Biologic therapy improves psoriasis by decreasing the activity of monocytes and neutrophils

Keiichi Yamanaka[1,4], Yoshinori Umezawa[2,4], Akisa Yamagiwa[1,4], Hidehisa Saeki[2], Makoto Kondo[1], Esteban C Gabazza[3], Hidemi Nakagawa[2], and Hitoshi Mizutani[1]

[1]Department of Dermatology and [3]Immunology, Mie University, Graduate School of Medicine, Tsu, Mie, Japan

[2]Department of Dermatology, The Jikei University School of Medicine, Minato-ku, Tokyo, Japan

[4]These three authors equally contributed to this project.

Running title: How dose biologics work?

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Correspondence to: Kei-ichi Yamanaka, MD, PhD

Associate Professor of Dermatology,

Mie University, Graduate School of Medicine,

2-174 Edobashi, Tsu, Mie 514-8507, Japan

23 TEL: +81-59-231-5025

24 FAX: +81-59-231-5206

25 E-mail: yamake@clin.medic.mie-u.ac.jp

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31 **Abbreviations:** Psoriasis Area and Severity Index, PASI; Body Surface Area, BSA;
32 Dermatology Life Quality Index, DLQI; cutaneous leucocyte-associated antigen
33 receptor, CLA; granulocyte and monocyte apheresis, GMA; The mean fluorescent
34 intensity, MFI

Abstract

Therapy with monoclonal antibodies to TNF-α and interleukin-12/23 p40 subunit has significantly improved the clinical outcome of patients with psoriasis. These antibodies inhibit the effects of the target cytokines and thus the major concern during their use is the induction of excessive immunosuppression. Recent studies evaluating the long-term efficacy and safety of biologic therapy in psoriasis have shown no significant appearance of serious adverse effects including infections and malignancies. However, the immunological consequence and the mechanism by which the blockade of a single cytokine by biologics can successfully control the activity of psoriasis remain unclear. In the current study, we investigated the effect of biologic therapy on cytokine production of various lymphocytes subclass and on the activity of monocytes and neutrophils in psoriasis patients. Neutrophils, monocytes and T cells were purified from heparinized peripheral venous blood by Ficoll density gradient centrifugation, and IFN-γ, TNF-α, and IL-17 production from lymphocytes was measured by flow cytometer. The activation maker of neutrophils and the activated subsets of monocytes were also analyzed. Biologic therapy induced no significant changes in the cytokine production by lymphocytes from the skin and gut homing T cells. However, neutrophil activity and the ratio of activated monocytes population increased in severe psoriasis patients were normalized in psoriasis patients receiving biologic therapy. The present study showed that biologic therapy ameliorates clinical symptoms and control the immune response in patients with psoriasis.

Key word: Infliximab, Ustekinumab, psoriasis, monocyte, neutrophil

Introduction

Psoriasis is a chronic immune-mediated skin disorder characterized by frequent clinical relapse; patients with moderate-to-severe psoriasis generally require specific topical and/or systemic therapy including phototherapy, methotrexate, cyclosporine, and retinoid [1, 2]. However, the disease control with this kind of therapy is usually difficult especially in severe psoriasis patients because of cytotoxicity-related adverse effects, treatment failure, or patient dissatisfaction[3, 4].

Tumor necrosis factor-α (TNF-α), which is mainly secreted by monocytes and macrophages, and to a lesser degree by T cells, is involved in the pathogenesis of psoriasis in conjunction with the Th1 cytokine interferon-γ (IFN-γ). The inflammatory response induced by TNF-α plays a central role in several immune-mediated diseases. The blockade of TNF-α with infliximab (Remicade®), a chimeric murine monoclonal IgG1 against TNF-α, has been shown to be highly effective for improving both skin symptoms and arthritis.

The heterodimers IL-12 and IL-23 with a common p40 subunit also play important roles in the pathogenesis of psoriasis [5]. The binding of the subunits to their respective receptors activates specific intracellular signaling pathways [6, 7]. Ustekinumab (Stelara®), a fully human IgG1κ monoclonal antibody, binds to the common p40 subunit of IL-12 and IL-23, and blocks the activation of their receptors.

Infliximab and ustekinumab show inhibitory effect on the activity or production of TNF-α and IFN-γ; therefore the major concern with biologic therapy using these

compounds is the induction of excessive immunosuppression. The long-term efficacy and safety of biologic therapy has been recently evaluated in patients with moderate-to-severe plaque-type psoriasis and psoriatic arthritis, and the results showed no significant appearance of serious adverse effects such as infection or malignancy [8-15]. In addition, we have recently reported that T cell immune response and T cell receptor diversity remain unaffected in psoriasis patients treated with ustekinumab [16]; however, the immunological effects induced by biologic therapy requires further investigations. Psoriasis is an inflammatory skin disease characterized by activation of a network of immune factors including T cells, dendritic cells, neutrophils, monocytes, and their secreted cytokines. The fact that suppression of a single cytokine with a monoclonal antibody can control more than 75% of psoriasis skin activity is still unclear.

In the present study, we investigated the immune response in psoriasis patients. In particular, the production of cytokines from lymphocytes and the activation of monocytes and neutrophil were evaluated in psoriasis patients receiving biologic therapy.

