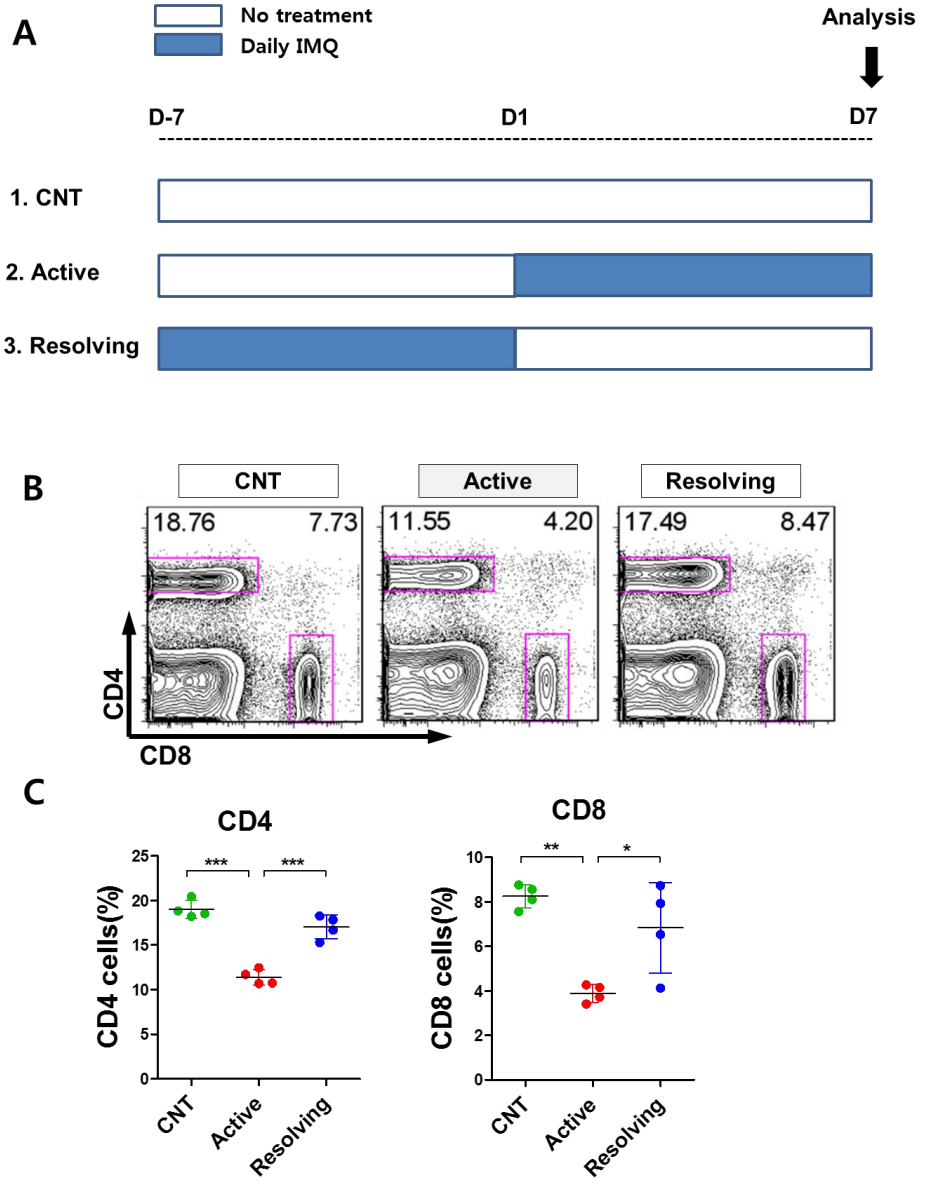


Figure 3. Decreased splenic CD4 T cells and CD8 T cells in IMQ-induced psoriasiform mice. (A) The experiment was designed with three groups (n=4)

as follows: (i) untreated control group (CNT), (ii) IMQ-applied group for 7 consecutive days (active group), and (iii) spontaneous resolving after 7 day application of IMQ (resolving group). (B) Harvested spleens were minced on a mesh with the plunger of a syringe. Erythrocytes were lysed with RBC lysis solution. Single cells obtained from spleen were stained with the fluorescence conjugated antibodies to CD4 and CD8. (C) The ratio of splenic CD4 T cells and CD8 T cells among viable splenic cells in active group significantly decreased compared with CNT and resolving group (n=4 each group). *P < 0.05, **P < 0.01, ***P < 0.001. Bar, mean ± SD.

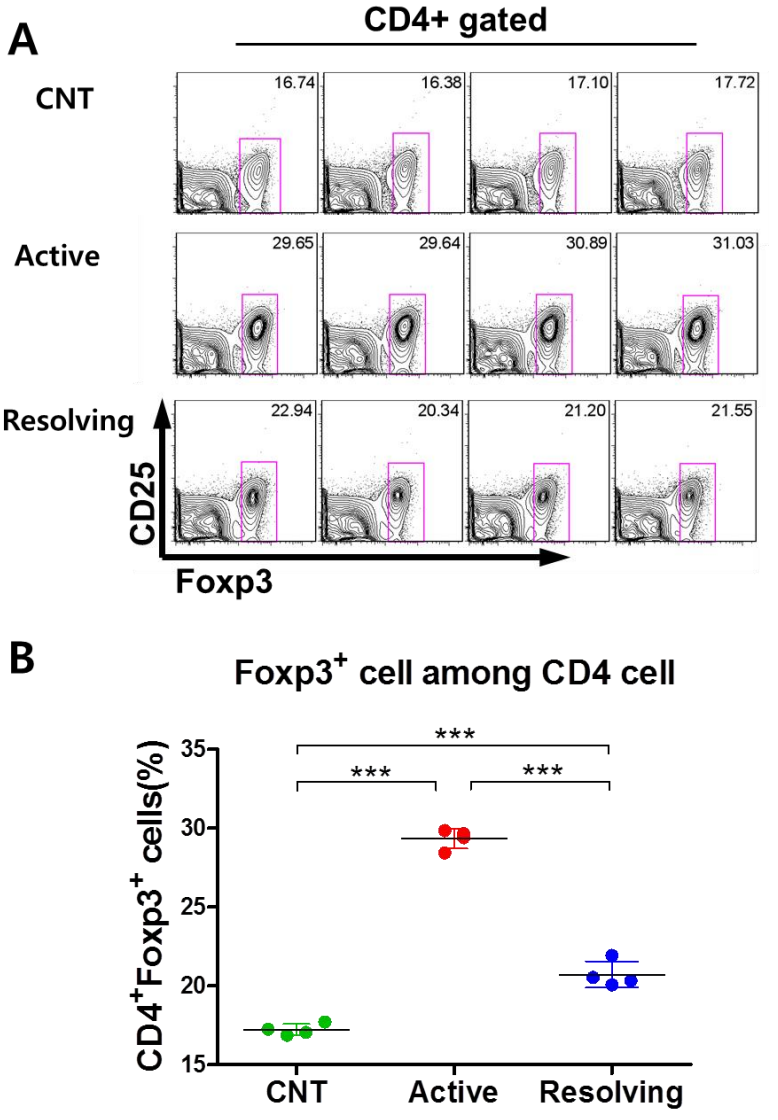


Figure 4. Increased splenic Treg cells among $CD4^+$ T cell in active period. (A) Single cells obtained from spleen were gated with CD4 positive cell and then analyzed the expression of CD25 and Foxp3. (B) The ratio of splenic $Foxp3^+$ cells among viable splenic $CD4^+$ cells in active group significantly increased compared with control (CNT) and resolving group (n=4, each group). ***$P < 0.001$. Bar, mean ± SD.

