



## Original Article

# Anti-IL-17A and IL-23p19 antibodies but not anti-TNF $\alpha$ antibody induce expansion of regulatory T cells and restoration of their suppressive function in imiquimod-induced psoriasiform dermatitis



Teruo Shimizu, Masahiro Kamata\*, Saki Fukaya, Kotaro Hayashi, Atsuko Fukuyasu, Takamitsu Tanaka, Takeko Ishikawa, Takamitsu Ohnishi, Yayoi Tada\*

Department of Dermatology, Teikyo University School of Medicine, Tokyo, Japan

## ARTICLE INFO

## Article history:

Received 15 January 2019

Received in revised form 1 July 2019

Accepted 18 July 2019

## Keywords:

Psoriasis

Regulatory T cell

IL-17, IL-23

TNF- $\alpha$

## ABSTRACT

**Background:** Psoriasis is a chronic inflammatory skin disease. Anti-TNF $\alpha$ , IL-17A and IL-23p19 antibodies are effective for psoriasis. However, the contribution of regulatory T cells (Treg) in their effectiveness remains to be elucidated.

**Objective:** We investigated the effects of TNF $\alpha$ , IL-17A and IL-23p19 inhibition on Tregs in imiquimod-induced psoriasiform dermatitis.

**Methods:** Psoriasiform dermatitis was induced by imiquimod application on murine shaved back skin for six days. Mice were treated with anti-TNF $\alpha$ , IL-17A or IL-23p19 monoclonal antibodies every other day from one day before imiquimod application.

**Results:** Administration of anti-TNF $\alpha$ , IL-17A or IL-23p19 antibodies improved the clinical score and downregulated Th17-related cytokines and chemokines, while IL-23p19 antibodies upregulated IL-10 mRNA expression. Anti-IL-17A or IL-23p19 antibody-treated imiquimod-applied mice showed a significant increase in the number of Foxp3<sup>+</sup> IL-10<sup>+</sup> Tregs. Recipient mice adoptively transferred with Tregs derived from donor mice treated with antibodies demonstrated clinical and pathological improvement in imiquimod-induced psoriasiform dermatitis. Anti-IL-17A or IL-23p19 antibody-induced Tregs significantly increased the number of Foxp3<sup>+</sup> cells and IL-10 expression in imiquimod-induced psoriasiform dermatitis in recipient mice but anti-TNF $\alpha$  antibody-induced Tregs did not.

**Conclusion:** Anti-IL-17A or IL-23p19 antibody inhibits the IL-17/IL-23 signaling pathway, and induces expansion of Tregs and their suppressive capacity in imiquimod-induced psoriasiform dermatitis.

© 2019 Japanese Society for Investigative Dermatology. Published by Elsevier B.V. All rights reserved.

## 1. Introduction

Psoriasis is a common chronic inflammatory skin disease characterized by clinical features of erythematous and scaly plaques, and pathological features of aberrant hyperproliferation of keratinocytes and infiltration of neutrophils, affecting approximately 2–3% of the world's population. A variety of cells such as keratinocytes, dendritic cells, Th1 cells, and Th17 cells are involved in the development of psoriasis and interact with each other through the production of cytokines [1–3]. The importance of the interleukin (IL)-17/IL-23 pathways in psoriasis pathogenesis has

been proven by the efficacy of biologics targeting those cytokines such as IL-17A [ixekizumab [4] and secukinumab [5]], IL-17RA [brodalumab [6]], IL-12/23p40 [ustekinumab [7]], IL-23p19 [guselkumab [8], risankizumab [9], and tildakizumab [10]].

Aside from directly inhibiting key cytokines involved in psoriasis by biologics, other therapeutic approaches are available. Topical corticosteroid, topical vitamin D3, and UVB irradiation are effective for psoriasis. Among them, UVB therapy and topical vitamin D3 are assumed to exert their therapeutic effect not only through inhibiting T cell activation but also expanding regulatory T cells (Tregs) [11–13]. IL-10 has an anti-inflammatory effect, inhibiting the production of pro-inflammatory cytokines. It is produced by Treg lymphocytes in addition to macrophages, dendritic cells, and B lymphocytes [14]. Relative IL-10 deficiency in serum and skin in patients with psoriasis is an essential factor in pathogenesis [15]. Furthermore, dysfunction of Treg is significant in the pathogenesis of psoriasis [16]. Indeed, we recently demonstrated that the vitamin D3 analog, maxacalcitol,

Abbreviations: Treg, regulatory T cell; IMQ, imiquimod; IL, interleukin.

\* Corresponding authors at: Department of Dermatology, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo, 173-8605, Japan.

E-mail addresses: [mkamata-tky@umin.ac.jp](mailto:mkamata-tky@umin.ac.jp) (M. Kamata), [ytada-tky@umin.ac.jp](mailto:ytada-tky@umin.ac.jp) (Y. Tada).

<https://doi.org/10.1016/j.jdermsci.2019.07.006>

0923-1811/© 2019 Japanese Society for Investigative Dermatology. Published by Elsevier B.V. All rights reserved.

ameliorated imiquimod (IMQ)-induced psoriasiform skin inflammation on mice by inducing functional Tregs and suppressing IL-17 responses [17]. Additionally, previous clinical studies indicated that therapies such as UVB irradiation and topical vitamin D3 induced expansion of Tregs, which resulted in long-term remission [18–20]. However, the effect of IL-17 or IL-23 inhibition by antibodies on Treg function and expansion has not been clarified yet.

We previously reported that inhibition of tumor necrosis factor (TNF)- $\alpha$  [21] or IL-17A [22] by antibodies was effective for IMQ-induced psoriasiform skin inflammation on mice. However, we have not examined the detailed mechanism including the induction and function of Tregs. In this study, we investigated the effect of IL-17, IL-23 and TNF- $\alpha$  inhibition on the induction and function of Tregs using the IMQ-induced psoriasiform mouse model.

## 2. Materials and methods

### 2.1. Mice

BALB/c mice were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). All mice for experiments were 6- to 10-week-old females and were maintained in specific pathogen-free conditions at the animal facility of Teikyo University School of Medicine. This study was approved by the Animal Research Committee of Teikyo University School of Medicine (Number 17-015).

### 2.2. Reagents

Goat anti-mouse CD3 $\epsilon$  (M-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Purified rat IgG2a, $\kappa$ , biotinylated rat anti-mouse MHC-class II (IA + IE) monoclonal antibody (mAb), purified or PE-conjugated rat anti-mouse/rat Foxp3 mAb, biotinylated anti-rat IgG, and HRP-conjugated streptavidin were purchased from Thermo Fisher Scientific (Waltham, MA). Goat IgG and biotinylated anti-goat IgG were obtained from Vector Laboratories (Burlingame, CA). Ultra-LEAF<sup>TM</sup> purified anti-TNF $\alpha$  (MP6-XT22), IL-17A (TC11-18H10.1) and IL-23p19 (MMp19B2) mAbs, rat IgG1, $\kappa$  (RTK2071, control for anti-TNF $\alpha$  and IL-17A mAbs), and mouse IgG2b, $\kappa$  (MPC-11, control for anti-IL-23p19 mAb), Alexa-647-conjugated anti-mouse CD4, IL-10 and CCR8, FITC-conjugated anti-mouse CD3 and CXCR3, PerCP/Cy5.5-conjugated anti-mouse CD4, PE-Cy7-conjugated anti-mouse CCR6 and BV-421, and conjugated anti-mouse CD25 were purchased from BioLegend (San Diego, CA).