Methods

Subjects and clinical manifestations

Forty-two psoriasis patients (male 28, female 14) and seven healthy volunteers (male 4, female 3) were enrolled in this study (Table 1). Criteria for inclusion of the patients in the study were as follows: Patients with Psoriasis Area and Severity Index (PASI)≧10, and/or Body Surface Area (BSA)≧10%, and/or Dermatology Life Quality Index (DLQI)≧10 were categorized as severe group. Cases that did not fulfill these criteria were categorized as moderate group.

Among 13 psoriasis patients receiving no biologic therapy, 8 were categorized in the severe group (male 5, female 3, PASI score 26.8) and 5 in the moderate group (male 3, female 2, PASI score 6.7). Twenty-nine patients with psoriasis received biologic therapy, 17 of them being treated with ustekinumab (male 12, female 5, PASI score 1.7) and 12 with infliximab (male 8, female 4, PASI score 0.9). The mean age of the patients was 46.1 years, and the mean disease duration was 5.8 years.

Psoriasis treatment protocol and blood sampling schedule

Ustekinumab was administered at a dose of 45 mg per body on day 0, after one month, and then every three months. Infliximab was administered at a dose of 5 mg/kg on day 0, after 2 and 6 weeks, and then every two months. Blood samples were taken from patients receiving no biologic therapy at their visit. Samples were also taken from patients receiving ustekinumab or infliximab on the day just before the injection. The investigational protocol was approved by the Institutional Review Board (IRB) of Mie

University Hospital (Permit Number 2096) and of the Jikei University Hospital (Permit Number 24-351-7117).

Antibodies and reagents

Lipopolysaccharide (LPS), Phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-hIFN-γ-PerCP mAb, anti-hIL-17-PerCP mAb, anti-hTNF-α-PerCP mAb, and brefeldin A were purchased from BioLegend (San Diego, CA, USA). Anti-hCD16 FITC, Anti-CLA-FITC, Anti-CCR4-PE, Anti-hCD62L-PE, Anti-hCD11b-PcrCP, Anti-hCCR6-APC, Anti-hCCR9-APC, Anti-hCD66b-APC, Anti-hCD4-PcrCP mAb were purchased from BD/PharMingen (San Diego, CA, USA). Foxp3-Alaxa647 mAb, Anti-hCD25-PE, and anti-hCD127-FITC mAb were from eBioscience (San Diego, CA, USA). Complete RPMI 1640 medium was made with 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, INC., South Logan, UT, USA), 2.0 mM L-glutamine, 100U/ml penicillin, and 100mg/ml streptomycin (Nacalai tesque, Kyoto, JAPAN).

Purification of neutrophils, PBMCs, $CD4^+$T cells and non-$CD4^+$T cells

Heparinized peripheral venous blood was separated by Ficoll density gradient centrifugation using Histopaque 1119 plus 1077 (Sigma-Aldlich, St. Louis, MO). Neutrophils and PBMCs were separately isolated. Purification of $CD4^+$ T cells was performed by negative selection using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec,

Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, PBMCs were incubated for 10 min with 20 μl of the antibody cocktail mixture followed by 15 min incubation with 20 μl of magnetic beads per 10^7 cells. Unconjugated $CD4^+$ T cells were then isolated from PBMCs by indirect magnetic labeling using MiniMACS separation LS columns. The purity of samples was between 96 and 99%.

Cell culture and intracellular staining of T cells

$CD4^+$ T cells suspended in complete RPMI 1640 culture medium, were plated into a flat-bottomed 24-well plate at 1×10^6 cells/well and incubated with PMA (25 ng/mL), ionomycin (1μg/mL) and brefeldin A (1 μg/mL) and cultured for 4 h at 37 °C in an atmosphere of 5%CO_2. Cultured $CD4^+$ T cells are 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 skin homing $CD4^+$ T cells, the cells were firstly stained with anti-human cutaneous leucocyte-associated antigen receptor (CLA)-FITC, anti-CCR4-PE, and anti-CCR6-APC mAbs. To detect cytokine production from gut homing $CD4^+$ T cells, the cells were firstly stained with anti-α4-integrin-FITC, anti-β7-integrin-PE, and anti-CCR9-APC mAbs. After treatment with the fixation and permeabilization wash buffer, the cells are incubated with anti-hIFN-γ-PerCP, anti-hIL-17-PerCP, or TNF-α -PerCP mAbs. Fluorescence profiles were assessed by flow cytometry using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA), and

the data were analyzed using FCS Express™ software (BD Biosciences).

Staining of naturally occurring regulatory T cells (nTregs)

For identification of nTreg ($FoxP3^{+}CD127^{low}CD25^{high}CD4^{+}$ T cells), magnetically collected $CD4^{+}$ T cells were directly stained with monoclonal antibodies to CD127-FITC, CD25-PE, and CD4-PerCP in cell surface staining buffer containing 0.1M PBS and 2%FCS. Intracellular staining with Foxp3-PECy5 antibody was performed according to the manufacture's instruction (eBioscience).