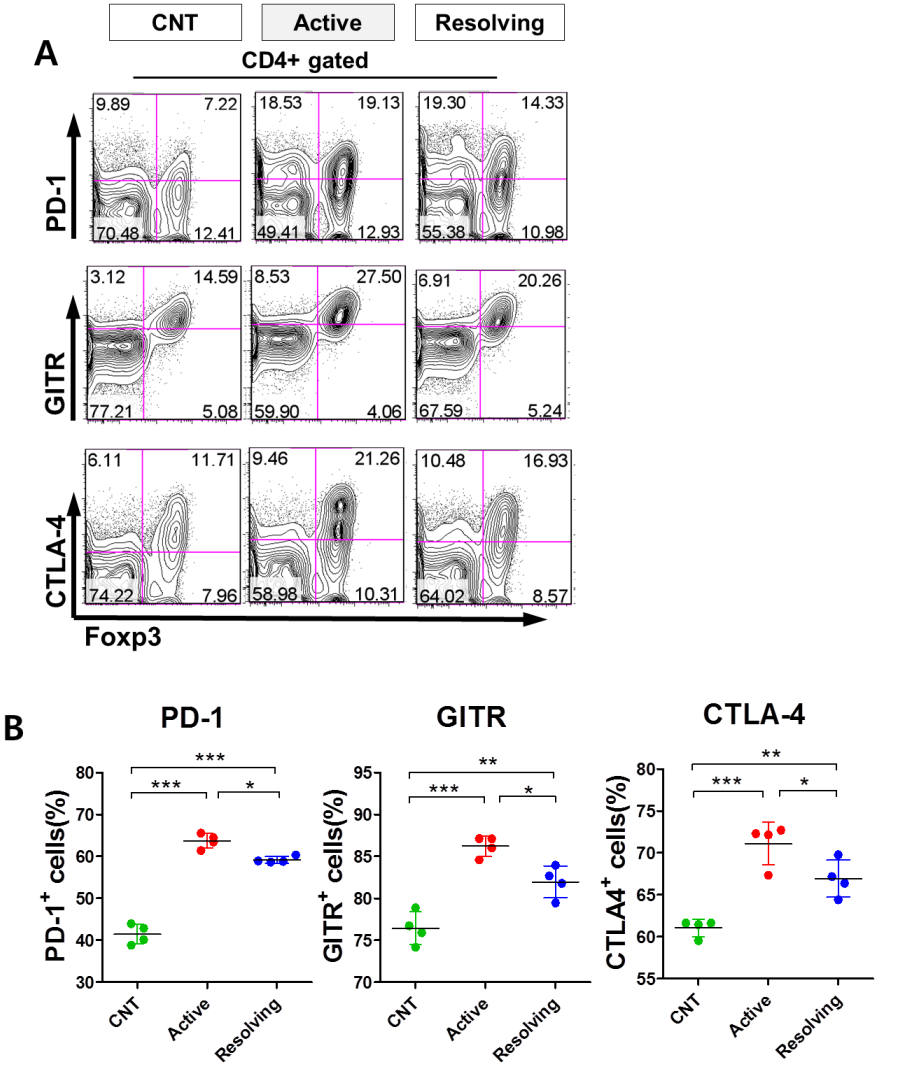


Figure 5. Suppressive markers of Treg cells, such as PD-1, GITR, and CTLA-4 are increased in active disease state. (A) Single cells obtained from spleen were gated with CD4 positive cell and then analyzed the expression of PD-1, GITR, and CTLA-4 in Foxp3$^+$ splenic cells. (B) The ratio of PD-1, GITR, and CTLA-4 expressing Foxp3$^+$ cell in active group significantly increased compared with control (CNT) and resolving group (n=4, each group). * P < 0.05, ** P < 0.01, *** P < 0.001. Bar, mean±SD.

3. Prolonged application of IMQ more than 1 week does not exacerbate skin inflammation.

As a recent study suggested IMQ-induced skin has biphasic responses in prolonged application of IMQ, I observe the skin change by applying IMQ for 14 consecutive days.[20] Experiment design was shown in Figure 6. Clinical parameters such as erythema, scaling, and thickness continuously increased over the first 7 d of application, but then reached a plateau by day 14. During the late phase (days 8~14) clinical parameters remained stable (Figure 7). In other word, the prolonged application of IMQ more than 1 week did not exacerbate skin inflammation in this model.

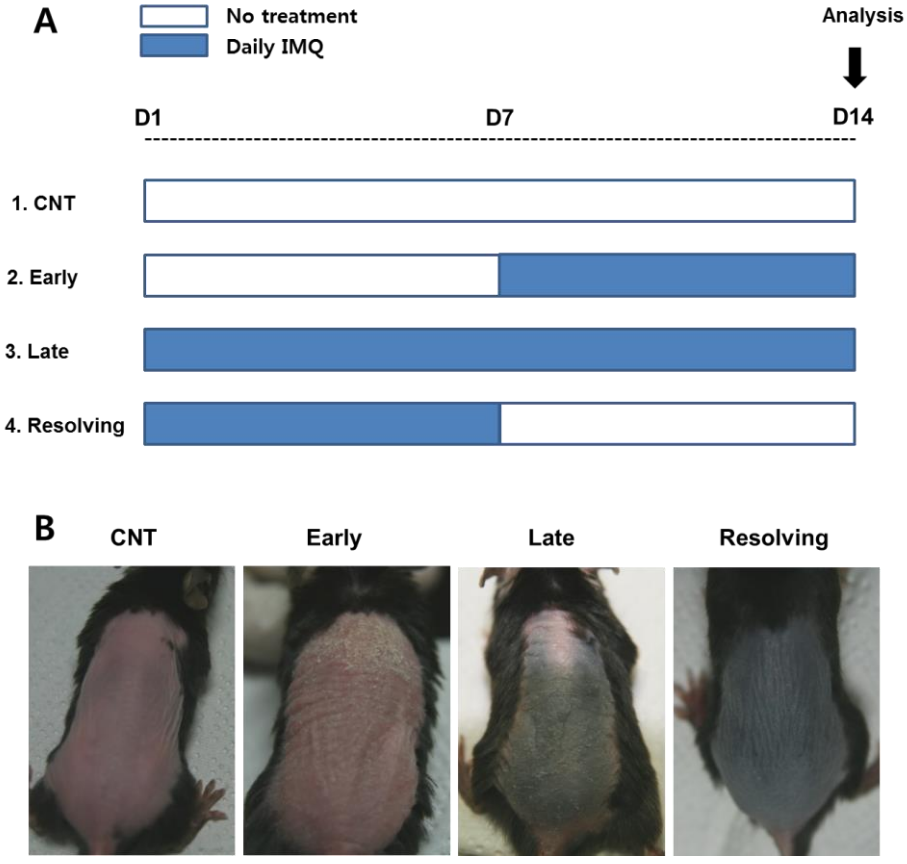


Figure 6. Experiment design for the evaluation of the change of Treg cells in prolonged IMQ application-induced animal model. (A) The experiment was designed with four groups (n=3) as follows: (i) untreated control (CNT) group, (ii) IMQ-applied group for 7 consecutive days (early response group), (iii), and IMQ-applied group for 14 consecutive days (late response group), (iv) spontaneous resolving after 7 day application of IMQ (resolving group). (B) Representative skin changes of each group on day 14.

