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Tocilizumab has no direct effect on cell lines infected with human T-cell leukemia virus type 1

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Abstract

Objective: It remains unclear whether human T-cell leukemia virus type 1 (HTLV-1) infection influences therapeutic responses in patients with rheumatic diseases and whether immunosuppressive treatments increase the risk of HTLV-1-related complications in HTLV-1 carriers with rheumatic diseases. We examined the effects of tocilizumab (TCZ), an interleukin (IL)-6 receptor antagonist, on two HTLV-1-infected T-cell lines (HCT-5 and MT-2) *in vitro*.

Methods: We evaluated production of cytokines and chemokines, expression of HTLV-1 associated genes, HTLV-1 proviral load (PVL), expression of HTLV-1 structural proteins, and apoptosis.

Results: There were no significant differences in cytokine and chemokine levels in the culture supernatants of HCT-5 and MT-2 cells treated with phosphate-buffered saline (PBS) or TCZ. No significant differences were detected in mRNA abundance of Tax or HBZ, PVL, expression of the HTLV-1 structural protein GAG, or apoptosis among HCT-5 and MT-2 cells treated with PBS or TCZ.

Conclusions: TCZ had no effect the cytokine profiles, HTLV-1 gene and protein expression, PVL, or apoptosis in HTLV-1-infected T-cell lines. Thus, TCZ treatment has no effect on HTLV-1 infection *in vitro*.

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Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection modifies the physiology of infected cells and induces inflammatory responses.[1] Therefore, it is clinically important to understand whether HTLV-1 infection influences therapeutic response in patients with rheumatic diseases and to assess whether immunosuppressive treatment increases risk of HTLV-1-related complications in HTLV-1 carriers with rheumatic diseases.

HCT-5 is an HTLV-1-infected T-cell line. We previously observed a time-dependent increase in levels of various cytokines and chemokines [interleukin (IL)-6, IL-8, inducible protein-10, macrophage-derived chemokine, macrophage inflammatory protein-1α, regulated on activation, normal T-cell expressed and secreted (RANTES), intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule-1, tumor necrosis factor (TNF)-α, interferon-γ and granulocyte macrophage colony stimulating factor (GM-CSF)] in culture supernatants from HCT-5 cells without any stimulation.[2] MT-2 is another HTLV-1-infected T-cell line[3] that spontaneously secretes various cytokines such as IL-6, IL-10, and TNF-α.[4] Our previous *in vitro* study suggested that treatment with TNF inhibitors had no effect on the HTLV-1-infected cell line HCT-5.[2]

Tocilizumab (TCZ) is an IL-6 receptor antagonist approved for the treatment of patients with rheumatoid arthritis (RA). In a clinical case report, a patient with RA developed adult T-cell leukemia/lymphoma (ATL) during long-term TCZ treatment.[5] In the present study, we examined the effects of TCZ on the HTLV-1-infected T-cell lines, HCT-5 and MT-2, *in vitro*.

Materials and methods

Cell lines

The HTLV-1-infected T-cell line, HCT-5, was previously established in our lab and was derived from cells isolated from the cerebrospinal fluid of a patient with HTLV-1-associated myelopathy (HAM).[6] This cell line is IL-2-dependent and was maintained in RPMI 1640 (Wako Pure Chemical Industries, Tokyo, Japan) containing 20% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific) supplemented with 100 U/mL of recombinant human IL-2 (kindly provided by Shionogi & Co., Osaka, Japan). A second HTLV-1-infected T-cell line., MT-2, was derived from normal human cord leukocytes from a healthy donor by co-cultivation with leukemic cells from a patient with ATL.[3] This cell line was maintained in RPMI 1640 containing 20% FBS and 1% penicillin/streptomycin. Jurkat cells (a human T-cell lymphoblast-like cell line) were used as a control. THP-1 cells (a human monocytic leukemia cell line) were used as a control to understand the effects of TCZ on each cytokine. These cell lines were maintained in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin. All cell lines were incubated in a humidified incubator

at 37°C with an atmosphere containing 5% carbon dioxide.

Reagents

TCZ (Centocor, Malvern, PA, USA) was always accompanied by 100 ng/mL of recombinant human IL-6 receptor α (PeproTech, Rocky Hill, NJ, USA) when added to cultures. Lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) was used to stimulate cytokine production in THP-1 cells.