Neutrophil activity

Neutrophils separated by Histopaque 1119 plus 1077 were gated by FACS and categorized as CD16 positive cells. Neutrophil activity was measured using anti-CD62L-PE, anti-CD11b-PerCp, and anti-CD66b-Alexa647 antibodies.

Monocyte activity

PBMCs suspended in complete RPMI 1640 culture medium were plated into a flat-bottomed 24-well plate at 1×10^6 cells/well and incubated with lipopolysaccharide (LPS, 1 μg/mL) and brefeldin A (1 μg/mL), and cultured for 4 h at 37 °C in an atmosphere of 5%CO_2. Cultured cells were washed twice with PBS containing 1%FBS, and stained with CD16 and CD14 antibodies, and the intracellular cytokines including IFN-γ, IL-17, and TNF-α production were measured.

183 ***Statistical analyses***

184 Statistical analysis was performed using the Kruskal-Wallis-test. Correlation was
185 tested by using the Spearman rank analysis. A $p<0.05$ was considered as statistically
186 significant.

187

Results

Biologic therapy induced no significant suppression of cytokine production from skin homing and gut homing CD4$^+$T cells

The lymphocyte count was not significantly different among the five groups (Fig. 1a). The production of IFN-γ, IL-17, and TNF-α by $CD4^+$ T cells stimulated with PMA and ionomycin was evaluated. The production of the three cytokines was not significantly different among the five groups. No significant suppression was found in the groups receiving biologic therapy (Fig. 1b). The skin homing T cells expressing $CLA^+CCR4^+CCR6^+CD4^+$T cells were also evaluated. No significant change in cytokine production was observed even in the severe psoriasis groups or biologic-treated well-controlled group (Fig. 1c). Cytokine production from gut homing $CD4^+$T cells expressing α4-integrin, β7-integrin, and CCR9 was also evaluated but no significant different was observed (Fig. 1d).

Naturally occurring regulatory T cells (nTregs)

The nTreg ratio ($FoxP3^+CD127^{low}CD25^{high}CD4^+$ T cells/ $CD4^+$ T cells) was evaluated. The nTreg ratio was significantly elevated in psoriasis patients receiving biologic therapy compared to patients with moderate psoriasis without biologic therapy and healthy volunteers (Fig. 2).

Biologic therapy decreases neutrophil activity

209 The neutrophil count was not different among the five groups (Fig. 3a). Ficoll separated
210 neutrophils were gated morphologically by FACS, and then CD16 positive cells were
211 gated. CD62L expression was decreased on neutrophils from severe psoriasis patients.
212 The CD62L expression was normal on neutrophils from the biologic therapy groups. In
213 addition, the expression of CD11b and CD66b was elevated in samples from the severe
214 group of patients, but their expression was normal in patients under biological therapy
215 (Fig. 3b).

216

217 ***Biologic therapy decreases monocyte activity***

218 The monocyte count was not significantly different among the 5 groups (Fig. 4a). The
219 percentage of $CD14^{high}$ activated monocytes was increased in severe psoriasis patients
220 compared to patients with moderate disease and normal controls. The $CD14^{high}$
221 activated monocyte ratio was normal in psoriasis patients receiving biologics (Fig. 4b).

222

223 ***The correlation between PASI score and the activity of neutrophils or monocytes***

224 The correlation between PASI score at the time of blood collection and the activity of
225 neutrophils or monocytes was analyzed. The mean fluorescent intensity (MFI) of
226 CD62L (Fig. 5a) and CD66b (Fig. 5c) levels of neutrophil activity was significantly
227 correlated with PASI score. The correlation did not show the significance between

228 CD11b level and PASI score (Fig. 5b). The other hand, the percentage of $CD14^{high}$
229 activated monocytes was significantly correlated with PASI score (Fig. 5d).

230

Discussion

Therapy with anti-TNF-α and anti-IL-12/23p40 antibodies has markedly improved the clinical outcome of patients with severe psoriasis. However, the strong inhibitory effect of these antibodies raised concern because the possible induction of excessive immunosuppression. The present study showed that these biologic therapies induce no significant changes in IFN-γ, IL-17, and TNF-α production from lymphocytes and that they decreased the activity of neutrophils and monocytes in patients with severe psoriasis.

Ustekinumab and infliximab target cytokines, thus we firstly investigated the effect on cytokine production from $CD4^+$ T cells but found that they cause no significant changes as previously reported[16]. Effector T cells home and infiltrate target organs by using their surface chemokine receptors. In the current study we found no significant difference in the cytokine production from skin homing $CLA^+CCR4^+CCR6^+$ $CD4^+$ T cells even in severe psoriasis patients irrespective of the treatment with biologics. Infliximab is also indicated in patients with the inflammatory bowel disorders. We found no significant difference in cytokine production in $\alpha4\beta7$ $integrin^+CCR9^+$ $CD4^+$ T cells.

$CD4^+CD127^{low}CD25^{high}Foxp3^+$ regulatory T cells (nTreg) play critical roles in the suppression of excessive inflammatory response in various diseases including psoriasis. nTreg also regulates the local and systemic immune responses maintaining the balance among Th1,Th2 and Th17/22 cells [17]. The number of nTreg increased in

252 patients under biologic therapy. Recent studies have shown that γδ-T cells also play a
253 role in the pathogenesis of psoriasis, but in the present study we found that biologics
254 induce no changes in γδ-T cells; in addition we found no changes in cytokine
255 production from $CD8^+$ T cells and NK cells (data not shown).