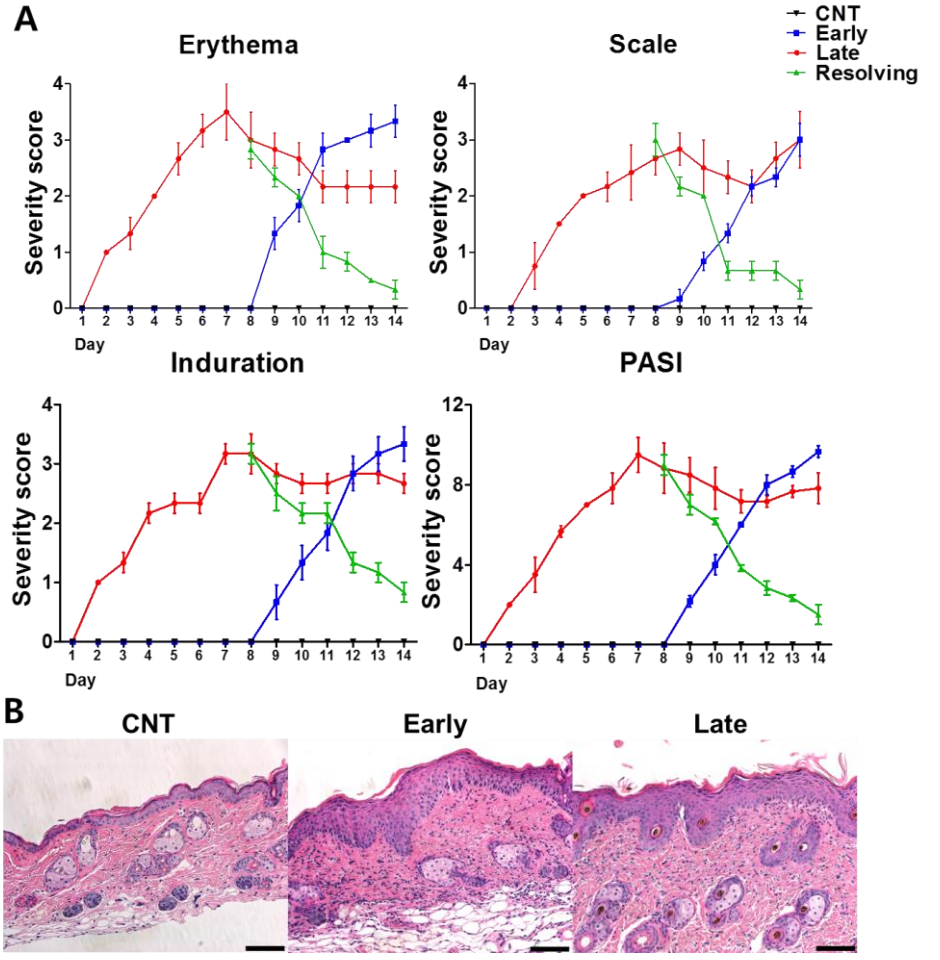


Figure 7. The change of clinical scores and histology during prolonged application of IMQ for 14 days. (A) Erythema, scaling, and induration of the back skin were scored daily on a scale from 0 to 4. The cumulative score (sum of scores for erythema, scale, induration) is depicted as modified PASI. Data are mean scores ± SD of three mice. (B) Histologic changes on the back skin. Representative photos from each group (n=3). Bars, 200 μm.

4. The change of T cells and Treg cells in spleen and lymph node during late response are similar with those in early response.

Analysis of $CD4^+$ T cell population in spleen over 14 days of continuous IMQ application showed similar results to early response (Figure 8), and there is no significant change in splenic Treg cells between early and late response (Figure 9A, 9B). And intracellular CTLA-4 expressions in $Foxp3^+$ $CD4^+$ Treg cells were not different between early and late response (Figure 9C-9E). In lymph node, CD4 and CD8 T cell ratio were not significantly changed in both early and late groups (Figure 10A). However, the ratio of $Foxp3^+$ $CD4^+$ Treg was similarly increased in both early and late period, but there was no statistically significant difference between early and late period (Figure 10B).

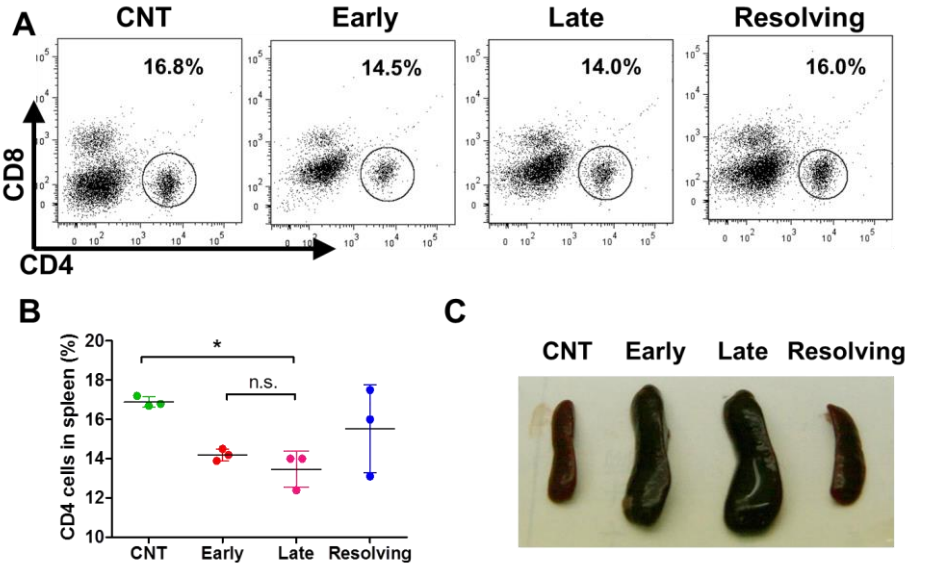
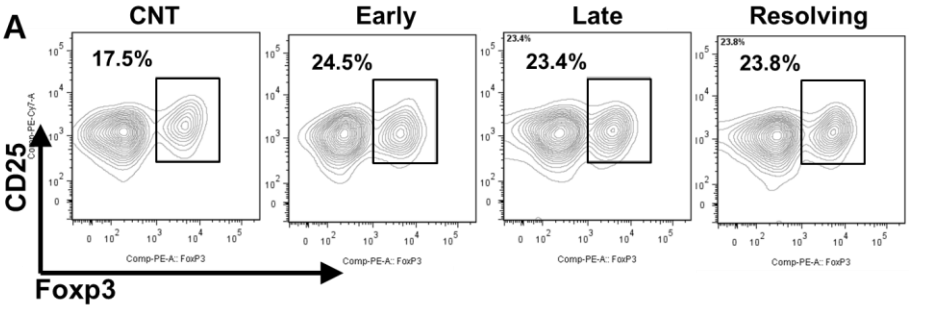
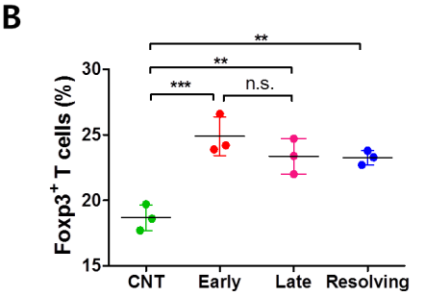
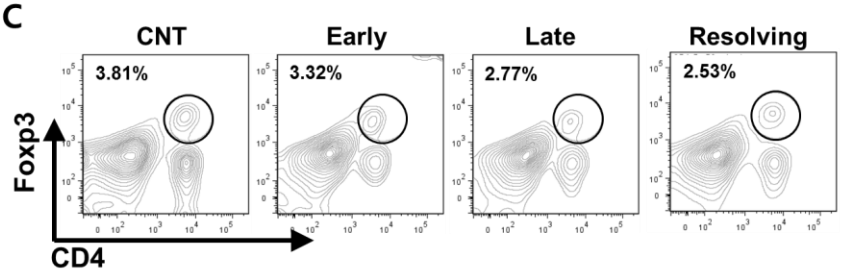
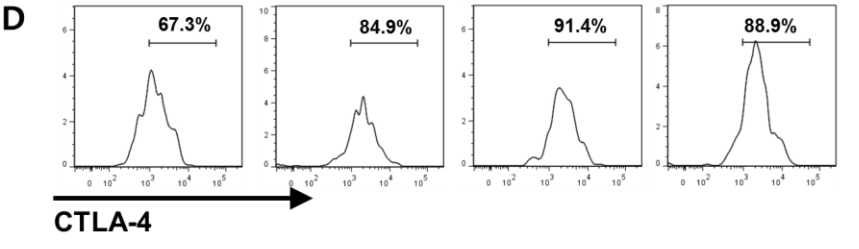


Figure 8. Decreased splenic CD4 cells in both early and late response. (A) Single cells obtained from spleen were stained with the fluorescence conjugated antibodies to CD4 and CD8. (B) The difference of splenic CD4 cells among viable splenic cells between early and late response was not statistically significant. * P <0.05; n.s., not significant. Bar, mean±SD. (C) Gross spleen size in four groups. The spleen size was increased in both early and late group. A representative mouse from three mice from each group.









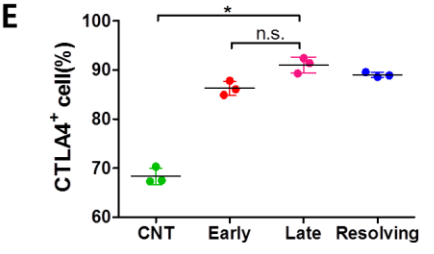


Figure 9. Increased splenic Treg among $CD4^+$ T cell in both early and late group. Single cells obtained from spleen were gated with CD4 positive cell and then analyzed the expression of CD25, Foxp3, and CTLA-4. (A, B) The ratio of splenic $Foxp3^+$ CD4 among viable splenic $CD4^+$ cells in both early and late groups significantly increased compared with untreated control (CNT). But the difference of Foxp3+ Treg between early and late groups was not statistically significance. (n=3, each group). (C, D) The ratio of CTLA-4 expressing Treg cell in late group increased compared with control. (E) However there was no statistically significant difference of CTLA-4 positive Treg cell between early and late period (n=3, each group, P=0.018 in Kruskal-Wallis test with Dunn's post-hoc test. ***** $P < 0.05$, ****** $P < 0.01$, *** $P < 0.001$. Bar, mean±SD.