IL-6 receptor analysis

We used flow cytometry to examine the cell surface expression of IL-6 receptor using phycoerythrin (PE)-conjugated anti-CD126 human monoclonal antibodies (BioLegend, San Diego, CA, USA). PE-conjugated mouse IgG1 was used as an isotype control (BioLegend). Flow cytometry was performed using a FlowSight Imaging Flow Cytometer (Merck-Millipore, Darmstadt, Germany).

Phosphorylation analysis of signal transducer and activator of transcription 3 (STAT3)

Flow cytometry was used to assess the intracellular phosphorylation of STAT3 using PE-conjugated anti-phosphorylated STAT3 (Tyr705) monoclonal antibodies (BioLegend). PE-conjugated mouse IgG1 was used as an isotype control. Intracellular phosphorylation was analyzed according to the manufacturer's instructions.

Cytokine enzyme-linked immunosorbent assays (ELISAs)

We used cytokine ELISAs to measure levels of GM-CSF, TNF-α, sICAM-1/CD54 and CCR5/RANTES in the culture supernatants of HCT-5, MT-2, and Jurkat cells treated with TCZ. GM-CSF, TNF-α, sICAM-1/CD54 and CCR5/RANTES Quantikine ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Levels of each cytokine/chemokine were determined according to the manufacturer's instructions. The optical densities were measured at 450 nm and 560 nm.

RNA extraction and quantitative reverse transcription PCR. We extracted total RNA from HCT-5, MT-2, and Jurkat cells using a Kingfisher Pure RNA Blood kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA was reverse transcribed to generate cDNA using a SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with 1 μg of total RNA at 25°C for 10 minutes, 42°C for 60 minutes and 85°C for 5 minutes in a final volume of 20 μL.

Abundance of Tax, HTLV-1 bZIP factor (HBZ) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA within cDNA was assessed using the LightCycler 480 probes Master Mix (Roche Diagnostics, Mannheim, Germany) and a LightCycler480 PCR System (Roche Diagnostics).

After 50 cycles, the absolute abundance of Tax, HBZ and GAPDH mRNA was interpolated from standard curves generated by the dilution method using pCR2.1-TOPO vectors (Life Technologies, Tokyo, Japan) containing Tax, HBZ and GAPDH inserts. GAPDH was used as an internal control for each sample to normalize the results and account for variability in the concentration and integrity of RNA and cDNA.

HTLV-I proviral load

We extracted genomic DNA from cells using Qiagen DNA Blood Mini kits (Qiagen, Crawley, UK). Quantitative polymerase chain reaction (qPCR) detection of

HTLV-1 was performed as described previously.[7–10] Briefly, primers were designed in the pX region and the template abundance was 30 ng per reaction. PVL was quantified using the Tax primer and probe. PVL was normalized using β-globin as a reference and presented as a percentage.

Immunofluorescence

HCT-5 and MT-2 cells were fixed for 10 minutes in phosphate-buffered saline (PBS) containing 4% paraformaldehyde at 4°C and immersed in methanol at −20°C for 10 minutes. After blocking in PBS containing 5% normal horse serum, the cells were incubated with diluted primary antibodies (10 μg/mL) for 1 hour at room temperature. Subsequently, cells were incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibodies supplemented with Hoechst dye 33258 for nuclear staining. After washing in PBS, the cells were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and scanned using a BIOREVO BZ-X710 fluorescence microscope (Keyence, Tokyo, Japan).

The following primary antibodies were used: mouse IgG1 as an isotype control (BD Biosciences, San Jose, CA, USA), monoclonal mouse anti-HTLV-1 p19 antibody (Chemicon, Hofheim, Germany), and monoclonal mouse anti-HTLV-1 p28 antibody (Chemicon).

Assessment of apoptosis

Apoptotic DNA was detected using an apoptotic DNA Ladder Kit (Sigma-Aldrich). Briefly, cultured HCT-5 and MT-2 cells were lysed and mixed with isopropanol in six-well plates. The plates were washed with PBS and prewarmed elution buffer was applied to extract DNA. The samples were applied to a 1% agarose gel containing ethidium bromide in 1× TBE buffer (90 mM Tris, 90 mM borate, 2 mM ethylenediaminetetraacetic acid) for DNA ladder detection. Apoptotic U937 cells in the kit were used as a positive control.

