Ustekinumab Improves Psoriasis without Altering T Cell Cytokine Production, Differentiation, and T Cell Receptor Repertoire Diversity

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Abstract

Ustekinumab is a fully human IgG1κ monoclonal antibody targeting interleukin (IL)-12/23 p40 subunit. The role of IL-12/23-mediated pathway in the mechanism of various inflammatory disorders especially psoriasis has been well recognized. Recently the long-term efficacy and safety of ustekinumab in patients with moderate-to-severe psoriasis has been evaluated in phase 2/3 clinical trials, and the results showed no significant risk for serious adverse effects, infections, or malignancies. Ustekinumab inhibits the function of the IL-12/23 p40 subunit, and therefore it is believed that inhibition of IL-12 p40 pathway decreases IFN-γ production. The major concern for the use of ustekinumab is the possibility of increased immunosuppression due to low IFN-γ production. However, the effects of ustekinumab on CD4+ T cell function have not been fully investigated so far. In this study, we explored changes in cytokine production by memory CD4+ T cells as well as in the differentiation of naïve T cells to helper T cell (Th) 1, Th2, or Th17 cells in psoriasis patients treated with ustekinumab. The effect of the treatment on T cell receptor repertoire diversity was also evaluated. The results showed that ustekinumab improves clinical manifestation in patients with psoriasis without affecting cytokine production in memory T cells, T cell maturation, or T cell receptor repertoire diversity. Although the number of patients is limited, the present study suggests that T cell immune response remains unaffected in psoriasis patients treated with ustekinumab.

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Introduction

Psoriasis is a chronic immune-mediated skin disorder with frequent clinical relapse [1]. The majority of patients with moderate-to-severe psoriasis require specific topical and systemic therapies including phototherapy (psoralen ultraviolet A therapy (PUVA) or narrow-band ultraviolet B (NB-UVB)), methotrexate [2], cyclosporine [2], and retinoids [3]. However, long-term follow-up during these therapies is generally difficult because of cytotoxicity-related adverse effects, treatment failure, or patient dissatisfaction [4,5].

Recently, several biologic agents (biologics) have been reported for the treatment of psoriasis [6–8]. Biologics have high target specificity and their use is associated with limited organ toxicity. However, the risk of cancer or infection during long-term use in patients with psoriasis has not been as yet investigated.

IL-12 and IL-23 play important roles in the pathogenesis of psoriasis [9]. In psoriasis patients, IL-12 and IL-23 are involved in immune response mediated by helper Th1 [10] and Th17 [11,12]. IL-12 and IL-23 are heterodimers with a common p40 subunit. The binding of the subunits to their respective receptors activates specific intracellular signaling pathways [13,14]. Ustekinumab (Stelara®, Janssen Biotech, Inc., Horsham, PA), a fully human IgG1κ monoclonal antibody, binds to the common p40 subunit of IL-12 and IL-23, and blocks activation of the receptors of these cytokines in dendritic cells and monocytes. Recent studies have shown significant effectiveness and safety of ustekinumab in moderate-to-severe plaque-type psoriasis during phase 2 [15] and phase 3 clinical trials [16–19]. However, IL-12 is known to have anti-cancer activity by promoting IFN-γ production, therefore there is risk of cancer development due to immunosuppression. The effects of ustekinumab on the production of IL-12/IL-23 are known but its effects on T cell function are not completely understood.

In the present study, we investigated the influence of ustekinumab on T cell cytokine production, differentiation of naïve T cells and on the T cell receptor repertoire diversity in psoriasis patients.
Materials and Methods

Subjects
Five psoriasis patients and five healthy volunteers were enrolled in this study. Patients with psoriasis eligible for the use of biologics were included in the study. Briefly, they fulfilled the rule of 10: Psoriasis Area and Severity Index (PASI) ≥ 10, and/or Body Surface Area (BSA) ≥ 10%, and/or Dermatology Life Quality Index (DLQI) ≥ 10. The phenotypical character and response to the biologics are shown in table 1.

Psoriasis Treatment Protocol and Blood Sampling Schedule
Ustekinumab was administered on weeks 0, 4, and 12. In principle, ustekinumab at a dose of 45 mg was administered intradermally during each therapy. Blood was sampled one month after the third administration after obtaining written informed consent from the subjects. Blood sampling was performed three times in two psoriasis patients and in one healthy volunteer (before the first administration, and one month after the second and third administration). The investigational protocol was approved by the Institutional Review Board (IRB) of Mie University Hospital (Permit Number 2096).