### 2.3. Development of IMQ-induced psoriasiform dermatitis and injection of antibodies

To induce psoriasiform dermatitis, 5% IMQ cream (beselna cream; Mochida Pharmaceuticals, Tokyo, Japan) was applied to the shaved back skin once a day for six consecutive days (days 0–5). Mice were intraperitoneally injected with 100  $\mu$ g neutralizing antibodies to cytokines or control IgG every other day from one day before imiquimod application until day 3. A blinded investigator performed daily evaluations for clinical severity scoring based on the Psoriasis Area and Severity Index (PASI). To be precise, erythema, scaling, and thickening were each scored independently on a scale from 0 to 4 (0, none; 1, slight; 2, moderate; 3, marked; 4, very marked), and the sum of the scores was used as the total clinical score (scale 0–12) [23].

### 2.4. Histological analyses

Infiltrating inflammatory cells were counted in five random grids per section under fields (x400). Epidermal thickness was measured using the Olympus cellSense Standard software (Olympus, Tokyo, Japan).

### 2.5. Quantitative real-time PCR (q-PCR)

Total RNA was isolated from day 2 mouse back skin samples using RNeasy Fibrous Tissue Mini-Kit (Qiagen, Valencia, CA), and cDNA was synthesized using ReverTra Ace<sup>®</sup> qPCR RT Master Mix (ToYoBo, Osaka, Japan). Gene expression in murine skin was quantified using the THUNDERBIRD<sup>®</sup> Probe qPCR Mix (ToYoBo, Osaka, Japan). Primers were available from Thermo Fisher Scientific (primers for IL-17 F, IL-22, IL-23p19, IL-12p40, and IL-6) and Integrated DNA Technologies (primers for IL-17A, IL-10, IL1 $\beta$ , TNF $\alpha$  CXCL-1, CXCL-2, Lipocalin-2, and GAPDH) (San Diego, CA). All samples were analyzed with technical duplicates and parallel for GAPDH gene expression as an internal control. The relative expression levels of each gene were determined by the  $2^{-\Delta\Delta CT}$  method.

### 2.6. Cell preparation and flow cytometric analysis

Murine inguinal lymph nodes were prepared from day 6 samples and minced through a 70  $\mu$ m mesh to obtain single cell suspensions. Skin samples were incubated in 2 U/ml Dispase (Thermo Fisher Scientific) for 90 min, and were separated into epidermis and dermis. Dermis was minced, and then incubated with 2 mg/ml of collagenase type 2 (Worthington, Lakewood, NJ) for 30 min. The digested tissues were centrifuged, resuspended in PBS, and filtered through a 70  $\mu$ m mesh. Single cell suspensions were stained with fixable viability dye (BioLegend, San Diego, CA), cell surface antibodies, then fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific), and stained with antibodies for intracellular staining. For IL-10 producing Treg analysis, isolated lymph node single cells ( $2 \times 10^6$  cells/ml) were resuspended in RPMI 1640 medium (NACALAI TESQUE, INC., Kyoto, Japan) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) complemented with antibiotic-antimycotic mixed solution (NACALAI TESQUE, INC., Kyoto, Japan) in the presence of LPS (10  $\mu$ g/ml; NACALAI TESQUE, INC., Kyoto, Japan), monensin and PMA-ionomycin cocktail (BioLegend, San Diego, CA) for four hours at 37°C according to the manufacturer's instructions. Thereafter, the cells were analyzed by FACS Aria III $\mu$  flow cytometer and FlowJo v10 software (Becton Dickinson, San Jose, CA).

### 2.7. CD4+CD25+Treg cell isolation and adoptive transfer

Anti-cytokines mAb- or control IgG-injected mice receiving IMQ application were sacrificed on day 6, and their inguinal lymph nodes were extracted. Single cell suspensions from the lymph nodes were generated by gentle dissection and filtered through a 70  $\mu$ m mesh. The CD4<sup>+</sup>CD25<sup>+</sup> Tregs were purified by MACS separation (Miltenyi Biotec, Bisley, Germany).  $1 \times 10^6$  CD4<sup>+</sup>CD25<sup>+</sup>Tregs were intravenously injected into BALB/c mice. Adoptive transfer of CD4<sup>+</sup>CD25<sup>-</sup> cells was also performed on other recipient mice as a control. After two days, IMQ cream was applied on the shaved dorsal murine skin for six consecutive days (days 0–5), with daily assessments for clinical severity scoring. Two days after the initial application of IMQ (day 2), mRNA was extracted from the skin of mice as described above for qPCR. Skin samples of mice at day 6 were obtained for histological analyses.

## 2.8. Statistical analyses

Data were obtained from three independent experiments. They are presented as mean  $\pm$  standard error (SEM). One-way analysis of variance (ANOVA) with Bonferroni's post-test was used for multiple comparisons. Statistical significance was set at  $p$  values  $< 0.05$  (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

## 3. Results

### 3.1. Anti-TNF $\alpha$ , IL-17A and IL-23p19 mAbs reduced psoriasisform inflammation clinically and pathologically

Mean clinical severity scores and representative data at day 6 are shown in Fig. 1. There was no significant difference in the total clinical score among antibodies-injected mice without IMQ treatment (Fig. 1B). IMQ-treated mice and IMQ-treated mice injected with control IgG showed greatly increased scores in erythema, scaling and skin thickening as IMQ application was repeated, whereas anti-TNF $\alpha$ , IL-17A or IL-23p19 mAb-injected mice with IMQ application had significantly reduced increase in severity scores at days 4–6.

H&E staining revealed that IMQ-treated mice and IMQ-treated mice injected with control IgG showed intense infiltration of inflammatory cells, and that anti-cytokines mAb injection suppressed infiltration of inflammatory cells (Supplementary Fig. 1A). The total number of infiltrating inflammatory cells and epidermal thickness were significantly lower and thinner, respectively, in IMQ-treated mice with anti-cytokines mAb injection than in IMQ-treated mice with control IgG injection (Supplementary Figs. 1B, C). Immunohistochemical staining revealed that anti-TNF $\alpha$ , IL-17A or IL-23p19 mAb injection significantly suppressed infiltration of CD3<sup>+</sup>T cells and MHC Class II<sup>+</sup>antigen-presenting cells in IMQ-induced psoriasisform dermatitis (Supplementary Figs. 2A, 3A). Anti-cytokines mAb injection significantly reduced the numbers of infiltrating CD3<sup>+</sup>cells (Supplementary Fig. 2B) and MHC-II<sup>+</sup>cells (Supplementary Fig. 3B) compared to those in control IgG-injected

mice. These findings indicate that anti-TNF $\alpha$ , IL-17A and IL-23p19 mAbs significantly reduced dermal infiltration of inflammatory cells, consequently improving IMQ-induced psoriasisform dermatitis clinically and histologically.

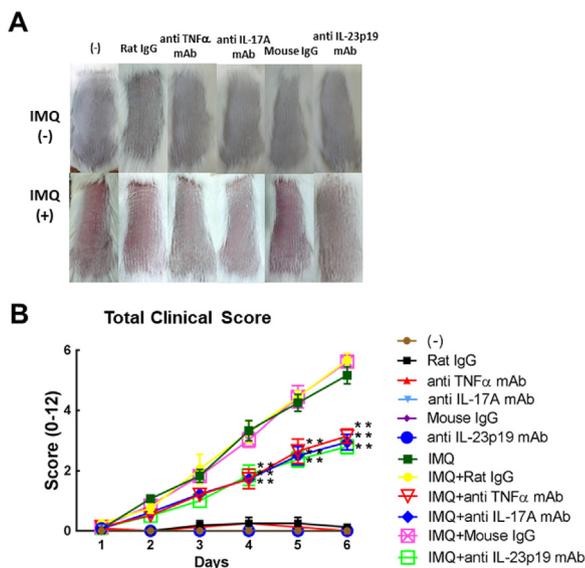
### 3.2. Anti-TNF $\alpha$ , IL-17A and IL-23p19 mAbs downregulated psoriasis-related cytokines and Anti-IL-23p19 mAb increased IL-10 gene expression in IMQ-treated murine psoriasisform dermatitis