Annexin V staining

Apoptosis was evaluated by staining with propidium iodide (PI) (MBL, Nagoya, Japan) and PE-conjugated annexin V.[11] After washing with PBS, cells were stained with PI and PE-conjugated annexin V for 15 minutes in ambient air. Flow cytometry was performed using a FlowSight Imaging Flow Cytometer (Merck-Millipore). PI-negative and annexin V-positive cells were defined as apoptotic cells. The overall percentage of apoptotic cells was calculated.

Statistical analysis

We used Student's t-tests to assess differences in levels of GM-CSF, TNF-α, sICAM-1/CD54, CCR5/RANTES, Tax, HBZ, and PVL as well as in the percentage of apoptotic cells. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using JMP Statistical Software, version 14 (SAS Institute, Cary, NC, USA).

Results

Expression of IL-6 receptor on unstimulated THP-1, Jurkat, HCT-5, and MT-2 cells

Flow cytometry was used to assess the cell surface expression of IL-6 receptor on each cell line (Figures 1a–d). Staining by an anti-IL-6 receptor antibody, but not by an isotype control antibody, was observed on THP-1 and MT-2 cells, but not on Jurkat and HCT-5 cells. To ensure that TCZ would act uniformly on each cell line, TCZ was accompanied by recombinant

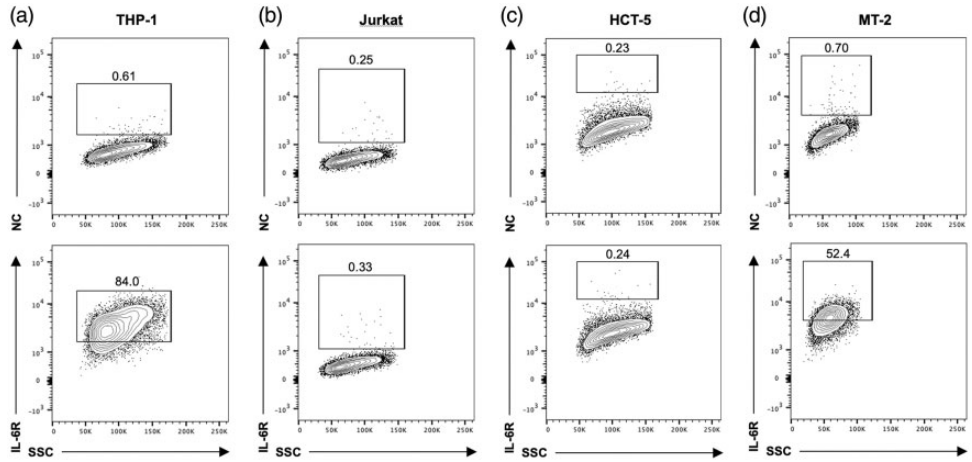

Figure 1. Expression of IL-6 receptor in unstimulated cells. Expression of IL-6 receptor (IL-6R) on unstimulated THP-1 (a), Jurkat (b), HCT-5 (c), and MT-2 (d) cells was assessed and compared with staining of an isotype control antibody.
NC, negative (isotype) control; SSC, side scatter.

human IL-6 receptor when added to cultures as previously described.[12]

Phosphorylation of STAT3 in HCT-5 and MT-2 cells

Flow cytometry was used to assess intracellular phosphorylation of STAT3 in HCT-5 and MT-2 cells (Supplementary Fig. 1A–D). Activation of STAT3 was observed in MT-2 cells, but only slightly in HCT-5 cells. In both cell lines, intracellular STAT3 was slightly further activated following the addition of IL-6 receptor.

No changes were observed in the cytokine/chemokine levels of HCT-5 and MT-2 cells treated with TCZ

Initially, we examined how TCZ affected levels of cytokines and chemokines in the culture supernatants of THP-1 cells. We measured levels of GM-CSF, TNF-α, ICAM-1 and RANTES in the culture supernatants of THP-1 cells by ELISA 48 hours following stimulation with LPS (1.0 μg/mL) and TCZ. Production of GM-CSF and ICAM-1 was significantly inhibited by TCZ. There was no significant decrease in the levels of TNF-α and RANTES in TCZ-treated cells (Figures 2a–d).