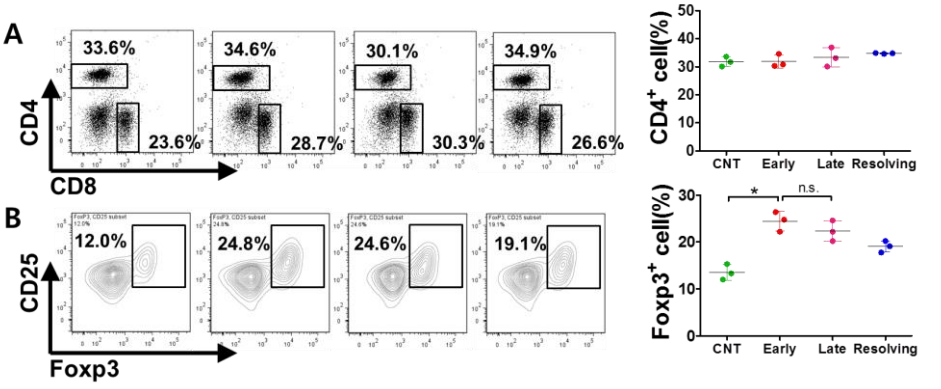


Figure 10. Increased lymph node Treg among CD4$^+$ T cell in both early and late group. (A) Single cells obtained from lymph node were gated with CD4 positive cell and then analyzed the expression of CD25 and Foxp3. CD4 and CD8 T cell ratio were not significantly different between early and late groups. (B) The ratio of LN Foxp3$^+$ CD4 among viable LN CD4$^+$ cells in early group significantly increased compared with untreated control. But the difference of Foxp3$^+$ Treg between early and late groups was not statistically significance. (n=3, each group). ***P* < 0.05. Bar, mean±SD.

5. Transient depletion of Treg cell in early response does not exacerbate skin inflammation

To understand the direct role of Treg cells in IMQ-induced mice model, Treg cell depletion model using Foxp3-DTR-eGFP mice was adopted. Foxp3-DTR-eGFP mice express a fusion protein consisting of the diphtheria toxin (DT) receptor and enhanced green fluorescent protein (eGFP) under the control of the Foxp3 gene locus inserted by BAC transgenesis.[21] Treg cells in both spleen and lymph node could be selectively depleted by administration of 1 μg of DT at 48 hr before observation (Figure 11). Using this model, DT was injected 48 hr before induction of IMQ-induced skin inflammation. Experiment design is shown in Figure 12A. Unexpectedly, the transient Treg depletion before the induction of skin inflammation did not a IMQ-induced inflammatory change (Figure 12B). Then total γδ T cells, the main source of IL-17 in this mice model, and IL-17-producing γδ T cells in skin (Figure 13), skin draining lymph nodes (SDLNs, Figure 14) and spleen (Figure 15) were measured. In the line with ear thickness change, total γδ T cells from total skin, SDLNs, and spleen were not different between IMQ group and IMQ+DT injected group. IL-17-producing γδ T cells also were not different between IMQ group and IMQ+DT injected group in total skin (Figure 13B) and spleen (Figure 15B). But in SDLNs IL-17-producing γδ T cells are slightly increased in IMQ+DT injected group than IMQ group (Figure 14B). Quantitative RT-PCR was also

conducted using total skin samples. IL-17A mRNA expressions were not different between IMQ group and IMQ+DT group (Figure 13C). IL-22 from CD4 T cell was not increased after IMQ application in both LN and spleen (Figure 14A, 15A).

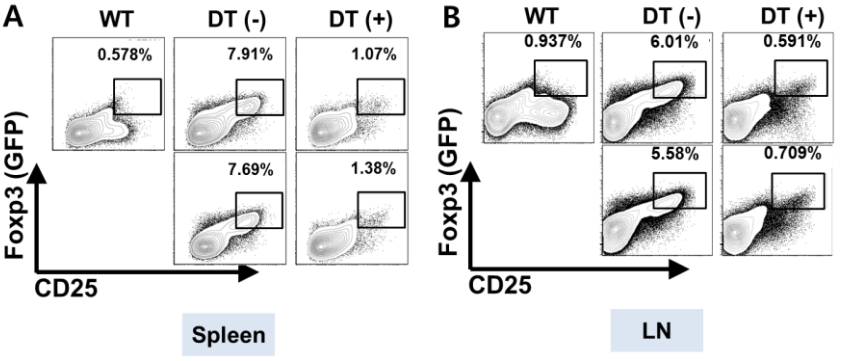


Figure 11. Depletion of Foxp3$^+$ Treg cells in Foxp3-DTR-eGFP mice. One μg of Diphtheria toxin (DT) was injected intraperitonially in both C57BL/6 wild type mice (WT) and the mice were sacrificed 2 days after DT injection. After DT injection, CD25hiFoxp3$^+$ Treg cells were successfully depleted in (A) spleen and (B) lymph node (LN). Wild type mice do not express green fluorescence (GFP) and flow cytometric analysis from Foxp3-DTR-eGFP mice without DT injection shows GFP-expressing CD25^{hi}Foxp3$^+$ cell in spleen and LN.

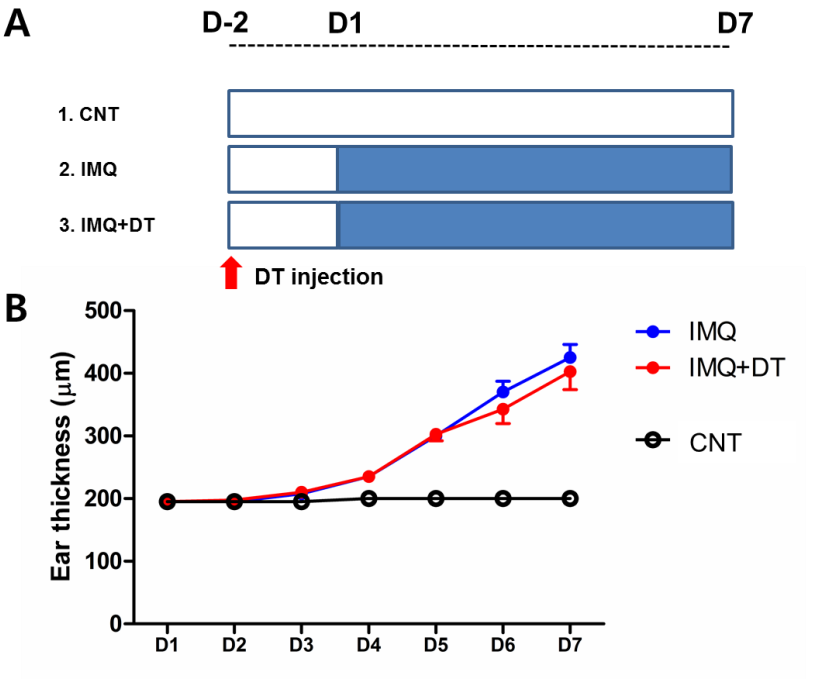


Figure 12. Transient depletion of Treg cell in early response does not exacerbate IMQ-induced skin inflammation. (A) Diphtheria toxin (DT) was injected in Foxp3-DTR-eGFP mice 48 hr before IMQ application to induce transient Tregs depletion. (B) There was no significant change of ear thickness depending on Treg cell depletion. Spots show the mean of ear thickness and bars indicate SD (n=3).

