Regular Article

Functional Mechanism(s) of the Inhibition of Disease Progression by Combination Treatment with Fingolimod Plus Pathogenic Antigen in a Glucose-6-phosphate Isomerase Peptide-Induced Arthritis Mouse Model

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We previously reported that combination treatment with fingolimod (FTY720) plus antigenic peptide of glucose-6-phosphate isomerase (residues 325–339) (GPI325–339) from the onset of symptoms significantly inhibited disease progression in a mouse model of GPI325–339-induced arthritis. In this study, we investigated the mechanism(s) involved. The model mice were treated from arthritis onset with FTY720 alone, GPI325–339 alone, or the combination of FTY720 plus GPI325–339. At the end of treatment, inguinal lymph nodes (LNs) were excised and examined histologically and in flow cytometry. Levels of apoptotic cells, programmed cell death-1-expressing CD4+forkhead box P3* nonregulatory T cells (non-Tregs), and cytotoxic T-lymphocyte antigen 4-expressing non-Tregs in inguinal LNs were markedly increased in the combination treatment group mice. Regulatory T cells (Tregs) were also increased. These results indicate that combination treatment with FTY720 plus GPI325–339 inhibits the progression of arthritis by inducing clonal deletion and anergy of pathogenic T cells and also by immune suppression via Tregs.

Key words fingolimod; FTY720; rheumatoid arthritis; pathogenic antigen; immune tolerance

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation and joint destruction. Biological agents provide effective therapeutic management of RA, but have various disadvantages, including induction of neutralizing antibodies and occurrence of relapse after discontinuation of the drug. Thus, a therapy able to induce complete remission is highly desirable. It has already been shown that intravenous administration (i.v.) of pathogenic antigens is effective for suppressing disease in several autoimmune animal models, including collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE). We also found that prophylactic administration of pathogenic antigens, that is, glucose-6-phosphate isomerase peptide (GPI325–339), significantly suppressed development of symptoms in GPI325–339-induced arthritis (an animal model of RA). However, although tolerance was easily induced in naive mice, primed mice were relatively resistant to the suppressive effect of pathogenic antigen. On the other hand, combination treatment with fingolimod (FTY720) plus GPI325–339 from the onset of symptoms efficiently suppressed progression of symptoms of GPI325–339-induced arthritis.

FTY720 is a synthetic structural analogue of myriocin (ISP-1), a compound from Isaria sinclairii. FTY720 has been reported to be effective not only in preclinical transplantation models, but also in various animal models of immunological diseases, including multiple sclerosis, myasthenia gravis, atopic dermatitis, and type 1 diabetes mellitus. The mechanism of action of FTY720 differs from that of established immunosuppressants, such as tacrolimus hydrate and cyclosporine. FTY720 is converted in vivo by sphingosine kinase 2 to FTY720 monophosphate (FTY720-P), which is the active form of the drug. FTY720-P acts as a high-affinity agonist for four sphingosine 1-phosphate receptors (S1P1, S1P3, S1P4, and S1P5). FTY720-P induces long-term down-regulation of S1P1 on lymphocytes and suppresses immune response by sequestering circulating mature lymphocytes from blood and peripheral tissues to secondary lymphoid tissues and thymus.

In the present study, we examined the functional mechanism(s) of disease suppression by combination treatment with FTY720 plus pathogenic antigen in the GPI325–339-induced arthritis mouse model.

MATERIALS AND METHODS

Animals and Ethics DBA/1 mice bred under specific pathogen-free conditions were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were given γ-ray-irradiated food (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and distilled water for injection (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). This study was performed according to the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and the protocol was approved by the Institutional Animal Care Committee of Setsunan University (approved number: 13-09-16-02-S-270, 13-02-16-02-S-448 and 14-07-16-02-S-163). Throughout the experimental procedures, every effort was made to minimize the number of animals used and their suffering.