Next, we examined changes in cytokine and chemokine levels in the culture supernatants of HCT-5, MT-2, and Jurkat cells. We used ELISA to measure levels of GM-CSF, TNF-α, ICAM-1 and RANTES 48 hours after the addition of PBS or TCZ. No significant differences in cytokine or chemokine levels were observed in the culture supernatants of HCT-5 (Figures 2e–h) or MT-2 (Figure 2i–l) cells treated with PBS or TCZ. TNF-α was not detected in MT-2 cell supernatants. No differences in cytokine or chemokine levels in Jurkat cell supernatants were observed (data not shown).

No changes were observed in HTLV-1-related mRNAs or PVL in HCT-5 and MT-2 cells treated with TCZ

To examine changes in HTLV-1-related gene expression following administration of TCZ, we measured mRNA levels of Tax and HBZ in HCT-5, MT-2, and

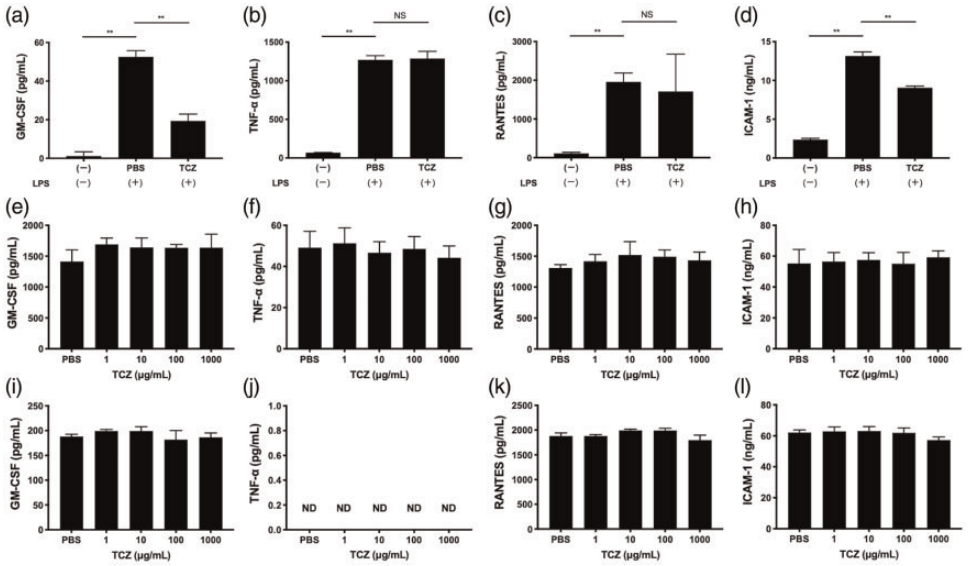

Figure 2. Cytokine/chemokine levels in cells treated with tocilizumab (TCZ). THP-1 cells were stimulated with lipopolysaccharide (LPS) (1 μg/mL) and either phosphate-buffered saline (PBS) or TCZ (1000 μg/mL) to assess the effects of TCZ. Levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) (a), tumor necrosis factor (TNF)-α (b), regulated on activation, normal T-cell expressed and secreted (RANTES) (c) and intercellular adhesion molecule (ICAM)-1 (d) were measured in culture supernatants (three independent experiments; $^{**}P < 0.01$, Student's t-test) 48 hours after stimulation. Levels of GM-CSF (e and i, HCT-5 and MT-2), TNF-α (f and j, HCT-5 and MT-2), RANTES (g and k, HCT-5 and MT-2), and ICAM-1 (h and l, HCT-5 and MT-2) in the culture supernatants of HCT-5 and MT-2 cells without LPS stimulation were measured 48 hours after the addition of PBS or different concentrations of TCZ (three independent experiments). Error bars represent standard deviations.
ND: not detected.

Jurkat cells 48 hours after the addition of PBS or TCZ. These are important genes for the expression of viral proteins and proliferation of HTLV-1.[13,14] No significant differences were observed in mRNA abundance of Tax or HBZ among HCT-5 (Figures 3a–b) or MT-2 (Figures 3d–e) cells treated with PBS or TCZ. No Tax or HBZ mRNA was detected in control Jurkat cells (data not shown).