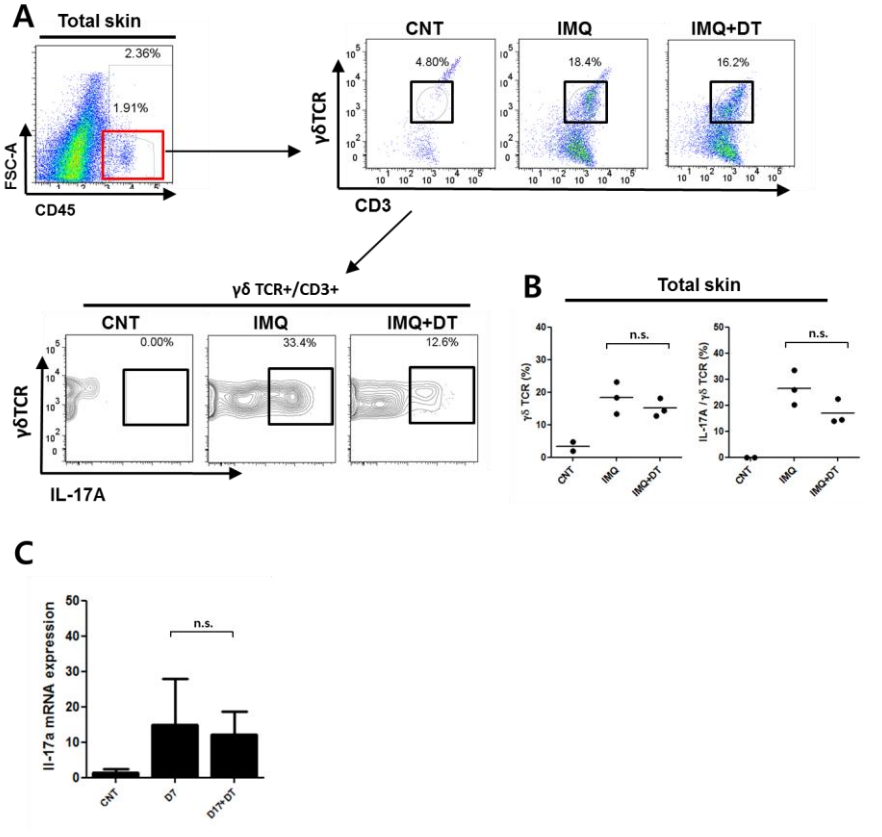


Figure 13. Depletion of Treg cell in early period did not affect the number of IL-17 producing γδ T cells in skin. Single cells obtained from total skin were gated with $CD3^+$ and $γδ^+$ cell and then analyzed the expression of IL-17A. (B) The ratio of γδ T cells and IL-17 producing γδ T cells in skin were not different between IMQ group and IMQ+DT group (n=3). (C) Quantitative RT-PCR was conducted using total skin samples. *IL17A* mRNA expressions were not different between IMQ group and IMQ+DT group. Bar, mean.

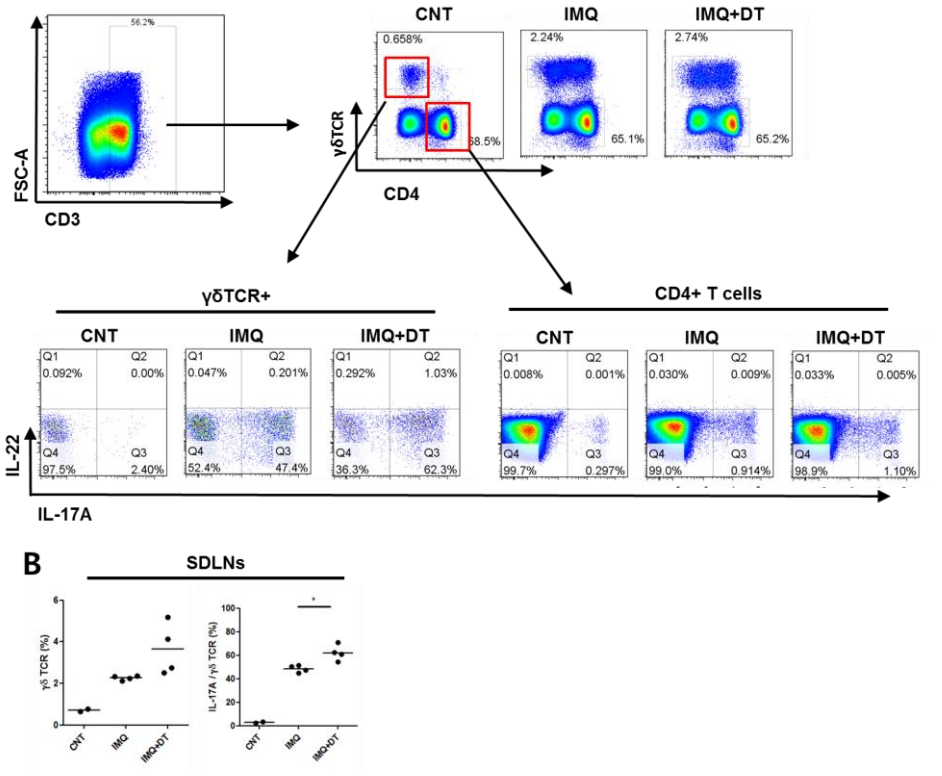


Figure 14. **Depletion of Treg cell in early period did not affect the number of IL-17 producing γδ T cells in lymph node.** Single cells obtained from skin draining lymph nodes (SDNLs) were gated with $\gamma\delta^+$ cells and $CD4^+$ cells respectively, then we analyzed the expression of IL-17A on each subset. (B) γδ T cells were main source of IL-17 compared with CD4, and the ratio of IL-17 producing γδ T cells but not total γδ T cells in SDLNs was slightly increased in IMQ+DT mice. Bar, mean. *P<0.05.

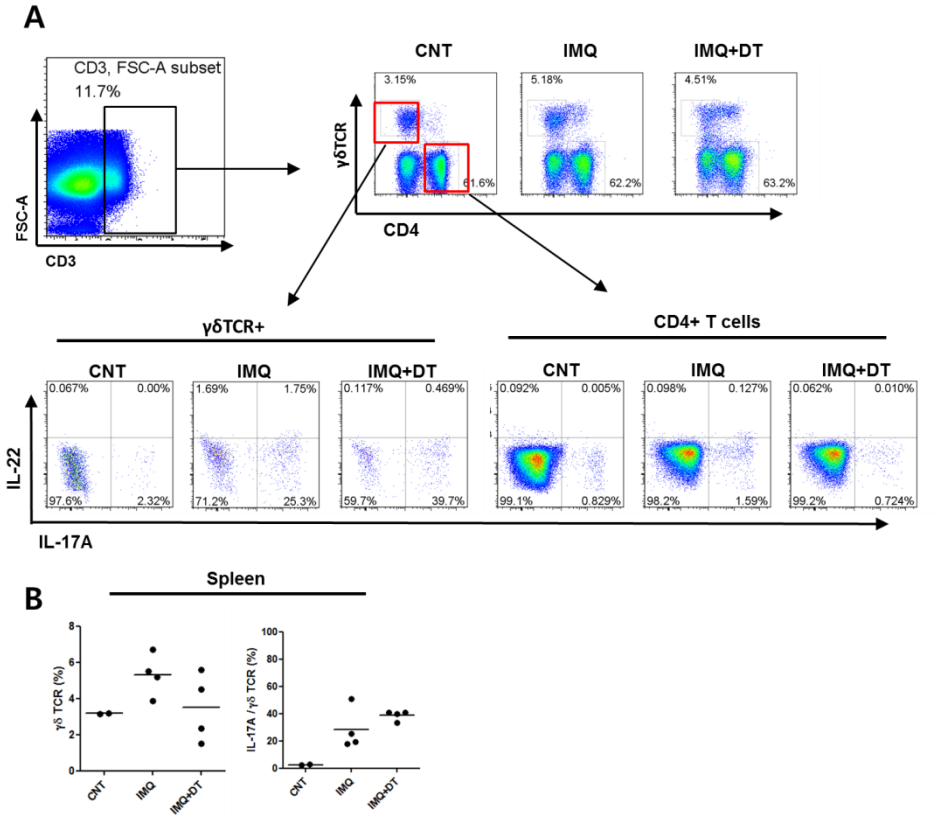


Figure 15. Depletion of Treg cell in early period did not affect the number of IL-17A producing γδ T cells in spleen. Single cells obtained from spleen were gated with $\gamma\delta^+$ cells and $CD4^+$ cells respectively, then we analyzed the expression of IL-17A on each subset. (B) γδ T cells were main source of IL-17 compared with $CD4^+$ cells, and the ratio of total γδ T cells and IL-17 producing γδ T cells were not different between IMQ group and IMQ+DT group. Bar, mean.

6. Transient depletion of Treg cell augments late response but not early response in IMQ-induced psoriasiform dermatitis model

To elucidate the effect of Treg cell depletion during late period, DT was injected on day 5 when the IMQ-induced skin change already developed. Experiment design is shown in Figure 16A. Contrary to early response, transient Treg cell depletion before the starting of late period, skin inflammation was exacerbated by supporting evidences from skin change (Figure 16B) and ear thickness change (Figure 16C).