Agents and Antibodies 2-Amino-2-[4-octylphenyl]-ethylpropane-1,3-diol hydrochloride (fingolimod; FTY720)
was kindly provided by Yoshitomi Pharmaceutical Industries, Ltd. (Present company name: Mitsubishi Tanabe Pharma Corporation, Osaka, Japan). Peptide 325(IWYINCFGCETHAML)339 of human glucose-6-phosphate isomerase (GPI₃₂₅₋₃₃₉) was purchased from Eurofins Genomics K. K. (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 monoclonal antibody (mAb; clone: GK1.5), Allophycocyanin/cyanine 7 (APC/Cy7)-conjugated anti-mouse CD4 mAb (clone: GK1.5) and phycoerythrin/cyanine 7 (PE/Cy7)-conjugated anti-mouse CD25 mAb (clone: PC61) were purchased from BioLegend, Inc. (CA, U.S.A.). APC-conjugated anti-mouse CD279 (programmed death-1; PD-1) mAb (clone: J43) and PE-conjugated anti-mouse Foxp3 mAb (clone: MF23) were purchased from BD Biosciences (CA, U.S.A.). APC-conjugated anti-mouse CD152 (cytotoxic T-lymphocyte antigen 4; CTLA-4) mAb (clone: UC10-4F10-11) was purchased from Tonbo Biosciences (CA, U.S.A.).

**Induction of GPI₃₂₅₋₃₃₉-Induced Arthritis**

DBA/1 mice (7–8-week-old males) were immunized by intracutaneous injection of GPI₃₂₅₋₃₃₉ (10 µg) with Freund’s complete adjuvant containing Mycobacterium tuberculosis H37Ra (BD Biosciences) at the base of the tail on day 0. Pertussis toxin (200 ng; PT; EMD Chemicals, Inc., CA, U.S.A.) was injected intraperitoneally on days 0 and 2, 5,10

**Administration Schedules of Test Drugs in GPI₃₂₅₋₃₃₉-Induced Arthritis Mice**

Administration of GPI₃₂₅₋₃₃₉ with FTY720 from the time of onset (when erythema and mild swelling to the tarsals or ankle joint was observed) for five days efficiently suppressed progression of symptoms of GPI₃₂₅₋₃₃₉-induced arthritis.5) To examine the functional mechanism(s) of disease suppression by combination treatment with FTY720 plus pathogenic antigen, GPI₃₂₅₋₃₃₉-induced arthritis mice were divided into four groups, and treated from the day of onset (days 9, 10) for five days. The placebo group was given water (orally, once a day) plus phosphate-buffered saline (PBS)(i.v., once a day). The FTY720 group was given FTY720 in water (1.0 mg/kg, orally, once a day) plus PBS (i.v., once a day). The GPI₃₂₅₋₃₃₉ group was given water (orally, once a day) plus GPI₃₂₅₋₃₃₉ in PBS (10 µg/mouse, i.v., once a day). The FTY720 plus GPI₃₂₅₋₃₃₉ combination treatment group was given FTY720 in water (1.0 mg/kg, orally, once a day) plus GPI₃₂₅₋₃₃₉ in PBS (10 µg/mouse, i.v., once a day). At the end of treatment, and were initially stained with fluorescence-labeled anti-mouse CD4 mAb in PBS containing 2% fetal calf serum (FCS) for 20 min at 4°C. Subsequently, the cells were stained with Annexin V and propidium iodide (PI) using the FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, Inc.) in accordance with the manufacturer’s instructions. Flow-cytometric analysis was performed with a BD FACS Aria II (BD Biosciences).

**Statistical Analysis**

The mean and standard deviation (S.D.) were calculated by IBM SPSS Statistics Version 22 software. The statistical significance of differences was evaluated by one-way ANOVA followed by Duncan’s test. A p<0.05 was considered statistically significant.

**RESULTS**

**TUNEL Assay in Inguinal Lymph Nodes**

The efficacy of combination treatment with FTY720 plus pathogenic antigen was already reported. We considered that the combination treatment might efficiently induce immune tolerance by sequestering circulating pathogenic lymphocytes in secondary lymphoid tissues, and the tolerance might be induced by apoptosis and/or anergy of pathogenic T cells and Tregs. To examine the effect of FTY720 plus pathogenic antigen on apoptotic cell death by histochemistry, GPI₃₂₅₋₃₃₉-induced arthritis mice were divided into four groups (FTY720 alone, GPI₃₂₅₋₃₃₉ alone, FTY720 plus GPI₃₂₅₋₃₃₉ and placebo groups), and were treated from the day of onset for 5d. Inguinal LNs were collected, and sections were stained with TUNEL method. TUNEL method has been used for the detection of apoptosis. The number of TUNEL− cells per unit area in inguinal LNs was markedly increased in the FTY720 plus GPI₃₂₅₋₃₃₉ group (mean±S.D.: 199±16.8) compared with the placebo (140±31.6) and FTY720 (138±19.3) groups (Fig. 1). GPI₃₂₅₋₃₃₉ alone tended to increase the number of TUNEL− cells per unit area (172±22.0), but was less effective than FTY720 plus GPI₃₂₅₋₃₃₉.