We next studied the effect of anti-TNF $\alpha$ , IL-17A or IL-23p19 mAb injection on psoriasis-related cytokine mRNA expression. The results are shown in Fig. 2. The mRNA levels of IL-17A were significantly lower in IMQ-applied mice treated with anti-cytokines mAb than in IMQ-applied mice treated with control IgG. IL-17 F expression was significantly reduced in anti-IL-17A or IL-23p19 mAb-injected mice compared to control IgG-injected mice, while there was no significant difference in IL17 F mRNA expression between anti-TNF $\alpha$  mAb-injected mice and control IgG-injected mice. Anti-TNF $\alpha$  or IL-23p19 mAb injection markedly reduced the mRNA expressions of IL-1 $\beta$  and one of the antimicrobial peptides, lipocalin-2, whereas anti-IL-17A mAb injection did not. CXCL-2 expression significantly decreased in only anti-IL-23p19 mAb-injected mice. Although IL-22, IL-23p19, IL-12p40, CXCL-1 and IL-6 expressions tended to be lower in anti-cytokines mAb-injected mice than in control IgG-injected mice, there were no significant differences. Interestingly, IL-23p19 mAb administration significantly increased IL-10 expression and IL-17A administration tended to increase IL-10 expression, while anti-TNF $\alpha$  mAb did not.

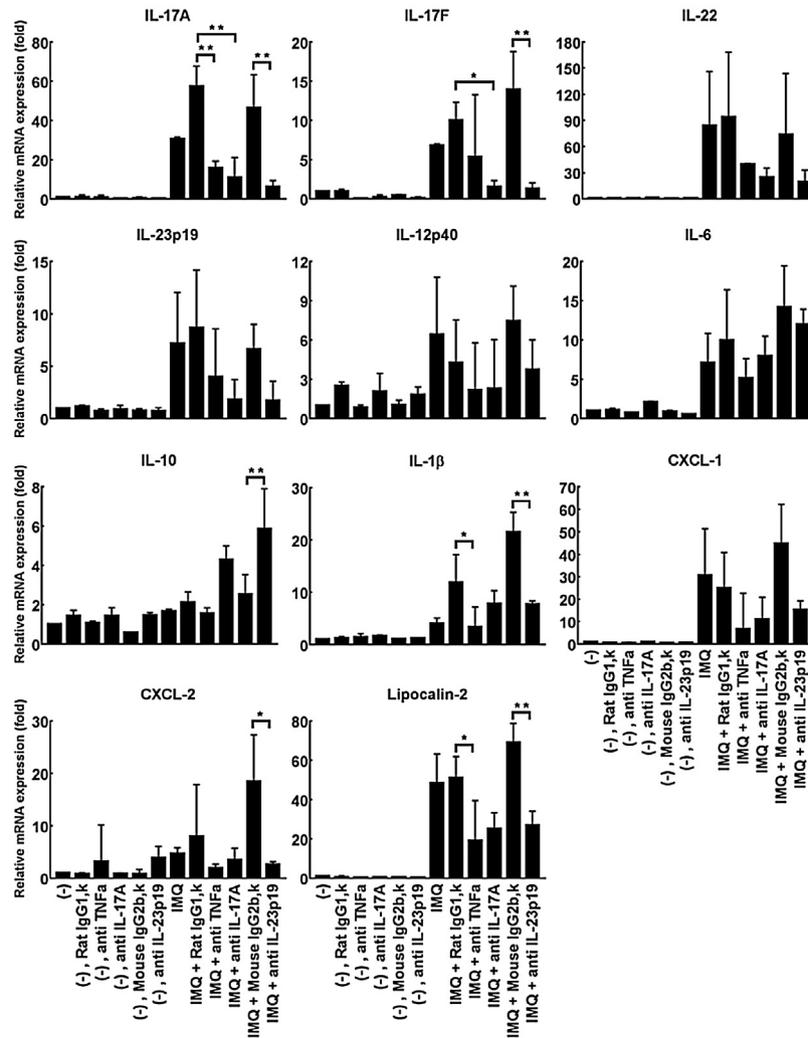
### 3.3. Tregs are induced in Anti-IL-17A and IL-23p19 mAb-injected IMQ-treated mice

In order to examine whether increased IL-10 expression in anti-IL-23p19 or IL-17A mAb-injected mice treated with IMQ is due to Treg induction, we examined Foxp3<sup>+</sup>Treg infiltration in each treatment group. Immunohistochemical staining showed that anti-IL-23p19 or IL-17A mAb injection but not anti-TNF $\alpha$  mAb injection induced significantly increased numbers of Foxp3<sup>+</sup>cells infiltrating to IMQ-treated skin compared to control IgG injection (Fig. 3A and B). Flow cytometric analyses showed that the proportion of CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs in CD3<sup>+</sup>CD4<sup>+</sup>T cells in inguinal lymph nodes of anti-IL-23p19 or IL-17A mAb-treated mice increased whereas that of anti-TNF $\alpha$  mAb-treated mice was comparable with that of isotype IgG-treated mice. (Supplementary Figs. 4A, B). In the skin, the proportion and count of CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs in CD3<sup>+</sup>CD4<sup>+</sup>T cells of anti-cytokines mAb-treated mice increased compared with that of isotype IgG-treated mice (Supplementary Figs. 4A, C, D). The proportion of IL-10 producing cells in CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs derived from inguinal lymph nodes of anti-TNF $\alpha$  Ab-injected mice was comparable with that of those cells derived from inguinal lymph nodes of control-IgG-injected mice, whereas those cells derived from inguinal lymph nodes of anti-IL-17A or IL-23p19 mAb-injected mice showed increased proportion of IL-10 producing cells (Supplementary Figs. 4E, F). These findings indicate that anti-IL-23p19 or IL-17A mAb administration induced functional Tregs which have an immunosuppressive effect by producing IL-10 in the lymph nodes, whereas anti-TNF $\alpha$  mAb did not.

Recent reports have identified many subpopulations of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs such as Th1-like Treg expressing CXCR3, Th2-like Treg expressing CCR8, and Th17-like Treg expressing CCR6 [24,25]. We investigated expression of those molecules on the surface of Tregs in the lymph nodes of IMQ-applied mice treated with anti-cytokines mAb (Supplementary Fig. 4E, G). The percentage of Tregs expressing CCR6 in the lymph nodes of



**Fig. 1.** Clinical effects of anti-cytokines monoclonal antibody (mAb) or control IgG on imiquimod (IMQ)-induced psoriasisform dermatitis. Antibodies-injected BALB/c mice were given daily applications of IMQ on the back skin as described in the Materials and Methods. (A) Clinical pictures showing the shaved back skin of antibodies-injected mice with or without IMQ treatment. (B) The total clinical scores of each group of mice. The data were obtained from three independent experiments. In each experiment, each group contained two mice ( $n = 6$ ). The data are shown as mean  $\pm$  SEM. \*\*  $p < 0.01$  (ANOVA with Bonferroni's post-test).



**Fig. 2.** Quantitative real-time PCR analyses of inflammatory cytokines, interleukin (IL)-10, chemokines and antimicrobial peptide in IMQ-treated skin of mice injected with anti-cytokines mAb. mRNA levels were determined at day 2 by quantitative real-time PCR for IL-17A, IL-17 F, IL-22, IL-23p19, IL-12p40, IL-6, IL-10, IL-1 $\beta$ , CXCL-1, CXCL-2, Lipocalin-2, normalized to GAPDH, and are expressed as fold induction relative to that of control. The data were obtained from skin samples of three independent experiments. In each experiment, each group contained two mice (n = 6). Values are presented as means  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$  (ANOVA with Bonferroni's post-test).

IMQ-applied mice treated with anti-TNF $\alpha$  mAb was significantly lower compared with those treated with control IgG whereas the percentage of those treated with anti-IL-23p19 mAb was significantly higher compared with those treated with control IgG. No significant differences were observed in the percentages of Tregs expressing CXCR3 or CCR8 among all groups (data not shown).