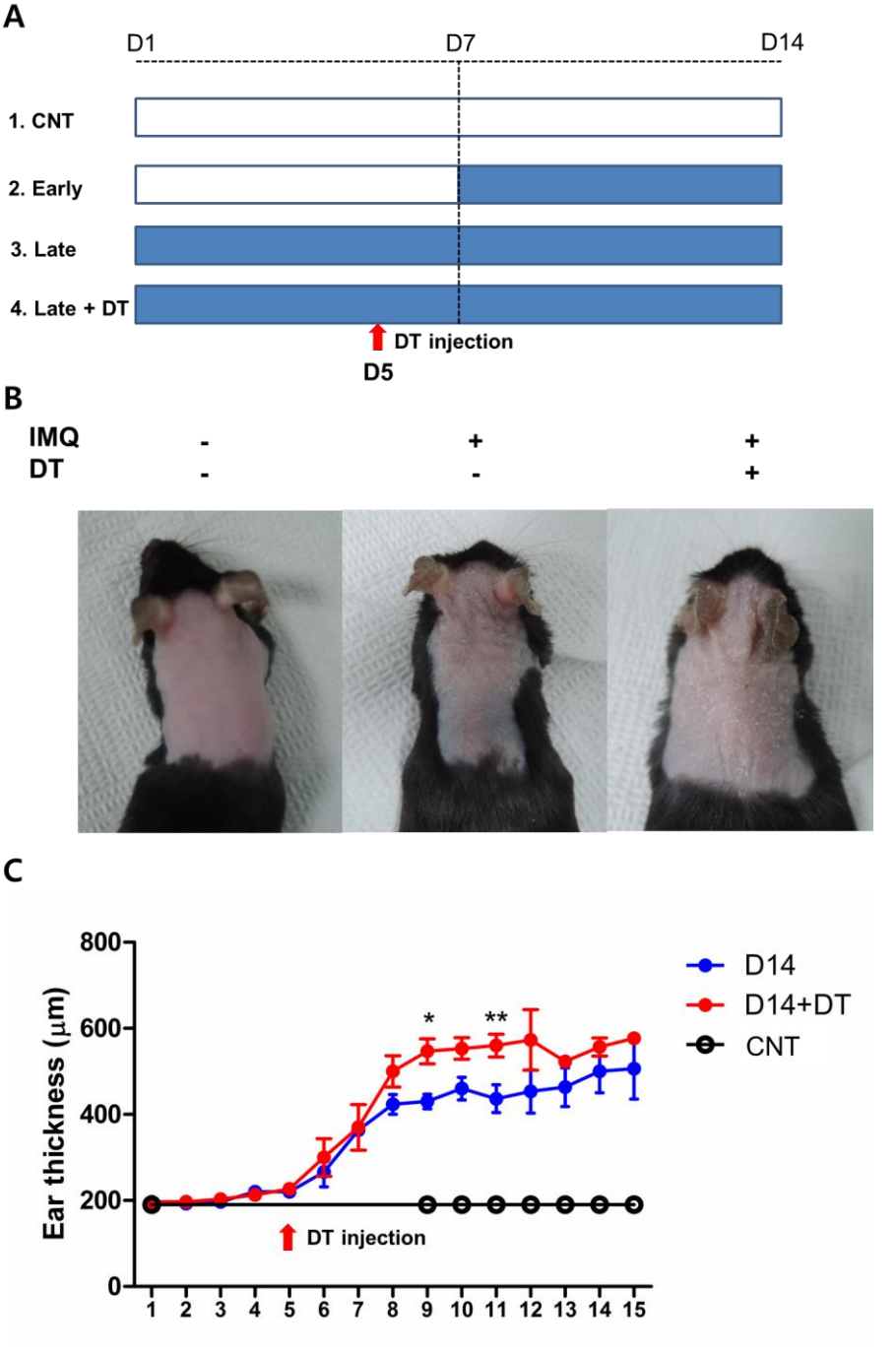


Figure 16. Transient depletion of Treg cell augments late response in IMQ-induced psoriasiform dermatitis model. (A) To deplete Treg during late period, diphtheria toxin (DT) was injected in Foxp3-DTR-eGFP mice on day 5. IMQ was applied for 14 consecutive days. (B) Clinical photo on day 9 in a representative mouse from each group (n=3) (C) The ear thickness was statistically increased in DT injected late response group on day 9 and day 11. Spots show the mean of ear thickness and bars indicate SD.

7. Treatment with anti-IL-17A monoclonal antibody rescues mice from the exacerbation of disease associated with Treg cell depletion.

Various biologics targeting IL-17A pathway shows excellent efficacies in human trials.[22-24] Based on these findings so far, I hypothesized that IL-17 could play a part in the increased disease activity in late phase observed following Treg depletion. More importantly, to test whether a state-of-the-art remedy, anti-IL-17A monoclonal antibody (mAb) can rescue Treg depletion induced skin inflammation or not, transient Treg-depleted mice were treated with anti-IL-17A mAb. Four hundred mg of anti-IL-17A mAb was injected twice after DT injection (Figure 17A). On D10, anti-IL-17A mAb treatment reduced ear swelling in mice both with and without DT injection (Figure 17B) although the reduction of ear swelling after anti-IL-17A mAb treatment was statistically significant only in DT injected mice (Figure 17C). Interestingly, therapeutic efficacy of anti-IL-17A mAb was better in Treg-depleted mice compared with Treg-competent mice (Figure 17D).

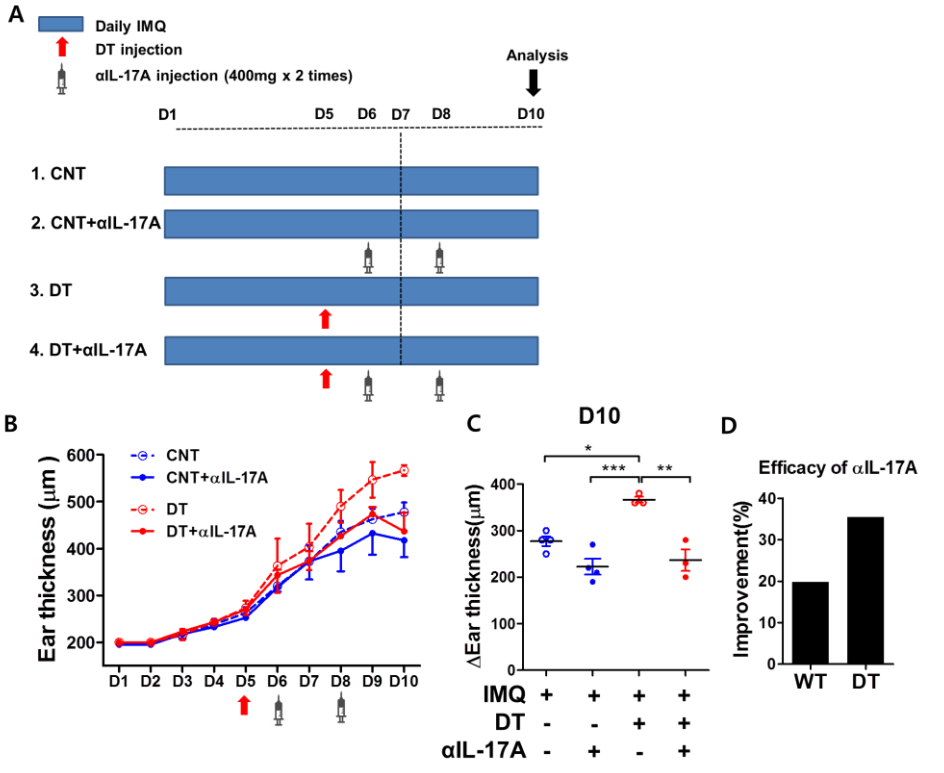


Figure 17. Treatment with anti-IL-17A mAb rescues mice from the exacerbation of disease associated with Treg cell depletion. (A) To deplete Treg cells during late period, diphtheria toxin was injected in Foxp3-DTR-eGFP mice on day 5. Four hundred mg of anti-IL-17A mAb (αIL-17A) was injected twice on day 6 and day 8 in treatment groups. (B, C) The ear thickness was significantly decreased in anti-IL-17A mAb treatment groups. Spots show the mean of ear thickness and bars indicate SD. (D) Therapeutic efficacy was defined as reduction of ear thickness per mean ear thickness of anti-IL17A mAb untreated mice. Anti-IL17A mAb reduced ear thickness by 19.8% in wild type (WT) group and by 35.5% in DT treated group. All mice were applied with IMQ cream daily.