We also measured PVLs in HCT-5, MT-2, and Jurkat cells 48 hours after addition of PBS or TCZ. No significant differences were observed in PVLs in HCT-5 (Figure 3c) and MT-2 (Figure 3f) cells treated with PBS or TCZ. There was no increase observed in the PVLs of control Jurkat cells (data not shown).

No changes were observed in GAG expression in HCT-5 and MT-2 cells treated with TCZ

To examine changes in HTLV-1 structural protein GAG expression following administration of TCZ, we performed HTLV-1 p19 and p28 staining 48 hours after the addition of PBS or TCZ. No differences in GAG expression were observed (Figure 4a).

Assessment of apoptosis in HCT-5 and MT-2 cells treated with TCZ

To assess apoptosis in HCT-5 and MT-2 cells treated with PBS or TCZ, we used apoptotic DNA ladder detection. No apoptotic

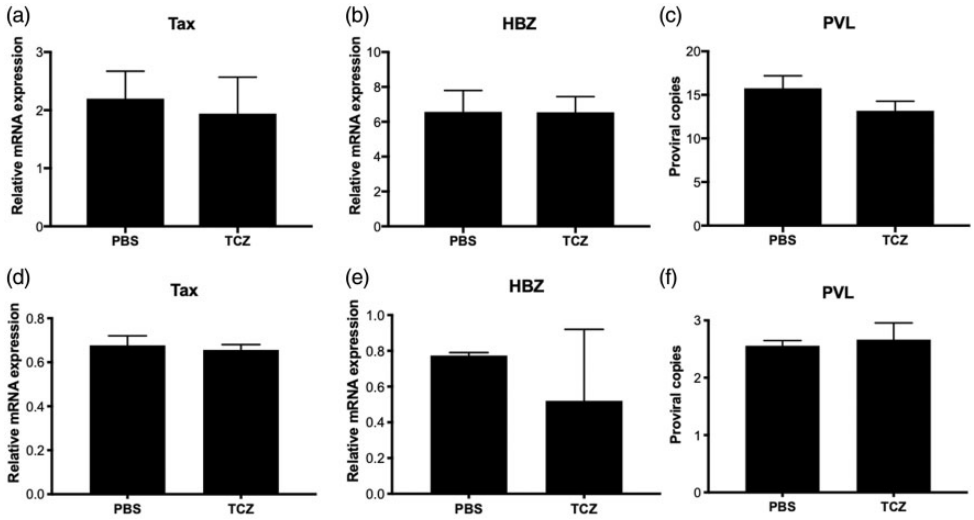

Figure 3. Human T-cell leukemia virus type I (HTLV-1)-related mRNAs and proviral loads (PVLs) in cells treated with tocilizumab (TCZ). Abundance of Tax mRNA (a and d, HCT-5 and MT-2) and HBZ mRNA (b and e, HCT-5 and MT-2) and PVLs (c and f, HCT-5 and MT-2) 48 hours after addition of phosphate-buffered saline (PBS) or TCZ (1000 μg/mL) (three independent experiments) was determined. Error bars represent standard deviations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-globin were used as housekeeping genes for normalization of mRNA and PVLs, respectively.

DNA ladders were detected 48 hours after the addition of PBS or TCZ (Figure 4b). In addition, we performed annexin V staining of HCT-5 and MT-2 cells 48 hours after the addition of PBS or TCZ (Figure 4c). There were no significant differences in the percentages of apoptotic HCT-5 and MT-2 cells treated with PBS or TCZ.

Discussion

The present *in vitro* study showed that TCZ had no effect on cytokine profiles, HTLV-1 gene and protein expression, PVL, or apoptosis in HTLV-1-infected T-cell lines.

A high baseline HTLV-1 PVL is an independent risk factor for development of ATL during treatment of HTLV-1 carriers with other diseases.[7] A recent study found no significant differences in PVL levels among RA patients receiving different types of biological therapies.[15] In the present study, we found that PVL, apoptosis, and mRNA abundance of Tax and HBZ (important genes for apoptosis resistance and proliferation of ATL cells, respectively) were not significantly changed following TCZ treatment. This finding suggests that TCZ treatment may not increase the risk of ATL development among HTLV-1 carriers.