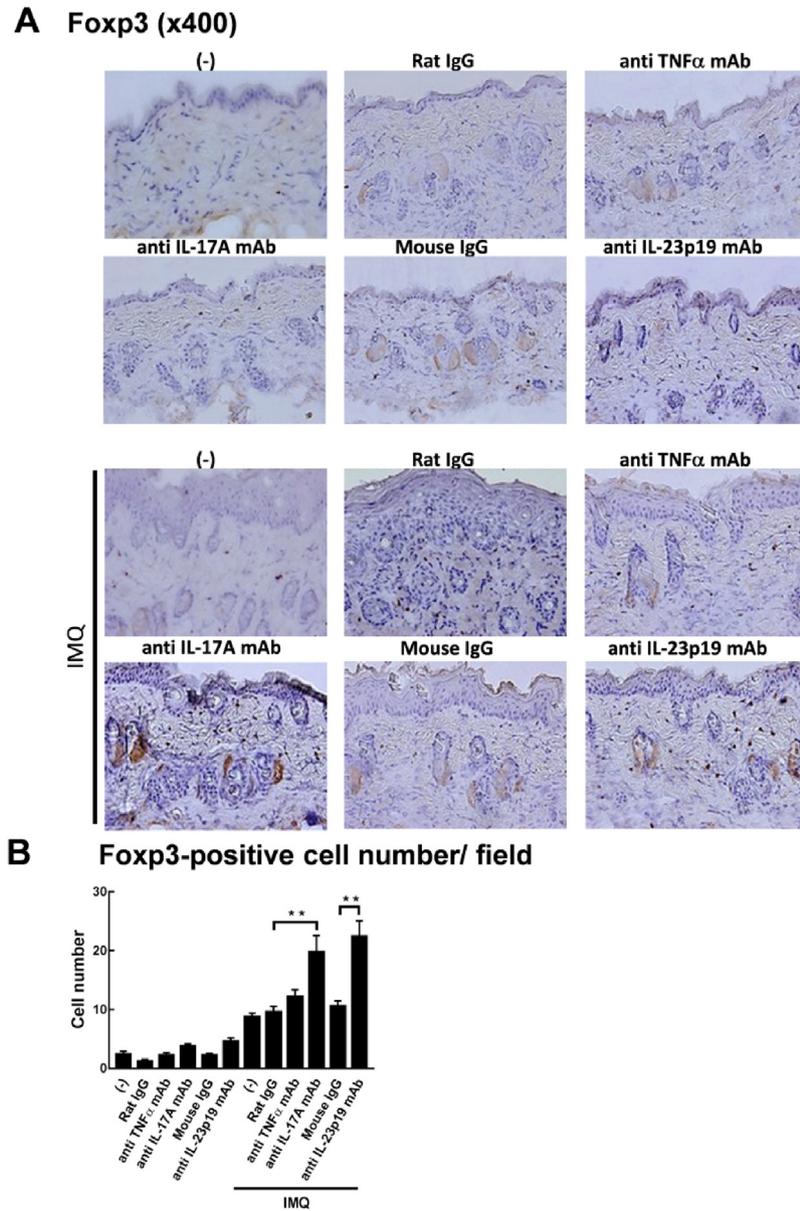
#### 3.4. Tregs derived from anti-cytokines mAb-injected mice ameliorate psoriasiform dermatitis

In order to further investigate the suppressive effects of Tregs induced by anti-cytokines mAb injection, we performed adoptive transfer experiments. Donor mice were treated with anti-cytokines mAb and IMQ as described above. On day 6, CD4<sup>+</sup>CD25<sup>-</sup> cells (control) and CD4<sup>+</sup>CD25<sup>+</sup> cells (Treg) isolated from the inguinal lymph nodes of donor mice were injected intravenously into recipient mice which were subsequently treated with IMQ cream for six consecutive days. Among IMQ-applied recipient mice, mice adoptively transferred with Tregs derived from anti-IL-17A or IL-23p19 mAb-injected donor mice showed significantly reduced clinical scores compared with those in mice adoptively transferred with control cells "from day 4", whereas mice

adoptively transferred with Tregs derived from anti-TNF $\alpha$  mAb-injected donor mice showed significantly reduced clinical scores compared with those in mice adoptively transferred with control cells "from day 5" (Figs. 4A, B).

Pathological assessment revealed that recipient mice adoptively transferred with Tregs derived from anti-TNF $\alpha$ , IL-17A or IL-23p19 mAb-injected donor mice demonstrated significantly reduced numbers of inflammatory cells infiltrating in the skin (Supplementary Figs. 5A, B) and showed decreased epidermal thickness (Supplementary Fig. 5C) compared with those in mice adoptively transferred with control cells. Recipient mice adoptively transferred with Tregs from mouse control IgG-injected donor mice also tended to show reductions in the number of infiltrating inflammatory cells and epidermal thickness. Recipient mice adoptively transferred with Tregs from anti-TNF $\alpha$  or IL-17A mAb-treated donor mice demonstrated decreased numbers of CD3<sup>+</sup> (Supplementary Figs. 6 A, B) and MHC-II<sup>+</sup> (Supplementary Figs. 7A, B) cells, whereas recipient mice adoptively transferred with Tregs from anti-IL-17A or IL-23p19 mAb-treated donor mice showed an increased number of Foxp3<sup>+</sup> cells infiltrating into IMQ-induced psoriasiform skin (Fig. 5A, B).

qPCR analyses revealed significant IL-17A, IL-17 F and IL-22 downregulation in the skin of recipient mice adoptively



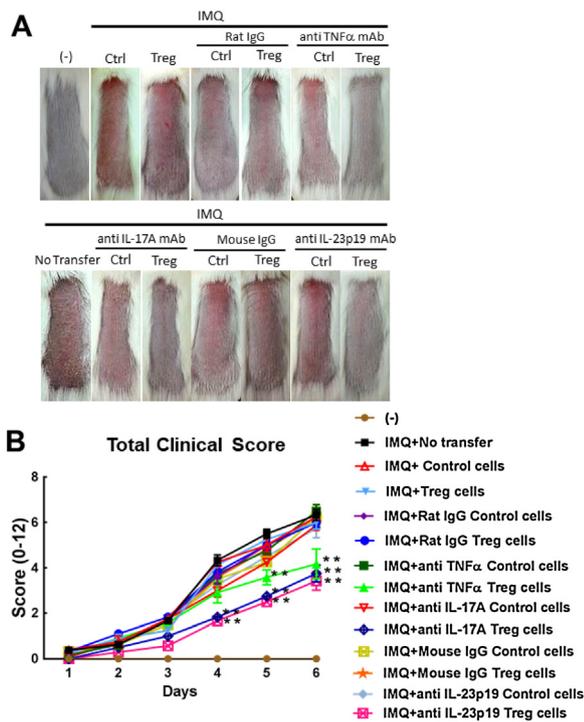
**Fig. 3.** Foxp3<sup>+</sup> cell infiltration in IMQ-induced psoriasiform skin of mice injected with anti-cytokines mAb or control IgG. (A) Immunohistochemical staining for Foxp3<sup>+</sup> cells was performed on skin sections at day 6 of IMQ treatment. (B) The Foxp3<sup>+</sup>-infiltrating dermal inflammatory cells were counted in five random grids per section under x400 high-power field. The data are shown as mean  $\pm$  SEM of samples pooled from three independent experiments. In each experiment, each group contained two mice (n = 6). \*\*  $p < 0.01$  (ANOVA with Bonferroni's post-test).

transferred with Tregs from anti IL-23p19 mAb-treated donor mice compared with the control mice. The results are shown in Fig. 6. Although those adoptively transferred with Tregs from anti-TNF $\alpha$  or IL-17A-treated donor mice showed that mRNA expression of those cytokines had a tendency to be down-regulated, there were no statistically significant differences between those mice and control mice. Consistently, recipient mice adoptively transferred with Tregs from anti-IL-17A or IL-23p19 mAb-treated donor mice demonstrated a significant increase in IL-10 mRNA expression in IMQ-induced psoriasiform dermatitis. As for mRNA expression of IL-23p19, IL-12p40, TNF $\alpha$  and IL-6, there were no significant differences among the groups. These results indicate that in addition to inhibition of psoriasis-related cytokines by administration of anti-IL-17A or IL-23p19 mAb, Treg induction and IL-10 production also contribute to improvement of IMQ-induced psoriasiform dermatitis.