256 Neutrophils accumulation is another characteristic finding in psoriatic lesions.
257 Neutrophils play a critical role in the remodeling of skin epidermis. Neutrophils are also
258 the first line defense against environment insults. Here we showed that neutrophil
259 activity was increased in the severe psoriasis group compared with the moderate
260 psoriasis group and normal control subjects, and that biologic therapy blocked
261 neutrophil activation in the patients. Interestingly two of three neutrophil activation
262 parameters showed the correlation with PASI score at the time of blood collection.

263 Monocytes also secrete several pro-inflammatory cytokines including TNF-α.
264 The role of monocyte in psoriasis is not completely clear. Monocytes are categorized
265 into three populations according to their cell surface expression of CD14 and CD16.
266 $CD16^+$ monocytes release high level of TNF-α, IL-6, and IL-10. Recent studies
267 suggested the role of $CD14^{high}$ $CD16^+$ monocytes in the pathogenesis of psoriasis; this
268 cell population is increased in patients with active generalized pustular psoriasis, and its
269 removal with adsorptive granulocyte and monocyte apheresis (GMA) has been shown to
270 improve clinical outcome in patients with pustular psoriasis, suggesting their important
271 role in the disease[18]. Patients with infectious disorders, malignancy, and autoimmune
272 diseases have also increased percentage of $CD14^{high}$ $CD16^+$ monocytes [19-21]. In

preliminary experiments, we found more significant production of TNF-α and IFN-γ in $CD14^{high}$ monocytes population than in $CD14^{low}$ monocytes, thus we defined $CD14^{high}$ monocytes as the major source of pro-inflammatory cytokines. Monocytes count remained unchanged during biologic therapy; however, the ratio of $CD14^{high}$ monocytes was increased in patients with severe psoriasis, and it was normal in patients receiving biologics suggesting that inhibition of monocyte activation is another mechanism for the beneficial effect of biologic therapy in patients with psoriasis.

The fact that antibodies against TNF-α and IL-12/23 effectively improve the clinical outcome of patients with psoriasis further strengthens the role of both cytokines in the pathogenesis of the disease. However, the precise mechanism to explain why one single biologic agent (infliximab or usutekimab) can successfully control most disease activity remains unclear. TNF-α receptor is expressed on a variety of cells including monocytes, neutrophils and lymphocytes. As previously reported, there was a significant infiltration of TNF-receptor positive cells in the skin lesions of psoriasis patients and they were decreased after clinical improvement of the disease [22]. Neutralization of TNF-α may reduce the activity of monocytes and neutrophils by blocking TNF-α mediated pro-inflammatory effects. On the other hand, it is known that IL-12/23 p40 receptor is expressed mainly on cells of the lymphocyte lineage. However, we found that blockade of IL-12/23 also decreases the activity of neutrophils and monocytes; the effect of this biologic on monocytes and neutrophils may be explained by the fact that IL-12/23 is in the upstream of IFN-γ and TNF-α network.

In brief, this study showed that biologic therapy improves the clinical symptoms in patients with psoriasis by stabilizing the innate and acquired immune system. The results of the present study may explain in part why mono functional biologics can block the complex cytokine network in psoriasis.

Conflict of interest: We received research contribution from Mitsubishi Tanabe Pharma Corporation.

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373
374

Figure legends

Figure 1. ***Biologic therapy induces no significant suppression of cytokine production from skin homing and gut homing CD4$^+$T cells***

(a) The lymphocyte count remained unchanged in the five groups. Severe or moderate biologics-free group is abbreviated as Sev or Mod, respectively. Patient receiving ustekinumab or infliximab therapy is abbreviated as Ust or Inf, respectively. Normal healthy control group is abbreviated as Nor. To determine cytokine production from $CD4^+$ T cells, the cells were stimulated with PMA and ionomycin. IFN-γ, IL-17, and TNF-α production from $CD4^+$ T cells remained unchanged among the five groups (b). Skin homing $CD4^+$ T cells were gated within $CLA^+CCR4^+CCR6^+CD4$ T^+ cells. The level of cytokine production was unchanged even in severe psoriasis patients and in biologic-treated well-controlled patients (c). Gut homing T cells express chemokine receptors such as α4–integrin, β7-integrin, and CCR9 on their cell membrane. The cytokine production form gut homing T cells was not different among the five groups (d).

Figure 2. ***Naturally occurring regulatory T cells (nTregs)***

The percentage of nTreg ($FoxP3^+CD127^{low}CD25^{high}CD4^+$ T cells/ $CD4^+$ T cells) was significantly elevated in psoriasis patients receiving biologic therapy compared to moderate psoriasis patients that received no biologic therapy or healthy volunteers.