HTLV-1 infection may lead to changes in inflammatory networks in patients with rheumatic disease. A previous study demonstrated elevated production of multiple cytokines and chemokines in the supernatants of HCT-5 and MT-2 cells,[2,4] and patients with HAM/tropical spastic paraparesis (HAM/TSP) had high circulating levels of TNF-α.[16] Therefore, changes in therapeutic responses related to HTLV-1 infection and HTLV-1-related adverse events in HTLV-1 carriers with rheumatic diseases are of clinical concern.

In a clinical case report, a patient with RA experienced exacerbation of HAM/TSP symptoms after both TCZ and abatacept

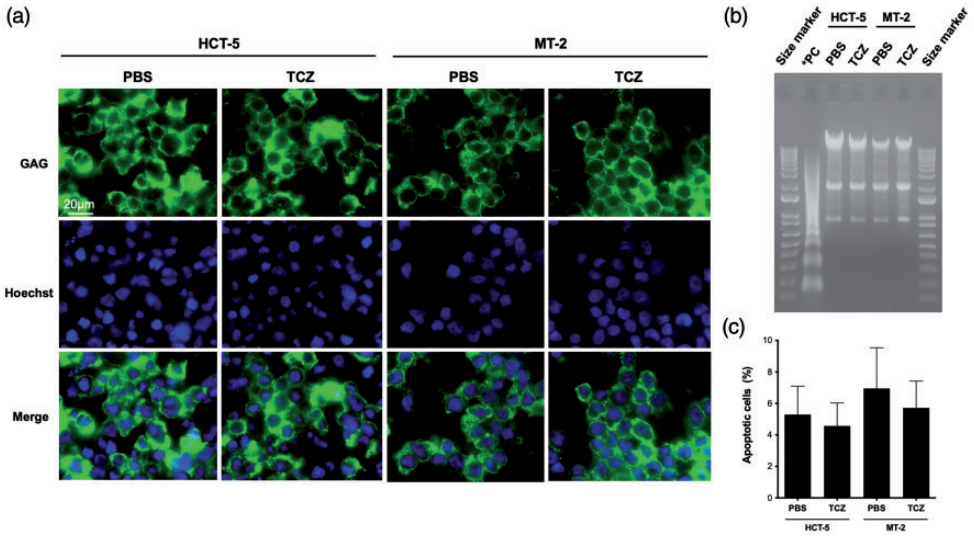

Figure 4. GAG expression and apoptosis in cells treated with tocilizumab (TCZ). (a) After HCT-5 and MT-2 cells were stimulated with phosphate-buffered saline (PBS) or TCZ (1000 μg/mL) for 48 hours, they were treated with mouse monoclonal anti-HTLV-I p19 and p28 antibodies followed by fluorescein isothiocyanate-labelled donkey anti-mouse secondary antibody. Hoechst 33258 was used for counterstaining of the nucleus (merged view). Bar: 20 μm (three independent experiments). (b) After HCT-5 and MT-2 cells were stimulated with PBS or TCZ (1000 μg/mL) for 48 hours, the cells were subjected to an apoptotic DNA ladder experiment. PC: positive control. U937 cells were treated with camptothecin. (c) After HCT-5 and MT-2 cells were stimulated with PBS or TCZ (1000 μg/mL) for 48 hours, we evaluated the percentage of apoptotic HCT-5 and MT-2 cells, defined as negative for propidium iodide staining and positive for phycoerythrin-conjugated annexin V staining. Error bars represent standard deviations.

treatments.[17] In addition, our previous study showed that the efficacy of TNF inhibitors was attenuated in anti-HTLV-1 antibody-positive patients with RA.[18] In the present study, we showed that TCZ had no effect on cytokine profiles, suggesting that TCZ has no effect on therapeutic response or on HTLV-1-related adverse events in HTLV-1 carriers with rheumatic disease.

This study had several limitations, several of which were shared with our previous study.[2] Because the cell lines used in this *in vitro* study were established from patients with HAM or ATL, the results do not necessarily reflect the clinical effects of TCZ therapy *in vivo*. It will be necessary to assess the efficacy and safety of TCZ therapy in HTLV-1 carriers with rheumatic diseases more extensively in the future.

Conclusion

Our results showed that TCZ had no effect on cytokine profiles, HTLV-1-related gene and protein expression, PVL, or apoptosis in the HTLV-1-infected T-cell lines HCT-5 and MT-2. Thus, TCZ treatment has no effect on HTLV-1 infection *in vitro*.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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Supplemental Material

Supplementary material for this article is available online.

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