#### 4. Discussion

In this study, we demonstrated that administration of anti-TNF $\alpha$ , IL-17A or IL-23p19 mAb improved IMQ-induced psoriasis dermatitis in a mouse model clinically and histologically, through reduction of Th17-related cytokines expression, which is compatible with previous reports using other murine psoriasis model [21,22,26] and clinical data of biologics in human [4–8,10].

Several papers reported that Tregs increased in peripheral blood or skin lesions in psoriasis [27,28], while a few studies reported that Tregs decreased in psoriasis [29]. Recent studies indicate that the number of Tregs in the skin lesion increases, whereas the suppressive functions of Tregs are impaired in psoriasis [30–32]. In addition, Jin et al. reported that IL-10-deficient mice treated with IMQ showed exacerbated inflammation compared with wild type mice [33], suggesting that endogenous IL-10 has the suppressive function in this



**Fig. 4.** Clinical effects of IMQ application on recipient mice adoptively transferred with CD4<sup>+</sup>CD25<sup>+</sup> Tregs or control cells derived from anti-cytokines mAb- or control IgG-treated donor mice. Donor mice given daily applications of IMQ with antibodies as described in the Materials and Methods were sacrificed at day 6. For adoptive transfer, control cells (CD4<sup>+</sup>CD25<sup>-</sup> cells) and Tregs (CD4<sup>+</sup>CD25<sup>+</sup> cells) were obtained from the inguinal lymph nodes of donor mice. Those cells were adoptively transferred to recipient mice followed by IMQ treatment for 6 days. (A) Clinical pictures showing the shaved back skin of recipient mice treated with IMQ for 6 days. Results are representative of at least three separate experiments. (B) The total clinical scores are shown for each group of mice. The data are shown as mean  $\pm$  SEM of three independent experiments. In each experiment, each group contained two mice (n = 6). \*\*  $p < 0.01$  (ANOVA with Bonferroni's post-test). Treg: CD4<sup>+</sup>CD25<sup>+</sup> cells; Ctrl: CD4<sup>+</sup>CD25<sup>-</sup> cells.

IMQ-induced psoriasisform dermatitis model. Considering them together, contribution of endogenous IL-10-producing Tregs to psoriasis is indicated. Similar to the previous literature, our experiments showed that IMQ application increased Foxp3-positive cells in skin sections, while it did not increase IL-10 mRNA expression. Furthermore, flow cytometric analysis revealed that IMQ application significantly increased the number of Tregs in lymph nodes of mice (data not shown) whereas it did not increase the number of IL-10-producing Tregs in the skin. As for the impact of anti-cytokines mAbs on Treg induction and restoration of their function, Maxwell et al. reported that in a mouse model of colitis IL-23 inhibition by anti-IL-23p19 mAb enhanced Tregs accumulation and reduced colonic inflammation [34]. Likewise, the results of our experiments using the mouse model of psoriasis demonstrated that in the IL-23p19 mAb-treated mice, IL-10 mRNA expression in the skin, the number of Tregs in the lymph nodes and the skin, and the number of IL-10-positive cells in the skin significantly increased compared with those in control IgG-treated mice. Furthermore, treatment with anti-IL-23p19 mAb had a slightly greater effect on functional Treg induction than treatment with IL-17A, which might indicate that IL-23p19 is located more upstream in the IL-17/IL-23 signaling pathway in the pathogenesis of psoriasis. These results suggest that administration of anti-IL-17A or IL-23p19 mAb should promote

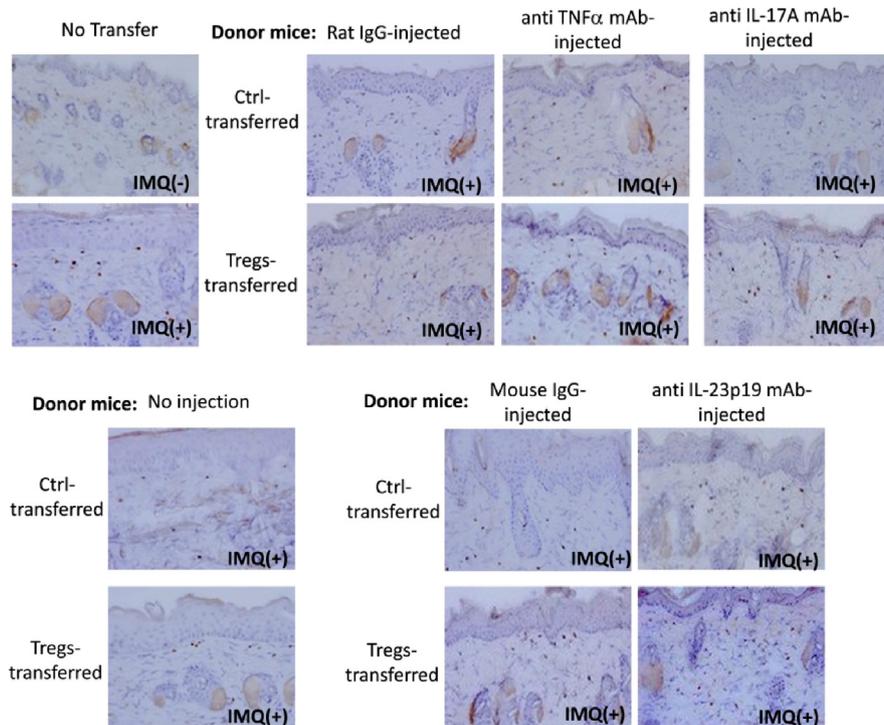
Tregs expansion and restoration of their suppressive activity. However, further investigations are necessary to clarify its detailed mechanism.

To prove the above hypothesis, we performed adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup>Tregs from lymph nodes of IMQ-applied mice treated with anti-cytokines mAb into recipient mice. The recipient mice adoptively transferred with Tregs from antibody-administered donor mice showed clinical and histological amelioration of IMQ-induced psoriasisform dermatitis. In addition, the recipient mice adoptively transferred with Tregs derived from anti-IL-17A and IL-23p19 mAb-injected donor mice, had increased IL-10 mRNA expression and increased number of Foxp3-positive cells, although to what extent Tregs induced by those antibodies in donor mice contributed to increased Tregs in recipient mice is unclear. Interestingly, recipient mice adoptively transferred with Tregs derived from the anti-TNF $\alpha$  antibody-treated donor mice, had no significant increase in Tregs. A previous study indicated that TNF $\alpha$  plays an important role in activation and expansion of Treg [35]. Blockade of TNF $\alpha$  by anti-TNF $\alpha$  mAb administration might have hindered activation and expansion of Tregs. The therapeutic effect of anti-TNF $\alpha$  mAb on psoriasis may be obtained only by inhibiting the key cytokine without Treg induction and restoration of their suppressive function, whereas that of anti-IL-17 or IL-23p19 mAb is gained by inhibiting the key cytokine and possible contribution of Treg induction and restoration of the function. Paradoxical reactions induced by inhibition of TNF $\alpha$  could be accounted for by this hindered activation and expansion of Tregs.