Figure 3. ***Biologic therapy decreases neutrophil activity***

(a) Neutrophil count was not different among groups. Neutrophil was firstly gated morphologically and then by CD16 staining by FACS. The neutrophil activity was assessed using three markers. The mean fluorescent intensity (MFI) of CD62L was decreased in activated neutrophils, being lower in severe psoriasis patients compared to moderate psoriasis patients and normal healthy volunteers. Patients receiving biologic therapy had normal levels. CD11b and CD66b were elevated in activated neutrophils; both were higher in severe psoriasis patients compared to moderate psoriasis patients and normal controls; the levels were normal in patients receiving biologic therapy (b).

Figure 4. ***Biologic therapy decreases monocyte activity***

(a) Monocytes count was not different among the five groups. Cultured monocytes were firstly gated morphologically and then gated by CD14 and CD16 staining. The percentage of $CD14^{high}$ activated monocytes was increased in patients with severe psoriasis compared to those with moderate psoriasis and normal controls; was significantly decreased in psoriasis patients receiving biologic therapy (b).

Figure 5. ***The correlation between PASI score and the activity of neutrophils or monocytes***

415 The MFI of CD62L (a) and CD66b (c) levels; the activation markers of neutrophil was
416 significantly correlated with PASI score at the time of blood collection. The correlation
417 between CD11b level and PASI did not reach the significance (b). The other hand, the
418 percentage of $CD14^{high}$ activated monocytes was significantly correlated with PASI
419 score (d). (•: severe patients, ▲: moderate patients, ✕: ustekinumab-treated patients;
420 □: infliximab -treated patients, +: normal control). X-axis shows PASI score and Y-axis
421 shows MFI levels of each activation marker (a-c), or the percentage of $CD14^{high}$
422 activated monocytes (d).

Figure 1. *Yamanaka et al.*

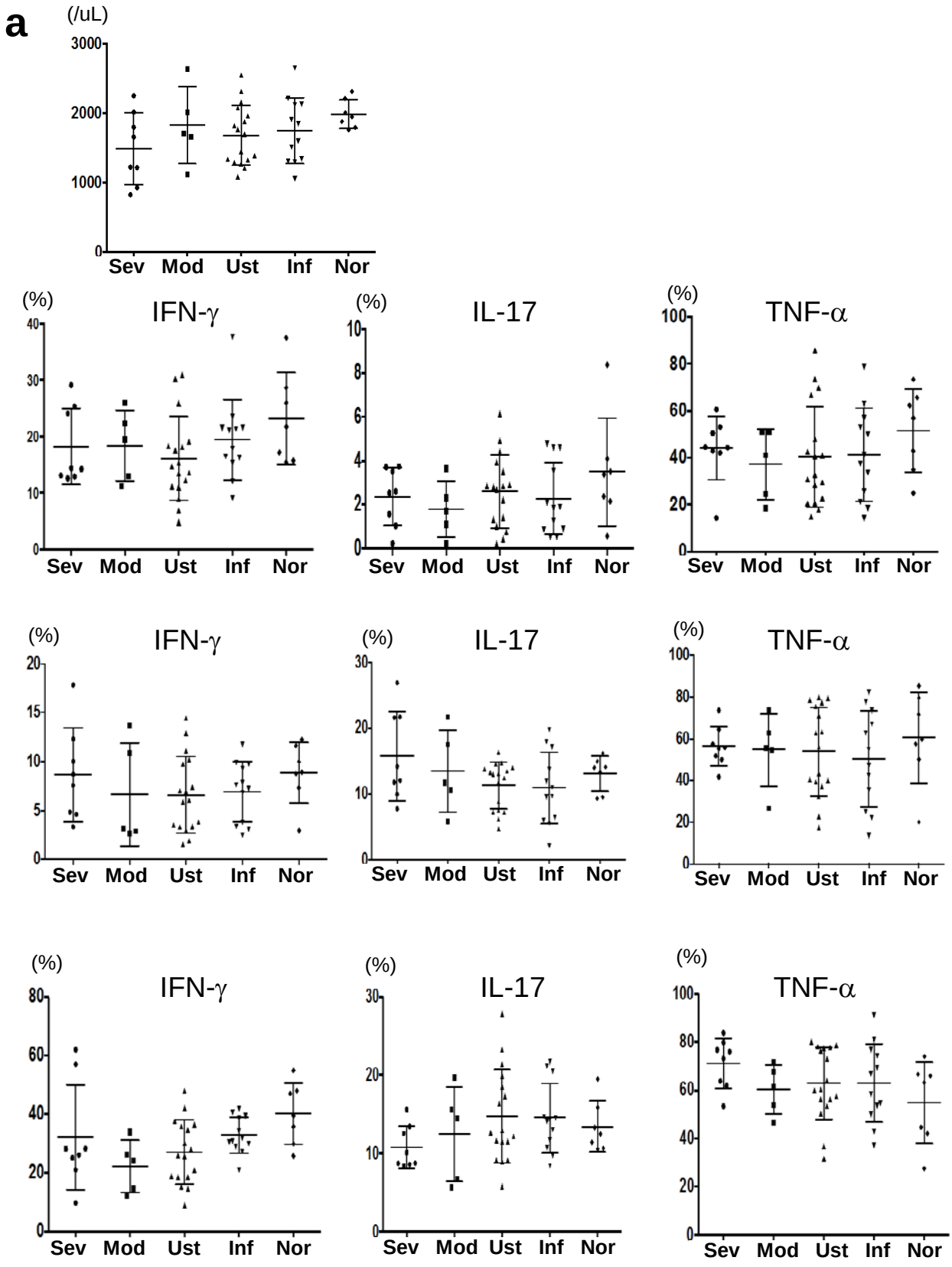
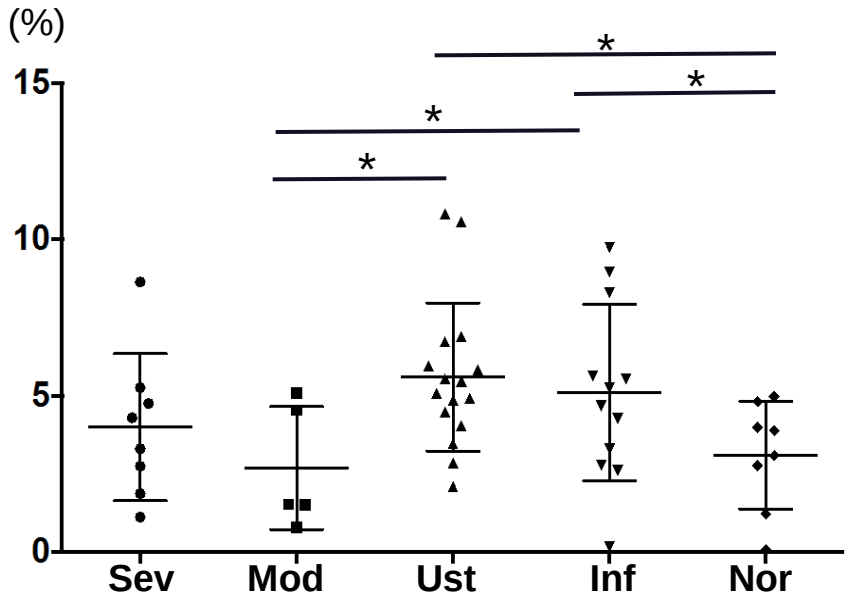