**Percentage of Annexin V− Cells in CD4⁺PI− Cells in Inguinal Lymph Nodes**

To examine the effect of FTY720 plus pathogenic antigen on apoptotic cell death by flow cytometry, the cells were collected from inguinal LNs of treated mice at the end of treatment, and stained with fluorescence-labeled anti-mouse CD4 mAb, Annexin V and PI. The percentage of Annexin V− cells in CD4⁺PI− cells was analyzed by flow cytometry. Annexin V is a protein that has a high affinity for phosphatidylinerine (PS). PS is predominantly localized in the inner membrane leaflet of viable cells, but is exposed at
the outer membrane leaflet during apoptosis. PI is selectively binds to nucleic acids. It is excluded from viable cells with a normal membrane, but is taken into dead cells with a broken membrane. Thus, Annexin V+PI- cells are in late apoptosis or necrosis, whereas Annexin V-PI+ cells are in early apoptosis. The percentage of Annexin V+ cells in CD4+PI- cells was significantly increased in the FTY720 plus GPI1325±339 (21.2±4.3) and GPI1325±339 alone (20.4±5.0) groups compared with the placebo (14.6±3.3) group (Fig. 2).

Percentage of CTLA-4-Expressing Non-regulatory T Cells in Inguinal Lymph Nodes CTLA-4, an inhibitory receptor for CD80/86, is expressed on activated T cells, and serves to attenuate T cell activation. CTLA-4 is an important molecule for anergy induction. To examine the pattern of CTLA-4 expression on non-regulatory T cells (non-Tregs; CD4+Foxp3- T cells), cells were collected from inguinal LNs of treated mice at the end of treatment and analyzed by flow cytometry. The percentage of CTLA-4+ cells in CD4+Foxp3- T cells was significantly increased in the FTY720 plus GPI1325±339 group (3.7±0.5) compared with the placebo (2.2±0.6) and FTY720 (2.2±0.4) groups (Fig. 3). The GPI1325±339 group (3.0±1.0) showed a moderate increase, but it was not statistically significant.

Percentage of PD-1-Expressing Non-regulatory T Cells in Inguinal Lymph Nodes PD-1, an inhibitory receptor for PD-1 ligand, is expressed on activated mouse T and B cells, and is involved in the maintenance of peripheral self-tolerance. PD-1 pathway plays a critical role in the induction and maintaining T cell anergy. To examine the pattern of PD-1 expression on non-Tregs, cells from inguinal LNs were analyzed by flow cytometry. The percentage of PD-1+ cells in CD4+Foxp3- T cells was significantly increased in the FTY720 plus GPI1325±339 group (5.6±2.0) compared with the placebo (2.1±0.5) and FTY720 (2.7±0.3) groups (Fig. 4). The GPI1325±339 group also showed an increase (4.0±1.3), but it was less than in the FTY720 plus GPI1325±339 group.

Percentage of Regulatory T Cells in Inguinal Lymph Nodes Regulatory T cells (Tregs) play an important role in inducing immune tolerance. To examine the percentage of Tregs (CD4+Foxp3+ T cells), cells were collected from inguinal LNs at the end of treatment and analyzed by flow cytometry. The percentage of Foxp3+CD25+ cells in CD4+ T cells was significantly increased in the FTY720 plus GPI1325±339 group (17.7±1.3) compared with the placebo (13.6±0.8), FTY720 (15.1±1.1) and GPI1325±339 (12.7±1.6) groups (Fig. 5).