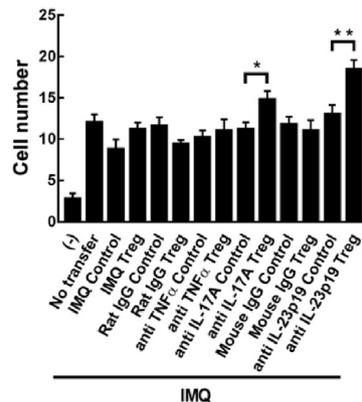
Results in Fig. 5 showed that the number of Tregs infiltrating the skin of recipient mice adoptively transferred with Tregs derived from anti-TNF $\alpha$  Ab-treated donor mice did not increase compared with control, while IMQ-induced dermatitis was alleviated in those mice. Based on results of clinical improvement by adoptive transfer of Tregs derived from TNF $\alpha$ -treated donor mice, we can only say that those adoptively transferred Tregs have the capacity to alleviate the dermatitis "via unknown mechanism" and that it is not due to increased number of Tregs at day 6. We speculate that Tregs derived from anti-TNF $\alpha$  Ab-treated donor mice also have the capacity to suppress expression of IL-17, IL-22, and/or IL-23, which is supported by the results in Fig. 6 that mRNA expressions of those cytokines at day 2 tended to decrease although statistically significant differences were not observed. However, the capacity and function of Tregs derived from TNF $\alpha$ -treated donor mice might be somewhat impaired and not fully restored, at least compared with Tregs derived from anti-IL-17A or IL-23p19 Ab-treated donor mice. Those subtle difference might account for the slight difference of clinical score and the number of Tregs at day 6, for example, because they might not be able to survive until day 6. Although Tregs derived from anti-TNF $\alpha$  Ab-treated donor mice were not increased at "day 6", Tregs might increase or play an inhibitory role at earlier day. (We did not evaluate the number of infiltrating inflammatory cells into the skin at earlier days, since its number is usually too small to evaluate.)

Our data demonstrated that inhibition of IL-23 or IL-17 in murine IMQ-induced psoriasisform dermatitis model induced Treg expansion whereas inhibition of TNF $\alpha$  did not. As for its possible mechanism, Bovenschen et al. showed that Foxp3<sup>+</sup>Tregs of psoriasis patients easily differentiated into IL-17A-producing cells under the stimulation of IL-23 [36]. Maxwell et al. reported that in an Abcb1a(-/-) mouse model of colitis, IL-23 inhibition enhanced Treg accumulation which resulted in attenuated disease by decreasing colonic inflammation [34]. Furthermore, accumulating evidences indicate plasticity of Treg and IL-17 producing cells [37–39]. In psoriasis, inhibition of IL-23 might restore impaired Treg or promote differentiation of IL-17

## A Foxp3 (x400)



## B Foxp3-positive cell number/ field



**Fig. 5.** Foxp3<sup>+</sup> cells infiltrating into IMQ-induced psoriasiform skin after adoptive transfer. (A) Representative images of immunohistochemical staining for Foxp3<sup>+</sup> cells on skin sections at day 6 of IMQ treatment (magnification, x400). (B) The dermal Foxp3<sup>+</sup> cells were counted in five random grids per section under x400 high-power field. The data are shown as mean ± SEM of samples pooled from three independent experiments. In each experiment, each group contained two mice (n = 6). \* *p* < 0.05, \*\* *p* < 0.01 (ANOVA with Bonferroni's post-test).

producing cells to Treg. The mechanism of Treg induction by anti-IL-17 mAb is unknown. Our data showed that IL-23p19 mRNA expression in mice treated with anti-IL-17 mAb tended to decrease, which suggests that inhibition of IL-17 might have resulted in decrease of IL-23 then led to Treg induction. Meanwhile, as for inhibition of TNF $\alpha$ , Chen et al. demonstrated that interaction of TNF with TNF receptor type 2 promoted expansion and function of murine CD4<sup>+</sup>CD25<sup>+</sup>Tregs [40]. Their data support that inhibition of TNF $\alpha$  did not induce Treg expansion in our results.

In summary, our results suggest that anti-IL-17A or IL-23p19 mAb treatment not only inhibits the IL-17/IL-23 signaling pathway, which is important for the development of psoriasis, but also induces expansion of Tregs and activation of their suppressive capacity.

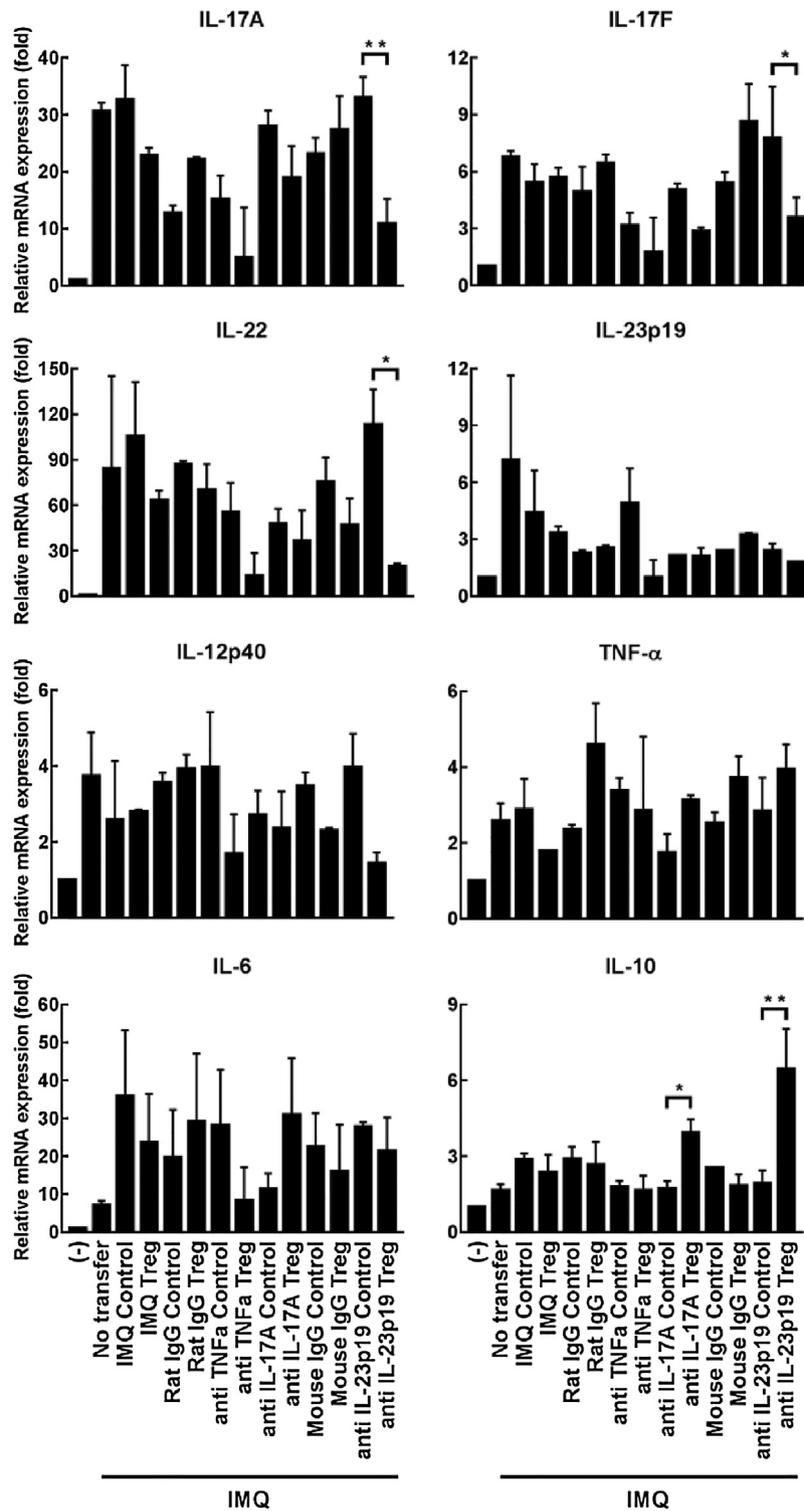
### Funding source

This study was performed through joint research agreement between Teikyo University and Eli Lilly Japan K.K., with funding from Eli Lilly Japan K.K.