Figure 2. *Yamanaka et al.*



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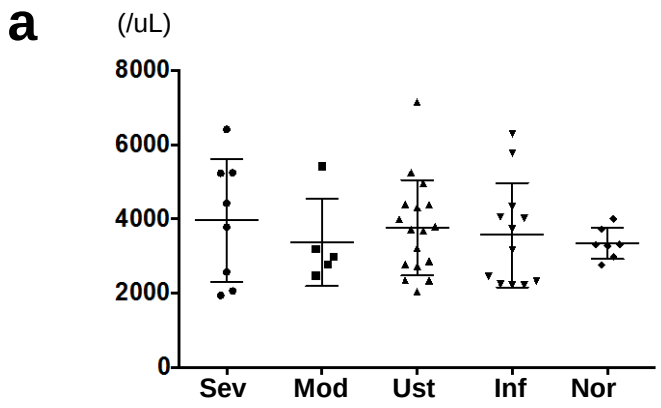

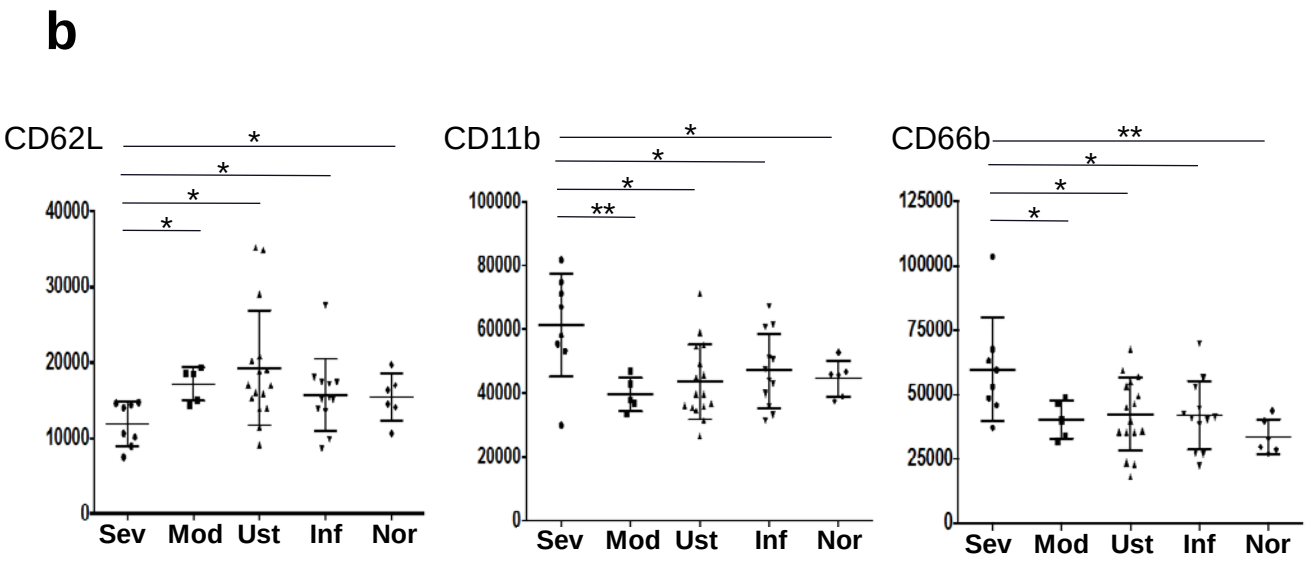

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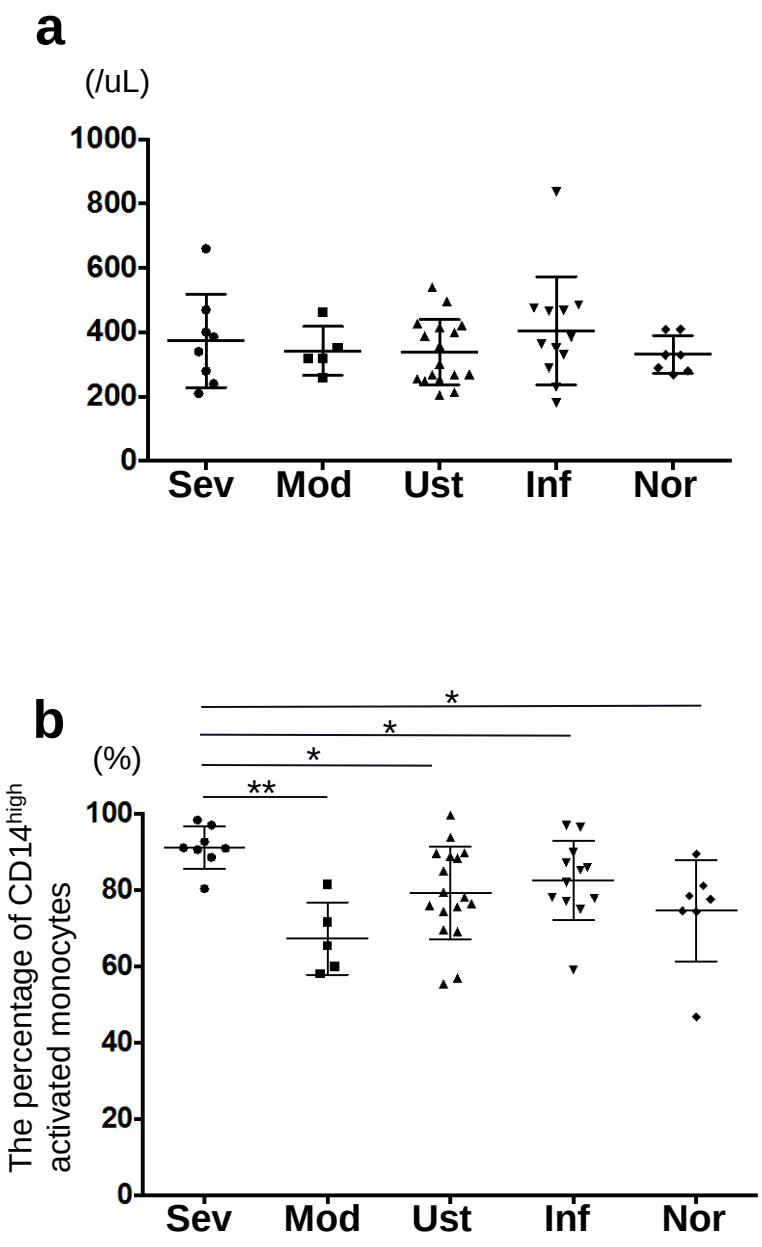