FTY-4 is required for Tregs immune suppression. Therefore, we next examined the percentage of Foxp3+ CTLA-4+ cells
in CD4$^+$ T cells. The percentage of Foxp3$^+$ CTLA-4$^+$ cells in CD4$^+$ T cells was significantly increased in the FTY720 plus GPI$_{325-339}$ (7.0±0.6) and FTY720 groups (6.8±1.3) compared with the GPI$_{325-339}$ (5.0±0.7) group (Fig. 6). Although the both groups tended to increase compared with the placebo (5.9±0.6) group, the differences were not statistically significant.

DISCUSSION

We previously reported that combination treatment with FTY720 plus GPI$_{325-339}$ from the onset of symptoms significantly suppressed progression of symptoms compared with FTY720 alone or pathogenic antigen alone in a mouse model of GPI$_{325-339}$-induced arthritis. It has been shown that, after i.v. administration of antigen, antigen-specific cells are rapidly accumulated in the paracortical regions of all lymph nodes, and most of the cells then rapidly disappear, leaving behind a population that is hyporesponsive to antigenic stimulation. In the case of combination with FTY720, FTY720 efficiently induces sequestration of circulating antigen-specific lymphocytes in secondary lymphoid tissues. In the present study, the functional mechanism(s) of the combination treatment with FTY720 plus pathogenic antigen was examined, focusing on induction of apoptosis and anergy of pathogenic T cells and modulation of Tregs.

Regarding the mechanism of i.v. tolerance, it has been reported that induction of apoptosis-mediated clonal deletion of autoantigen-reactive T cells together with anergy of remaining cells is a major mechanism for the improvement of EAE, an animal model of multiple sclerosis. In the present study, apoptotic cells were efficiently increased in the FTY720 plus GPI$_{325-339}$ group. Also, the GPI$_{325-339}$ group showed a moderate increase. Thus, i.v. administration of GPI$_{325-339}$ appears to induce clonal deletion of pathogenic lymphocytes (cf. apoptosis on Figs. 1, 2).

It is known that CTLA-4 and PD-1 receptors are negative regulators of T cell function. CTLA-4 is not expressed on resting T cells and appears only after T cell activation. CTLA-4 has significantly higher binding affinity for the B7 ligands (CD80 and/or CD86) on antigen-presenting cells than for CD28. Thus, CTLA-4 on effector T cells down-regulates T cell activation by competing with CD28. PD-1 is expressed on activated mouse T and B cells, and PD-1 ligand 1 (PD-L1) is expressed on mouse macrophages, dendritic cells (DCs), T and B cells, and so on. The PD-1–PD-L1 pathway inhibits T cell receptor-mediated lymphocyte proliferation and cytokine secretion. PD-1 is a more potent suppressor of CD3/
CD28-mediated changes in the T-cell transcriptional profile than is CTLA-4. In the present study, the ratio of both CTLA-4 and PD-1 in CD4+Foxp3+ T cells was increased in the FTY720 plus GPI325–339 group. Thus, in the case of combination treatment, hyporeactivity of pathogenic T cells might be efficiently induced in secondary lymphoid tissues via CTLA-4 signaling and/or the PD-1–PD-L1 pathway. It has been shown that S1P1–mTOR axis. Thus, activation of GPI325–339-specific T cells might be efficiently induced in secondary lymphoid tissues (Fig. 7). In conclusion, in the case of no treatment, activated-T lymphocytes are recruited from secondary lymphoid tissues by S1P signaling pathway, and symptoms of GPI325–339-induced arthritis are deteriorated. While, in the case of combination treatment with FTY720 plus GPI325–339, the circulating activated-T lymphocytes is sequestered in secondary lymphoid tissues by FTY720 and the cells are exposed large amount of GPI325–339. Accordingly, TUNEL+ cells, annexin V+ cells, CTLA-4 and/or PD-1 expressing pathogenic T cells are increased by vigorous antigenic stimulation. Thus, apoptosis and anergy of pathogenic antigen-specific T cells may be efficiently induced in secondary lymphoid tissues (Fig. 7). In addition, Tregs balance in secondary lymphoid tissues may be increased by vigorous antigenic stimulation. Thus, apoptosis and anergy of pathogenic antigen-specific T cells may be efficiently induced in secondary lymphoid tissues (Fig. 7).