### Declaration of Competing Interest

Dr Masahiro Kamata received grant for research from Torii Pharmaceutical, Eisai, Maruho, Novartis Pharma, and honoraria for lecture from Maruho, LEO Pharma, Eisai, AbbVie, Kyowa Hakko Kirin, Eli Lilly, Taiho Pharmaceutical, Mitsubishi Tanabe Pharma, and Janssen Pharmaceutical.

Dr Yayoi Tada received grant for research from Maruho, LEO Pharma, Eisai, AbbVie, Kyowa Hakko Kirin, Taiho Pharmaceutical,



**Fig. 6.** Quantitative real-time PCR analyses of cytokine mRNA levels in IMQ-treated skin of recipient mice adoptively transferred with CD4<sup>+</sup>CD25<sup>-</sup> control cells or CD4<sup>+</sup>CD25<sup>+</sup> Tregs from donor mice treated with anti-cytokine mAb. mRNA levels were determined at day 2 by quantitative real-time PCR for IL-17A, IL-17 F, IL-22, IL-23p19, IL-12p40, tumor necrosis factor (TNF)- $\alpha$ , IL-6, and IL-10, normalized to GAPDH, and are expressed as fold induction relative to that of control. The data were obtained from skin samples of three independent experiments. In each experiment, each group contained two mice (n = 6). Values are presented as means  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$  (ANOVA with Bonferroni's post-test).

Celgene, and Eli Lilly, and honoraria for lectures from Maruho, LEO Pharma, Eisai, AbbVie, Kyowa Hakko Kirin, Eli Lilly, Taiho Pharmaceutical, Mitsubishi Tanabe Pharma, and Janssen Pharmaceutical.

#### Acknowledgement

The authors wish to acknowledge Ms. Maiko Sakuragi for her assistance in performing some experiments.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jdermsci.2019.07.006>.