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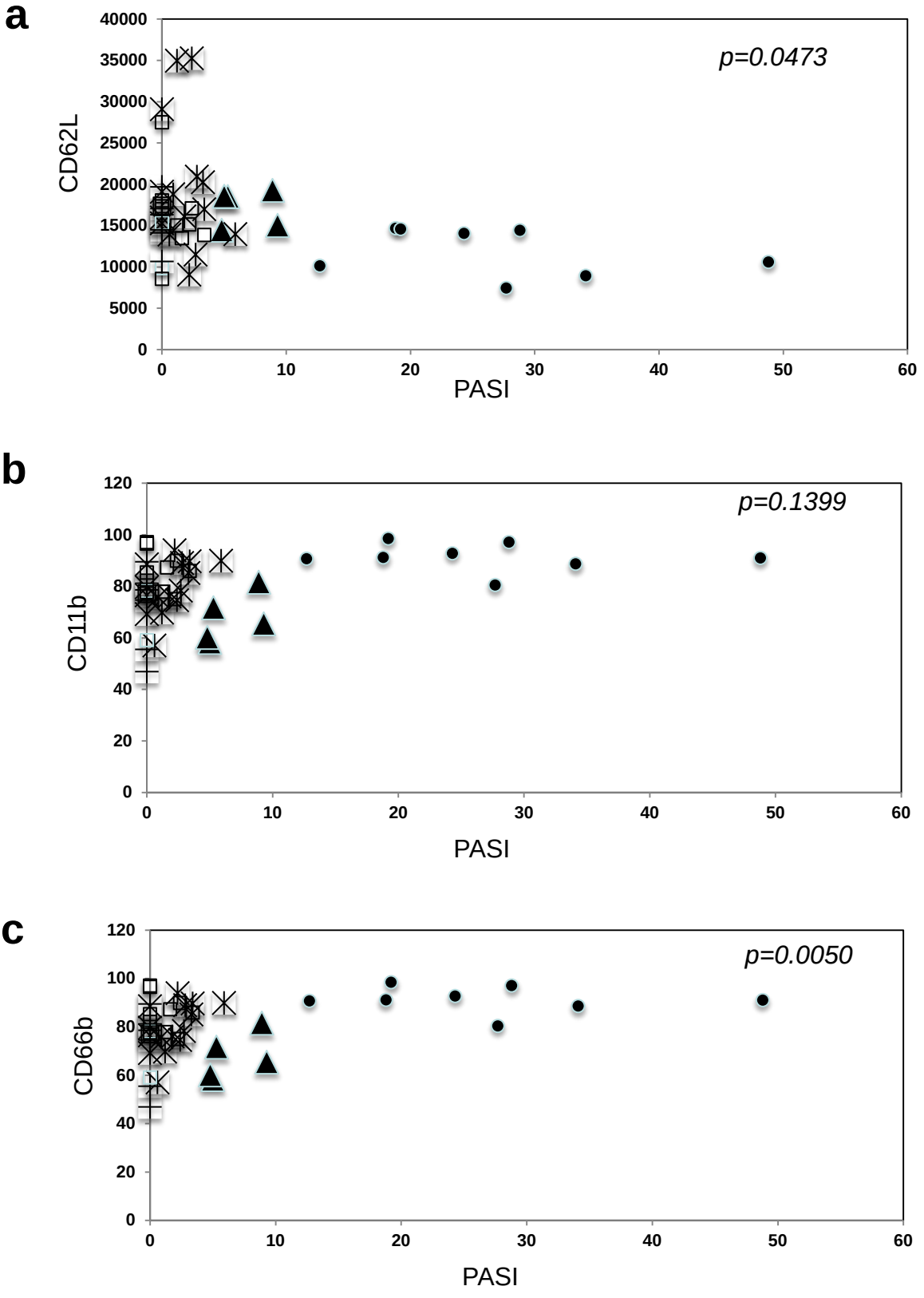

Figure 5. *Yamanaka et al.*

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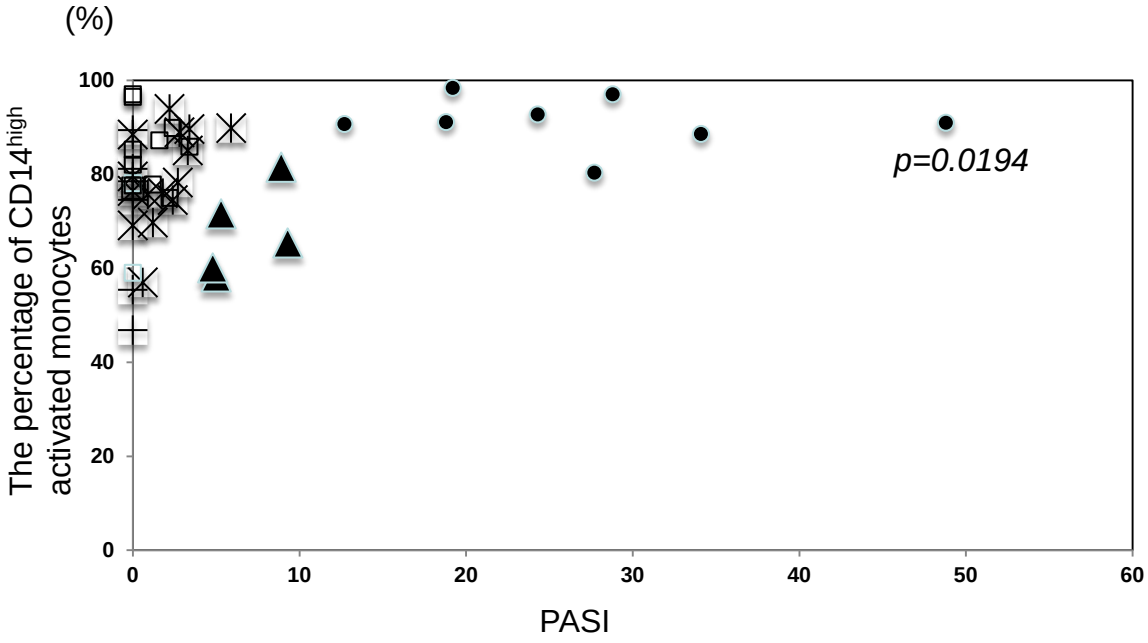

Table 1. *Yamanaka et al.*

	severe	mild	Stelara	Remicade	normal
n; total	8	5	17	12	7
n; male	5	3	12	8	4
n; female	3	2	5	4	3
age	47.8	42.1	50.1	40.8	42.2
Disease duration	7.1 years	4.4 years	6.2 years	5.1 years	none
PASI score	26.8	6.7	1.7	0.9	0