Although the therapeutic mode of anti-IL-17A mAb is uncertain in lesional skin, but anti-IL-17A mAb treated IMQ mice without Treg depletion showed significantly lower IL-17A-producing γδ T cells in total skin suspension (Figure 18). However, this difference was not observed in Treg-depleted mice with or without anti-IL-17A mAb.

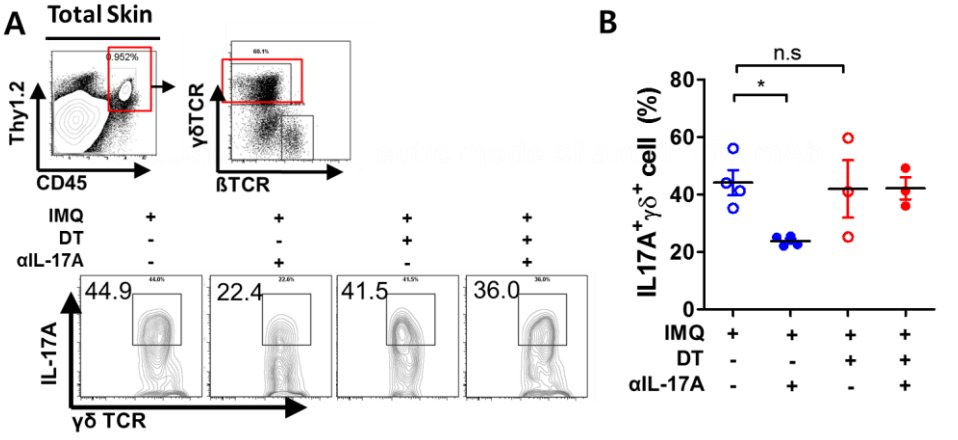


Figure 18. Therapeutic mode of anti-IL-17A mAb. (A) Cell suspension from total back skin was gated as γδ T cells, then expression of IL-17A was analyzed using flow cytometry. (B) Anti-IL-17A mAb treated IMQ mice without Treg depletion showed significantly lower IL-17A- producing γδ T cells. However there are no significant differences between anti-IL-17A mAb treated and untreated mice in Treg depleted groups. *P<0.05; n.s., not significant; αIL-17A, anti-IL17A monoclonal antibody. Bars, mean.

After 4 to 5 days after DT injection Treg cells start to repopulate. To avoid misinterpretation by complicated cellular change during the restoration of Treg cells, DT injections were repeated in injection interval of every other day up to three times (Figure 19A). However, even in the setting of prolonged depletion of Treg cells, the changes of ear thickness, in terms of the therapeutic response to anti-IL-17A mAb, were similar with the results from the experiment using single injection of DT (Figure 17B, Figure 19A). Interestingly, in the model of prolonged Treg depletion, skin from DT injected mice with or without anti-IL-17A mAb treatment showed decreased IL-17A mRNA expression but increased IFN-γ mRNA expression (Figure 19B, C). These results suggest anti-IL-17A mAb treatment did not directly affect the expression of these cytokines within cutaneous tissue level although anti-IL17A mAb partially abrogated ear swelling.

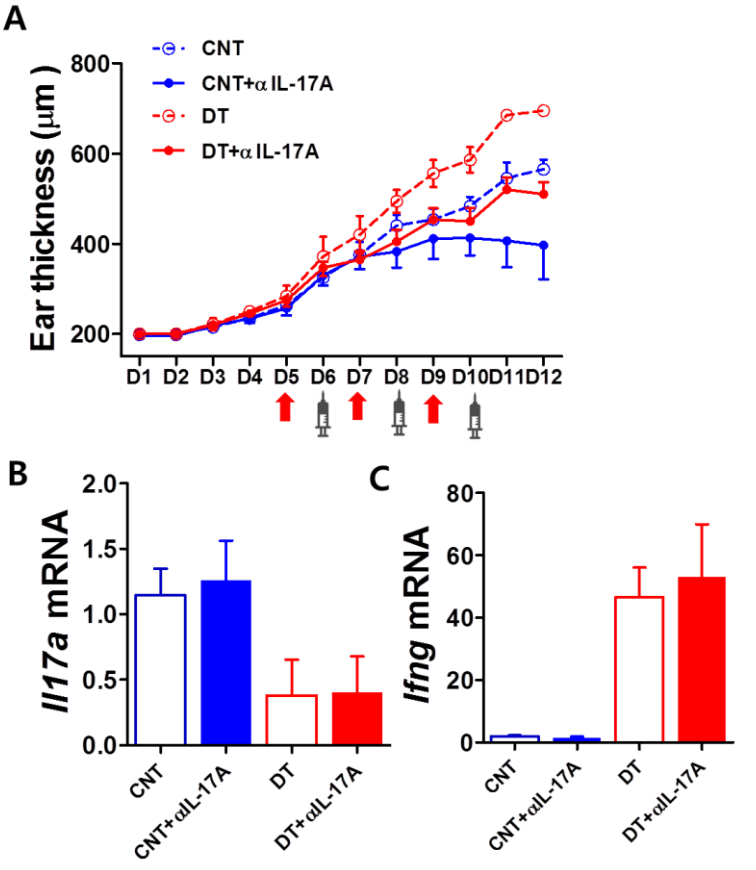


Figure 19. Therapeutic effect of anti-IL-17A mAb in model with continuous depletion of Treg cells. (A) To induce complete depletion of Treg cells during late period, diphtheria toxin was injected in the interval of every other day on day 5, day 7, and day 9. Four hundred mg of anti-IL-17A mAb (αIL-17A) was injected thrice on day 6, day 8, and day 10 in treatment groups. The ear thickness was significantly decreased in anti-IL-17A mAb treatment groups. Spots show the mean of ear thickness and bars indicate SD. (B, C) mRNA expression of IL-17A and IFN-γ were measure using back skins from mice.

IV. DISCUSSION

To best knowledge, this is the first study to characterize the different response to depletion of Treg cells in prolonged application of IMQ using Foxp3-DTR-eGFP mice. In this study, transient depletion of Treg cells augmented late response but not early response in IMQ-induced psoriasiform skin inflammation. From this observation, Treg cells are important in controlling ongoing inflammation in IMQ-induced skin inflammation.

In human psoriasis, dysregulation of the Treg compartment has been reported, and inflammatory cytokine milieu such as IL-6, IL21, and IL-23 critically modulated the suppressive function of Treg cells.[5-7,12] In addition, the balance between Treg cells and responder T cells was broken by the increased number of Th17 cells in psoriasis.[14,25,26] Collectively, recent studies suggest Treg cells do not have direct pathogenic roles in the development of psoriasis but the functions of Treg cells have been vulnerably altered by surrounding milieu.

In human psoriasis and in mouse models, the IL-23/IL-17 axis has a major role in disease.[2,27] Both intradermal injection of IL-23 and the topical application of IMQ, a TLR7/8 agonist, induce psoriasis-like epidermal thickening and dermal inflammation and are considered representative animal model for psoriasis.[2,28] Despite of species difference, long-term application of IMQ on mouse skin can recapitulate the early and late phases of human psoriasis.[20] In this study, splenic and lymph nodal Treg cells were increased after IMQ application, and the Treg cell markers, PD-1, CTLA-4, and GITR

which are related with their suppressive functions were also increased.[29,30] Increased Treg cells possessing these suppressive phenotypes in IMQ-induced animal model favor the competent suppressive role of Treg cells are still preserved.

Previous studies have shown that Treg cells are able to limit the accumulation of Th17 cells at inflammatory sites and draining lymph nodes.[31] Interestingly, in graft versus host disease mice model, Treg lymphocytes are able to completely prevent the inflammatory disease but they could not ameliorate the disease when given late in the course.[31] The potent disease-preventing effect of Treg cells in these models is likely because of the ability of Treg cells to block initial activation and, as a consequence, antigen-induced T cell expansion after recognition of the systemic antigen.[31] Contrary to this classical antigen specific Treg response, action mechanism of Treg cells in IMQ-induced model is still elusive. Similar to effector T-cell subsets, the function of Treg cells was shown to be dependent on activation via the T-cell receptor/CD3 complex.[33] However, once appropriately activated, regulatory function of Treg cells can be antigen nonspecific.[34,35] Many of Treg cells act their suppressive roles by secretion of the immunosuppressive cytokines IL-10 and/or TGF-β.[36,37] Thus, activated Treg cells create a tolerogenic milieu to down-regulate aberrant immune responses even in late period of IMQ-induced inflammation.