## References

- R.G. Langley, G.G. Krueger, C.E. Griffiths, Psoriasis: epidemiology, clinical features, and quality of life, *Ann. Rheum. Dis.* 64 (Suppl 2) (2005) ii18–23; discussion ii24–5.
- W.A. Myers, A.B. Gottlieb, P. Mease, Psoriasis and psoriatic arthritis: clinical features and disease mechanisms, *Clin. Dermatol.* 24 (5) (2006) 438–447.
- A. Di Cesare, P. Di Meglio, F.O. Nestle, The IL-23/Th17 axis in the immunopathogenesis of psoriasis, *J. Invest. Dermatol.* 129 (6) (2009) 1339–1350.
- R.G. Langley, B.E. Elewski, M. Lebwohl, K. Reich, C.E. Griffiths, K. Papp, L. Puig, H. Nakagawa, L. Spelman, B. Sigurgeirsson, E. Rivas, T.F. Tsai, N. Wasel, S. Tyring, T. Salko, I. Hampele, M. Notter, A. Karpov, S. Helou, C. Papavassilis, Secukinumab in plaque psoriasis—results of two phase 3 trials, *N. Engl. J. Med.* 371 (4) (2014) 326–338.
- C.E. Griffiths, K. Reich, M. Lebwohl, P. van de Kerkhof, C. Paul, A. Menter, G.S. Cameron, J. Erickson, L. Zhang, R.J. Secrest, S. Ball, D.K. Braun, O.O. Osuntokun, M.P. Heffernan, B.J. Nickoloff, K. Papp, Comparison of ixekizumab with etanercept or placebo in moderate-to-severe psoriasis (UNCOVER-2 and UNCOVER-3): results from two phase 3 randomised trials, *Lancet* 386 (9993) (2015) 541–551.
- M. Lebwohl, B. Strober, A. Menter, K. Gordon, J. Weglowska, L. Puig, K. Papp, L. Spelman, D. Toth, F. Kerdel, A.W. Armstrong, G. Stingl, A.B. Kimball, H. Bachelez, J.J. Wu, J. Crowley, R.G. Langley, T. Blicharski, C. Paul, J.P. Lacour, S. Tyring, L. Kirck, S. Chimenti, K. Callis Duffin, J. Bagel, J. Koo, G. Aras, J. Li, W. Song, C.E. Milmont, Y. Shi, N. Erond, P. Klekotka, B. Kotzin, A. Nirula, Phase 3 Studies Comparing Brodalumab with Ustekinumab in Psoriasis, *N. Engl. J. Med.* 373 (14) (2015) 1318–1328.
- C.L. Leonardi, A.B. Kimball, K.A. Papp, N. Yeilding, C. Guzzo, Y. Wang, S. Li, L.T. Dooley, K.B. Gordon, Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1), *Lancet* 371 (9625) (2008) 1665–1674.
- K. Reich, A.W. Armstrong, P. Foley, M. Song, Y. Wasfi, B. Randazzo, S. Li, Y.K. Shen, K. B. Gordon, Efficacy and safety of guselkumab, an anti-interleukin-23 monoclonal antibody, compared with adalimumab for the treatment of patients with moderate to severe psoriasis with randomized withdrawal and retreatment: Results from the phase III, double-blind, placebo- and active comparator-controlled VOYAGE 2 trial, *J. Am. Acad. Dermatol.* 76 (3) (2017) 418–431.
- K.A. Papp, A. Blauvelt, M. Bukhalo, M. Gooderham, J.G. Krueger, J.P. Lacour, A. Menter, S. Philipp, H. Sofen, S. Tyring, B.R. Berner, S. Visvanathan, C. Pamulapati, N. Bennett, M. Flack, P. Scholl, S.J. Padula, Risankizumab versus Ustekinumab for Moderate-to-Severe Plaque Psoriasis, *N. Engl. J. Med.* 376 (16) (2017) 1551–1560.
- K. Reich, K.A. Papp, A. Blauvelt, S.K. Tyring, R. Sinclair, D. Thaci, K. Nograles, A. Mehta, N. Cichanowitz, Q. Li, K. Liu, C. La Rosa, S. Green, A.B. Kimball, Tildrakizumab versus placebo or etanercept for chronic plaque psoriasis (reSURFACE 1 and reSURFACE 2): results from two randomised controlled, phase 3 trials, *Lancet* 390 (10091) (2017) 276–288.
- K. Loser, A. Mehling, S. Loeser, J. Apelt, A. Kuhn, S. Grabbe, T. Schwarz, J.M. Penninger, S. Beissert, Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells, *Nat. Med.* 12 (12) (2006) 1372–1379.
- T. Furuhashi, C. Saito, K. Torii, E. Nishida, S. Yamazaki, A. Morita, Photo(chemo) therapy reduces circulating Th17 cells and restores circulating regulatory T cells in psoriasis, *PLoS One* 8 (1) (2013)e54895.
- F. Baeke, T. Takiishi, H. Korf, C. Gysemans, C. Mathieu, Vitamin D: modulator of the immune system, *Curr. Opin. Pharmacol.* 10 (4) (2010) 482–496.
- W.D. Docke, K. Asadullah, G. Belbe, M. Ebeling, C. Hofflich, M. Friedrich, W. Sterry, H.D. Volk, Comprehensive biomarker monitoring in cytokine therapy: heterogeneous, time-dependent, and persisting immune effects of interleukin-10 application in psoriasis, *J. Leukoc. Biol.* 85 (3) (2009) 582–593.
- K. Asadullah, R. Sabat, M. Friedrich, H.D. Volk, W. Sterry, Interleukin-10: an important immunoregulatory cytokine with major impact on psoriasis, *Curr. Drug Targets Inflamm. Allergy* 3 (2) (2004) 185–192.
- A. Owczarczyk-Saczonek, J. Czerwinska, W. Placek, The role of regulatory T cells and anti-inflammatory cytokines in psoriasis, *Acta Dermatovenerol. Alp. Pannonica Adriat.* 27 (1) (2018) 17–23.
- C.S. Hau, T. Shimizu, Y. Tada, M. Kamata, S. Takeoka, S. Shibata, A. Mitsui, Y. Asano, M. Sugaya, T. Kadono, S. Sato, S. Watanabe, The vitamin D3 analog, maxacalcitol, reduces psoriasisform skin inflammation by inducing regulatory T cells and downregulating IL-23 and IL-17 production, *J. Dermatol. Sci.* (2018).
- J.M. Camarasa, J.P. Ortonne, L. Dubertret, Calcitriol shows greater persistence of treatment effect than betamethasone dipropionate in topical psoriasis therapy, *J. Dermatolog. Treat.* 14 (1) (2003) 8–13.
- M. Lebwohl, A. Yoles, K. Lombardi, W. Lou, Calcipotriene ointment and halobetasol ointment in the long-term treatment of psoriasis: effects on the duration of improvement, *J. Am. Acad. Dermatol.* 39 (3) (1998) 447–450.
- M. Karakawa, M. Komine, T. Takekoshi, N. Sakurai, Y. Minatani, Y. Tada, H. Saeki, K. Tamaki, Duration of remission period of narrowband ultraviolet B therapy on psoriasis vulgaris, *J. Dermatol.* 38 (7) (2011) 655–660.
- A. Mitsui, Y. Tada, S. Shibata, M. Kamata, C. Hau, A. Asahina, S. Sato, Deficiency of both L-selectin and ICAM-1 exacerbates imiquimod-induced psoriasis-like skin inflammation through increased infiltration of antigen presenting cells, *Clin. Immunol.* 157 (1) (2015) 43–55.
- S. Shibata, Y. Tada, C.S. Hau, A. Mitsui, M. Kamata, Y. Asano, M. Sugaya, T. Kadono, Y. Masamoto, M. Kurokawa, T. Yamauchi, N. Kubota, T. Kadowaki, S. Sato, Adiponectin regulates psoriasisform skin inflammation by suppressing IL-17 production from gammadelta-T cells, *Nat. Commun.* 6 (2015) 7687.
- L. van der Fits, S. Mourits, J.S. Voerman, M. Kant, L. Boon, J.D. Laman, F. Cornelissen, A.M. Mus, E. Florencia, E.P. Prens, E. Lubberts, Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis, *J. Immunol.* 182 (9) (2009) 5836–5845.
- B.M. Hall, N.D. Verma, G.T. Tran, S.J. Hodgkinson, Distinct regulatory CD4+T cell subsets; differences between naive and antigen specific T regulatory cells, *Curr. Opin. Immunol.* 23 (5) (2011) 641–647.
- B.M. Hall, T cells: soldiers and spies—the surveillance and control of effector T cells by regulatory T cells, *Clin. J. Am. Soc. Nephrol.* 10 (11) (2015) 2050–2064.
- K. Nakajima, T. Kanda, M. Takaishi, T. Shiga, K. Miyoshi, H. Nakajima, R. Kamijima, M. Tarutani, J.M. Benson, M.M. Ellosa, L.L. Gushall, M.F. Naso, Y. Iwakura, J. DiGiovanni, S. Sano, Distinct roles of IL-23 and IL-17 in the development of psoriasis-like lesions in a mouse model, *J. Immunol.* 186 (7) (2011) 4481–4489.
- K.X. Yan, X. Fang, L. Han, Z.H. Zhang, K.F. Kang, Z.Z. Zheng, Q. Huang, Foxp3+ regulatory T cells and related cytokines differentially expressed in plaque vs. guttate psoriasis vulgaris, *Br. J. Dermatol.* 163 (1) (2010) 48–56.
- L. Zhang, X.Q. Yang, J. Cheng, R.S. Hui, T.W. Gao, Increased Th17 cells are accompanied by FoxP3(+) Treg cell accumulation and correlated with psoriasis disease severity, *Clin. Immunol.* 135 (1) (2010) 108–117.
- W.J. Yun, D.W. Lee, S.E. Chang, G.S. Yoon, J.R. Huh, C.H. Won, M.W. Lee, S.E. Kim, B.J. Kim, K.C. Moon, J.H. Choi, Role of CD4CD25FOXP3 regulatory T cells in psoriasis, *Ann. Dermatol.* 22 (4) (2010) 397–403.
- H. Sugiyama, R. Gyulai, E. Toichi, E. Garaczi, S. Shimada, S.R. Stevens, T.S. McCormick, K.D. Cooper, Dysfunctional blood and target tissue CD4+CD25high regulatory T cells in psoriasis: mechanism underlying unrestrained pathogenic effector T cell proliferation, *J. Immunol.* 174 (1) (2005) 164–173.
- K. Zhang, X. Li, G. Yin, Y. Liu, X. Niu, R. Hou, Functional characterization of CD4+CD25+ regulatory T cells differentiated in vitro from bone marrow-derived haematopoietic cells of psoriasis patients with a family history of the disorder, *Br. J. Dermatol.* 158 (2) (2008) 298–305.
- D.C. Soler, H. Sugiyama, A.B. Young, J.V. Massari, T.S. McCormick, K.D. Cooper, Psoriasis patients exhibit impairment of the high potency CCR5(+) T regulatory cell subset, *Clin. Immunol.* 149 (1) (2013) 111–118.
- S.P. Jin, S.J. Koh, D.A. Yu, M.W. Kim, H.T. Yun, D.H. Lee, H.S. Yoon, S. Cho, H.S. Park, Imiquimod-applied Interleukin-10 deficient mice better reflects severe and persistent psoriasis with systemic inflammatory state, *Exp. Dermatol.* 27 (1) (2018) 43–49.
- J.R. Maxwell, Y. Zhang, W.A. Brown, C.L. Smith, F.R. Byrne, M. Fiorino, E. Stevens, J. Bigler, J.A. Davis, J.B. Rottman, A.L. Budelsky, A. Symons, J.E. Towne, Differential Roles for Interleukin-23 and Interleukin-17 in Intestinal Immunoregulation, *Immunity* 43 (4) (2015) 739–750.
- A. Baeyens, D. Saadoun, F. Billiard, A. Rouers, S. Gregoire, B. Zaragoza, Y. Grinberg-Bleyer, G. Marodon, E. Piaggio, B.L. Salomon, Effector T cells boost regulatory T cell expansion by IL-2, TNF, OX40, and plasmacytoid dendritic cells depending on the immune context, *J. Immunol.* 194 (3) (2015) 999–1010.
- H.J. Bovenschen, P.C. van de Kerkhof, P.E. van Erp, R. Woestenenk, I. Joosten, H.J. Koenen, Foxp3+ regulatory T cells of psoriasis patients easily differentiate into IL-17A-producing cells and are found in lesional skin, *J. Invest. Dermatol.* 131 (9) (2011) 1853–1860.
- H.J. Koenen, R.L. Smeets, P.M. Vink, E. van Rijssen, A.M. Boots, I. Joosten, Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells, *Blood* 112 (6) (2008) 2340–2352.
- T. Wang, X. Sun, J. Zhao, J. Zhang, H. Zhu, C. Li, N. Gao, Y. Jia, D. Xu, F.P. Huang, N. Li, L. Lu, Z.G. Li, Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood, *Ann. Rheum. Dis.* 74 (6) (2015) 1293–1301.
- M. Tarique, C. Saini, R.A. Naqvi, N. Khanna, A. Sharma, D.N. Rao, IL-12 and IL-23 modulate plasticity of FoxP3(+) regulatory T cells in human Leprosy, *Mol. Immunol.* 83 (2017) 72–81.
- X. Chen, M. Baumele, D.N. Mannel, O.M. Howard, J.J. Oppenheim, Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells, *J. Immunol.* 179 (1) (2007) 154–161.