Antibodies and Reagents
Phytohemagglutinin (PHA), Phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified anti-human CD3 mAb, anti-CD28 mAb, anti-hCD93-FTTC mAb, anti-h-TCR γ/δ-FTTC mAb, anti-hIFN-γ-PerCP mAb, anti-hIL-4-PerCP mAb, anti-hIL-12-PerCP mAb, anti-hTNF-α-PerCP mAb, and brefeldin A were purchased from R&D Systems (Brea, CA, USA). Anti-hIL-4 mAb, anti-hIL-12 mAb, anti-hIFN-γ mAb, and anti-hCD45RO-PE mAb were purchased from BD/PharMingen (San Diego, CA, USA). Foxp3-PECy5 mAb, and anti-hCD127-FITC mAb were from BioLegend (San Diego, CA, USA). Anti-hCD4-FITC mAb, anti-hCD8a-FITC mAb, and anti-hTCR-PerCP mAb were from Beckman Coulter (Brea, CA, USA). Anti-CD4 mAb and/or Dermatology Life Quality Index (DLQI) ≥ 10. The phenotypical character and response to the biologics are shown in table 1.

Table 1. Background of five patients and five healthy controls.

| Disease duration | 12 years | 16 years | 10 years | 1 year | 14 years | none | none | none | none | none | none |
|------------------|----------|----------|----------|--------|----------|------|------|------|------|------|------|
| PASI score       |          |          |          |        |          |      |      |      |      |      |      |
| Pre therapy      | 16.8     | 13.2     | 17.2     | 49.2   | 7.3      | 0    | 0    | 0    | 0    | 0    | 0    |
| Post 1st infusion| 3.8      | 4.4      | 4.8      | 4.2    | 6.1      | 0    | 0    | 0    | 0    | 0    | 0    |
| Post 2nd infusion| 5.7      | 2.0      | 2.0      | 0.9    | 14.7     | 0    | 0    | 0    | 0    | 0    | 0    |
| WBC (before)     | 6930     | 6530     | 5200     | 8150   | 6070     | 4600 | 6340 | 7200 | 5830 | 8280 |
| Lymphocyte (%)   | 28.9     | 26.5     | 24.7     | 13.7   | 32.3     | 28.5 | 33.0 | 27.7 | 32.8 | 37.4 |
| WBC (Post 1)     | 6850     | 7080     | 5360     | 7160   | 8560     | 5010 | 6020 | 7080 | 5920 | 8100 |
| Lymphocyte (%)   | 27.4     | 32.5     | 34.3     | 15.5   | 22.5     | 30.1 | 30.3 | 29.1 | 31.2 | 36.2 |
| WBC (Post 2)     | 7120     | 6500     | 5230     | 6700   | 6750     | 4670 | 6730 | 6830 | 6310 | 8310 |
| Lymphocyte (%)   | 31.3     | 38.3     | 26.4     | 13.9   | 20.6     | 29.2 | 29.1 | 30.6 | 28.5 | 36.4 |

The PASI score of the patients was high before ustekinumab therapy, and improved dramatically after the treatment. However, the PASI score of case 5 was increased at one month after the third therapy. WBC counts and the ratio of lymphocytes in all patients and controls were preserved during all the course of the study.

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(1 μg/mL) and brefeldin A (1 μg/mL) for 24 h at 37°C in an atmosphere of 5% CO₂.

**Generation of Th1 Cells in vitro**

Th1 cells were generated by culturing naïve CD4⁺ T cells (1 × 10⁶/mL) with PHA (1 μg/mL), rhIL-12 (50 ng/mL), and anti-hIL-4 mAb (500 ng/mL) in a flat-bottomed 24-well plate in 1 mL of complete RPMI1640 culture medium at 37°C with 5% CO₂. The stimulated T cells were collected and washed on day 3 and expanded in the same culture medium with 100 U/mL of rhIL-2 for an additional 3 days. On day 6, the cells were stimulated with PMA (25 ng/mL) and ionomycin (1 μg/mL) in the presence of brefeldin A (1 μg/mL) for 8 h [20,21].

**Generation of Th2 Cells in vitro**

Th2 cells were generated by culturing naïve CD4⁺ T cells (1 × 10⁶/mL) with PHA (1 μg/mL), rhIL-4 (200 ng/mL), and anti-hIL-12 mAb (10 μg/mL) in a flat-bottomed 24-well plate in a complete RPMI1640 culture medium at 37°C and an atmosphere of 5% CO₂. The stimulated T cells were washed on day 3 and expanded in the same culture medium with the addition of 100 U/mL of rhIL-2 for an additional 3 days. On day 6, the cells were stimulated with PMA (25 ng/mL), ionomycin (1 μg/mL) and brefeldin A (1 μg/mL). The cells were incubated for additional 8 h [20,21].