In another point of view, one might argue that insufficient depletion of Treg cell can be related with negligible effect on early response. As Foxp3-null mice induce lethal lymphoproliferative autoimmune syndrome, transient Treg cell-depletion model was adopted in this study.[21,38-40] In this model, 24 h after DT injection almost $CD4^+CD25^+eGFP^+$ cells disappeared in blood and the near-total depletion lasted at least 4 days after DT injection.[21] Transient DT treatment, although sufficient to deplete Treg cells in spleen and LN, but leave a residual number of $eGFP^+FoxP3^+$cells, and these cells eventually repopulate the Treg niche.[21,39] Because this study focused on analyzing the effects of transient Treg depletion on inflammation in the late response of IMQ-induced skin change, this mouse was suitable for our purpose, despite its limitations. Without Treg cell depletion, Treg cell numbers in IMQ-induced skin inflammation expanded in spleen and these cells were found to be highly activated characterized by increased PD-1, CTLA-4, and GITR as prescribe above. When Foxp3 mRNA level was analyzed 1 week after DT injection, Foxp3 mRNA level significantly increased in Treg depletion mice which suggest rapid repopulation of $Foxp3^+$ Treg in this model. This result also indicates that Treg cells are important in the controlling systemic inflammation provoked by topical application of IMQ. However, the fact showing severe inflammation in the back skin despite the expansion of Treg cells could be the result of a dysregulation of the Th17/Treg balance. In support of this, neutralizing anti-IL-17 mAb treatment could partially ameliorate the detrimental effects of

Treg depletion during late response in IMQ-induced mice model. This result suggests current drug developments targeting IL-17/IL-23 pathway looks more feasible strategy than modulating Treg cell itself in practical view.[22-24,41] In addition to Th17 pathway, Th1 pathway characterized by IFN-γ can be another important target of controlling by Treg cells.

There are several limitations of this study in lack of ample analysis on tissue resident T cells subsets and dendritic cell compartment. Recently, resident memory T cells (TRM) are a recently described as important subset of memory T cells that persist long-term in peripheral tissues.[42-45] In addition to $CD8^+$ TRM, $CD4^+$ TRM has been also described, we can expect the important role of interaction between TRM and recruiting of non-resident Treg cells or even the presence of resident Treg subsets in chronic skin inflammation.[46,47] Other immune regulatory subset includes DCs. For example, tolerogenic DCs are characterized by low constitutive expression of positive costimulatory molecules compared with inhibitory factors, as well as by their ability to suppress a broad range of effector T cell responses.[48,49] Immature DCs and semi-mature developmental stages of DC differentiation and some subtypes of DCs, such as resting plasmacytoid DC, are prone to induce T cell anergy and Treg development.[50] So, to understand Treg dynamics in IMQ-induced model on the whole, various immune components should be parallel investigated together.

Taken together, these findings show that Tregs are important for the controlling of on-going inflammation in IMQ-induced skin inflammation. And transient Treg cell depletion acts, at least partly, through increasing IL-17 producing γδ T cell and inflammatory cytokines such as IFN-γ. These results suggest possibility that Treg cells therapy may be of benefit in psoriasis as a supportive therapeutics to current anti-psoriatic treatments based on anti-IL-17/IL-23 strategies. Further investigations to reveal the detailed dynamic change of Treg cells and their functional phenotypes on different time points in IMQ-induced skin change will be needed for the comprehensive understanding of psoriasis.

V. CONCLUSION

In this study, repetitive application of IMQ on the mice skin successfully induced psoriasiform dermatitis model. In active inflammation, splenic $CD4^{+}Foxp3^{+}$ Treg cells were significantly increased and their expression of markers related with suppressive function are increased on Treg cells. However, prolonged application of IMQ more than one week did not exacerbate IMQ-induced skin inflammation and did not changed splenic and lymph node Treg cells and their suppressive markers.

The transient Treg depletion before the induction of skin inflammation did not worsen IMQ-induced inflammation. On the contrary to this, Treg cell depletion before the starting of late period, skin inflammation was augmented. The treatment with anti-IL-17A mAb partially rescued mice from the exacerbation of disease associated with Treg cell depletion.

Taken together, these findings show that Tregs are important for the controlling of on-going inflammation in IMQ-induced skin inflammation. These results suggest possibility that Treg cells therapy may be of benefit in psoriasis as a supportive therapeutics to current anti-psoriatic treatments based on anti-IL-17 strategies. Further investigations to reveal the detailed dynamic change of Treg cells and their functional phenotypes on different time points in IMQ-induced skin change will be needed for the comprehensive understanding of psoriasis.

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ABSTRACT(IN KOREAN)

Imiquimod 유발 건선양 피부염 모델에서 면역조절 T 세포의 역할

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건선은 두꺼운 은백색의 각질을 동반한 광범위한 홍반성 반을 특징으로 하는 만성 염증성 면역피부질환이다. 다양한 피부 면역세포 중에서도 T 세포와 T 세포 유래 염증물질이 병인에 중요한 것으로 알려져 있다. 다양한 T 세포 중 Foxp3를 발현하는 면역조절세포(이하 Treg 세포)는 만성적인 염증의 억제를 통한 면역조절에 중요한데, 건선에서도 이러한 Treg 세포의 기능의 이상이 보고된 바 있다. 그러나 아직까지 Treg 세포를 기반으로 한 치료나 동물모델에서의 Treg 세포에 대한 연구는 희소하였다. 이 연구는 imiquimod (이하 IMQ) 유발 건선 동물모델을 이용하여 Treg 세포의 기능과, Treg 세포 고갈 시에 나타나는 변화를 통해 Treg 세포의 건선에서의 기능을 연구하고자 하였다.

먼저 마우스의 등과 귀에 IMQ 크림을 1주간 도포하여 IMQ 유발 건선양 피부염을 성공적으로 유발하였다. 활성 피부병변을 가진 마우스의 경우 대조군에 비해 비장과 림프절 내 Treg 세포

의 비율이 증가하였고, Treg 세포의 면역억제 기능과 관련된 마커인 PD-1, CTLA-4, 및 GITR 의 발현증가가 확인되었다. 흥미롭게도 IMQ 크림을 1주 이상 2주간 도포하더라도 피부병변의 악화를 보이지 않았다. 또한 2주간 IMQ를 매일 도포한 마우스의 경우 1주간 도포한 마우스와 비교하여 비장 및 림프절 내 Treg 세포의 수 및 마커 발현의 유의한 변화는 관찰되지 않았다.

이러한 IMQ 유발 마우스 모델에서의 Treg 세포의 기능을 확인하기 위해 일과성 Treg 세포의 고갈 모델인 Foxp3-DTR-eGFP 마우스를 이용하여 추가 실험을 진행하였다. 흥미롭게도 IMQ 도포 전, 즉 피부염증반응을 유발하기 전 디프테리아 독소(이하 DT)를 처리하여 Treg 세포를 고갈시키는 경우 건선양 피부염의 초기반응은 악화를 보이지 않은 반면, 피부염증이 충분히 발생한 시기에 DT를 이용하여 Treg 세포를 고갈시키는 경우 후기반응은 악화됨을 확인하였다. 대표적인 건선 치료약제인 항 IL-17A 단클론 항체를 이용한 치료모델에서는 이러한 Treg 고갈에 의한 후기 염증반응의 악화가 부분적으로 상쇄됨을 확인하였다.

결론적으로 이러한 IMQ 유발 건선양 피부염 모델에서 Treg 세포는 병변의 발생 초기보다는 염증이 어느 정도 유발된 상황에서는 진행중인 염증의 조절에 중요한 역할을 함을 알 수 있었고, Treg의 고갈 시 나타나는 피부염 악화의 일부에는 IL-17A와 IFN- γ 등의 염증성 사이토카인이 관여함을 확인하였다. 이는 향후 Treg 세포를 조절할 수 있는 약제가 현재의 IL-17/IL-23 축 기반의 약물에 보조적인 치료제로 활용될 수 있음을 시사한다.

향후 사람 및 동물모델 건선에서의 임상경과 및 치료에 따른 Treg 세포의 변화에 대한 연구를 통해 Treg 세포의 병인에서의 역할 및 치료 타깃으로의 효용성에 대한 추가적인 연구가 필요하다.

핵심되는 말 : 동물모델, 디프테리아 독소, 이미퀴모드, 인터루킨 17, 건선, 면역조절 T 세포