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. Lancet. 2010;376:1094–1010.
2) Guamanovskaya ML, Myers LK, Rosloniec EF, Stuart JM, Kang AH. Intravenous tolerization with type II collagen induces interleukin-4 and interleukin-10-producing CD4+ T cells. Immunology, 97.
12) Tsuji T, Yoshida Y, Iwatsuki R, Inoue M, Fujita T, Kohno T. Thera-

10) Kohno T, Tsuji T, Hirayama K, Iwatsuki R, Hirose M, Watabe K,

4) Jiang Z, Li H, Fitzgerald DC, Zhang GX, Rostami A. MOG

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8) Brinkmann V, Pinschewer D, Chiba K, Feng L. FTY720: a novel

5) Yoshida Y, Tsuji T, Watanabe S, Matsushima A, Matsushima Y,

Fujita T. A novel immunomodulator, FTY720, prevents spontane-

ous autoimmunity in rats. I. FTY720 selectively decreases

the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J. Immunol., 160, 5037–5044 (1998).

17) Yanagawa Y, Sugahara K, Katoaka H, Kawaguchi T, Masubuchi Y,

Chiba K. FTY720, a novel immunosuppressant, induces sequestra-
tion of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. J. Immunol., 160, 5493–5499 (1998).

18) Iwanami K, Matsumoto I, Tanaka Y, Inoue A, Goto D, Ito S,

Tsutsuomi A, Sumida T. Arthritogenic T cell epitope in glucose-

6-phosphate isomerase-induced arthritis. Arthritis Res. Ther., 10, R130 (2008).

19) Auchincloss H, Turka LA. CTLA-4: not all costimulation is stimula-
tory. J. Immunol., 187, 3457–3458 (2011).

20) Perez VL, Van Parijs L, Buckians A, Zheng XX, Strom TB,

Abbas AK. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity, 6, 411–417 (1997).

21) Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsutbata T, Yagita H,

Hojio T. Expression of the PD-1 antigen on the surface of stimulat-

ced mouse T and B lymphocytes. Int. Immunol., 8, 765–772 (1996).

22) Fife BT, Guleria I, Gubbel B, Mager T, Tang Q, Bour-

Jordan H, Yagita H, Azuma M, Sayegh MH, Bluestone JA. Insulin-

induced remission in new-onset NOD mice is maintained by the PD-1/PD-L1 pathway. J. Exp. Med., 203, 2733–2747 (2006).

23) Kearney ER, Papenhausen A, Lob IVY, Jenkins MK. Visualization of peptide-speciﬁc T cell immunity and peripheral tolerance induction in vivo. Immunity, 1, 327–339 (1994).

24) Zhang GX, Liu TT, Ventura ES, Chen Y, Rostami A. Reversal of spontaneous progressive autoimmune encephalomyelitis by myelin basic protein-induced clonal deletion. Autoimmunity, 31, 219–227 (1999).

25) Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu. Rev. Immunol., 26, 677–704 (2008).

26) Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, Azuma M, Yagita H. Expression of programmed death 1 ligands by murine T cells and APC. J. Immunol., 169, 5538–5545 (2002).

27) Freeman GJ. Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura F, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR, Hojio T. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp. Med., 192, 1027–1034 (2000).

28) Passy RV, Chemnitz JM, Frauwirth KA, Lanfranco AK, Braunstein J, Kobayashi SV, Linsley PS, Thompson CB, Riley JL. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol. Cell. Biol., 25, 9543–9553 (2005).

29) Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell, 133, 755–757 (2008).

30) Ono M, Yaguchi H, Ohkura N, Kita bayashi I, Nagamura Y, No-

mura T, Miyachi Y, Tsukada T, Sakaguchi S. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. Nature, 446, 685–689 (2007).

31) Wu Y, Bode M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, Mathis D, Benoist C, Chen L, Rao A. FOXP3 controls regulatory T cell function through cooperation with NFA1. Cell, 126, 375–387 (2006).

32) Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fe-

hervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3 regulatory T cell function. Science, 322, 271–275 (2008).

33) Liu G, Yang K, Burns S, Shrestha S, Chi H. The SIP(1)–mTOR axis directs the reciprocal differentiation of Th(1) and Th(2) cells. Nat. Immunol., 11, 1047–1056 (2010).

34) Brinkmann V, Chen S, Feng L, Pinschewer D, Nikolova Z, Hof R, FTY720 alters lymphocyte homing and protects allografts without inducing general immunosuppression. Transplant. Proc., 33, 530–531 (2001).