**Generation of Th17 Cells in vitro**

Th17 cells were generated by culturing naïve CD4⁺ T cells (1 × 10⁶/mL) with rhIL-2 (10 U/mL), rhTGF-β (5 ng/mL), rhIL-6 (20 ng/mL), rhIL-1β (10 ng/mL), rhIL-23 (10 ng/mL), anti-hIL-4 mAb (1 μg/mL), anti-hIFN-γ mAb (1 μg/mL), anti-hCD3 mAb (4 μg/mL), and anti-hCD28 mAb (8 μg/mL) in a flat-bottomed 24-well plate in complete RPMI1640 culture medium at 37°C and an atmosphere of 5% CO₂. On days 3 and 5, the culture plate was centrifuged, and the media was removed and replaced with fresh media containing all cytokines mentioned above, and antibodies. On day 6, the cells were stimulated with PMA (25 ng/mL), ionomycin (1 μg/mL) and brefeldin A (1 μg/mL). The cells were incubated for 8 h at 37°C and an atmosphere of 5% CO₂ [20,22].

**Cell Surface and Intracellular Staining of CD4⁺ T Cells**

Cultured CD4⁺ T cells were collected and washed twice with PBS containing 1% FBS. Cell surface antigens and intracellular cytokines were stained according to the formal Cell Surface Immunofluorescence Staining Protocol and Intracellular Cytokine Staining Protocol (BioLegend). Briefly, for analyzing cytokine production from memory cells, the cells were firstly stained with anti-hCD4-FTTC mAb. To detect cellular differentiation of naïve T cells into cytokine-producing cells, the cells were firstly stained with anti-hCD45RA-FTTC and anti-hCD45RO-PE mAbs. After treatment with the fixation and permeabilization wash buffer, the cells were incubated with PerCP-conjugated anti-hIL-4, or IL-17A mAbs. Fluorescence profiles were assessed by flow cytometry using FACS Calibur (BD Biosciences, San Jose, CA), and the data were analyzed using Cell Quest Pro software (BD Biosciences).

**Statistical Analyses**

Statistical analysis was performed using the Mann-Whitney test. A P-value of less than 0.05 was considered as statistically significant.

**Results**

**Clinical Manifestations**

The PASI scores of the patients were 16.8 (patient 1), 13.2 (patient 2), 17.2 (patient 3), and 49.2 (patient 4) before ustekinumab therapy, and they improved to 3.8, 4.4, 4.8, and 4.2 at one month after the second therapy, and 5.7, 2.0, 2.0, and 0.9 at one month after the third therapy, respectively. However, the PASI score of case 5 was 7.3 before ustekinumab therapy, improved to 6.1 at one month after the second therapy, but returned to 14.7 at one month after the third therapy. The WBC count and the percentage of lymphocytes in all patients and controls were stable during all the study (Table 1).
suppress the percentage of CD45RA^{+}CD45RO^{+}IFN-γ^{+} cells, CD45RA^{+}CD45RO^{+}IL-4^{+} cells, or CD45RA^{+}CD45RO^{+}IL-17^{+} cells compared to untreated control group (Fig. 1B). Flow cytometric analysis showed abundant cytokine production from CD45RA^{+}CD45RO^{+}CD4^{+} T cells. The follow-up study in two patients and one healthy control showed no alteration of T cell maturation by ustekinumab treatment (Fig. 3).

**Naturally Occurring Regulatory T Cells (nTregs)**

No significant difference in nTreg (FoxP3^{+}CD127^{low}CD25^{high}CD4^{+} T cells/CD4^{+} T cells) ratio was found among patients and volunteers during the third sampling (Fig. 4).

**T Cell Receptor Repertoire Diversity**

Staining with antibody to TCR BV subfamily showed that all the subfamilies were preserved in the patients compared to normal volunteers, and with no particular collapse in T cell receptor repertoire diversity (Fig. 5).

**Discussion**

Recent studies have shown the therapeutic efficacy of several biologic agents for the treatment of refractory psoriasis. Increase in the risk of severe infection, malignancy, or in mortality rate during the use of the agents has not been reported; however, further statistical data are required to confirm the safety of these agents during long-term use [24]. Susceptibility to infection [25] and malignancy [26] has been reported during ustekinumab therapy due to inhibition of IL-12 and IL-23. Recent 2/3 phase clinical trials demonstrated the efficacy and safety of ustekinumab [15–19,27–29]. However, there are just a few data on changes in T cell immune response during ustekinumab therapy.

In the present study, we demonstrated cytokine production by Th1 and Th17 cells during ustekinumab therapy, suggesting that blockade of IL-12/IL-23p40 improves skin manifestations without decreasing cytokine production by Th1/Th17 cells (Fig. 1A and 2). In addition, TNF-α production from memory CD4^{+} T cells was...
Figure 2. Cytokine production by memory CD4+ T cells. Representative flow cytometry data from two patients and control are shown. (A) The percentage of CD4⁺CD45RO⁺IFN-γ T cells (B) The percentage of CD4⁺CD45RO⁺IL-4 T cells (C) The percentage of CD4⁺CD45RO⁺IL-17 T cells. All cytokine production remained unchanged during treatment.

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Figure 3. Differentiation of naïve CD4+ T cells to cytokine-producing mature cells (Th1/Th2/Th17). Representative flow cytometry data are shown. (A) The percentage of CD45RA<sup>−</sup>CD45RO<sup>+</sup>IFN-γ<sup>+</sup> cells in the CD4<sup>+</sup> T cell population (B) The percentage of CD45RA<sup>−</sup>CD45RO<sup>+</sup>IL-4<sup>+</sup> cells in the CD4<sup>+</sup> T cell population (C) The percentage of CD45RA<sup>−</sup>CD45RO<sup>+</sup>IL-17<sup>+</sup> cells in the CD4<sup>+</sup> T cell population. T cell maturation was not influenced by ustekinumab treatment.
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the same between patients treated with ustekinumab and normal controls (Fig. S1). Furthermore, IL-12/IL-23p40 blockade has limited effects on naive T cell development with normal T cell development milieu during ustekinumab therapy (Fig. 1B and 3).

Figure 4. Naturally occurring regulatory T cells. The percentage of nTreg (FoxP3⁺CD127lowCD25highCD4⁺ T cells/CD4⁺ T cells) was similar among the seven volunteers. Flow cytometry data from four patients and three healthy controls are shown.

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IL-17 secreted by Th17 cells plays a key role in the inflammatory response in various diseases; the level of IL-17 production and Th17 cell development remained unchanged during the course of treatment. CD4+CD127lowCD25+FoxP3+ regulatory T cells (nTreg) play critical role in the suppression of excessive inflammatory response in various diseases including psoriasis. nTregs also regulate local and systemic immune response by maintaining the balance among Th1, Th2, and Th17/22 cells. The function of nTreg was also conserved during the course of treatment (Fig. 4). In addition, the cytokine production by CD8+ T cells and γ/δ T cells was similar between patients and controls (Fig. S2 and S3).

In the present study, the skin manifestations of the patients markedly improved, despite unaltered cytokine production and T cell differentiation. The cytokine production and differentiation of T cells in response to infections and malignancies were preserved in the peripheral blood. On the other hand, the excessive production of inflammatory cytokines in the skin lesions was controlled during ustekinumab therapy.

Evaluation of the qualitative alteration in T cell immunity during ustekinumab therapy is also important. Clonal expansion or loss of some T cell clones can be associated with risk of malignancy and infection. TCR BV subfamily immune-staining with TCR BV antibodies is a reliable tool for analysis of T cell receptor diversity; collapse and restoration of T cell receptor diversity was reported in CTCL patients in advanced stages of disease [23,30]. In the present study, no significant alteration in TCR diversity after ustekinumab therapy was observed, suggesting that ustekinumab has no effects on immunological competence (Fig. 5).

In conclusion, the present data showed that ustekinumab improves clinical manifestations in patients with psoriasis without inducing immunosuppression. However, a study with a larger population and longer follow-up should be carried out to confirm these observations.

Supporting Information

Figure S1 The percentage of CD4+CD45RO+TNF-α+ T cells. Flow cytometry data are shown. (A) The percentage of CD8a+IFN-γ+ T cells (B) The percentage of CD8a+TNF-α+ T cells. The production of IFN-γ and TNF-α by CD8+ T cells was not suppressed in patients with psoriasis during ustekinumab treatment as compared to normal controls. (TIF)

Figure S2 Cytokine production by memory CD8+ T cells. Flow cytometry data are shown. (A) The percentage of CD8α+IFN-γ+ T cells (B) The percentage of CD8α+TNF-α+ T cells. The production of IFN-γ and TNF-α by CD8+ T cells was not suppressed in patients with psoriasis treated with ustekinumab. (TIF)

Figure S3 Cytokine production by γ/δ T cells. Flow cytometry data are shown. (A) The percentage of TCR γ/δ+IFN-γ+ T cells (B) The percentage of TCR γ/δ+IL-17+ T cells. The production of IFN-γ and IL-17 from γ/δ T cells was not suppressed in patients with psoriasis treated with ustekinumab. (TIF)

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Author Contributions

Conceived and designed the experiments: KY HT HM. Performed the experiments: KT KY MK KM RS. Analyzed the data: KT KY. Wrote the paper: KT KY EG HM.

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