東邦大学学術リポジトリ
Toho University Academic Repository

タイトル	Ibudilast Inhibits Th17 Cell Differentiation from Naïve Human T Cells
作成者（著者）	Masaru, Yanagihashi / Kiyokazu, Kawabe / Ken, Ikeda
公開者	The Medical Society of Toho University
発行日	2015.03
ISSN	21891990
掲載情報	Toho Journal of Medicine. 1(1). p.2-6.
資料種別	学術雑誌論文
内容記述	Original Article
著者版フラグ	publisher
メタデータのＵＲＬ	https://mylibrary.toho-u.ac.jp/webopac/TD72971324

Original Article

Ibudilast Inhibits Th17 Cell Differentiation from Naïve Human T Cells

Masaru Yanagihashi Kiyokazu Kawabe* and Ken Ikeda

Division of Neurology (Omori), Department of Internal Medicine, School of Medicine, Faculty of Medicine, Toho University

ABSTRACT

Background: T helper cells that secrete interleukin (IL)-17 (Th17 cells) are important in the pathogenesis of autoimmune diseases such as multiple sclerosis (MS). In addition, Th17 cells are associated with secondary inflammation induced by cerebral infarction. The nonselective phosphodiesterase inhibitor ibudilast ameliorates inflammation and neurodegeneration in patients with ischemic stroke and MS and is used in Japan to treat bronchial asthma and cerebrovascular disorders. Several reports have shown that ibudilast decreases production of tumor necrosis factor α, IL-1β, IL-6, and interferon-γ and suppresses differentiation of T helper 1 (Th1) cells. However, it is not clear if ibudilast suppresses Th17 differentiation and IL-17 production. Thus, we investigated if ibudilast inhibits differentiation of human Th17 cells.

Methods: Naïve T cells were isolated from 5 healthy volunteers, using a magnetic beads kit. Differentiation of Th17 cells was then stimulated *in vitro* using treatments with anti-CD3, anti-CD28, IL-1β, IL-6, IL-21, IL-23, and transforming growth factor-beta1 (TGF-β1), with or without ibudilast, for 1 week. Populations of Th17 cells were measured using flow cytometry with anti-IL-17 antibodies. The activity of signal transducer and activator of transcription 3 (STAT3) was analyzed in naïve T cells, using enzyme-linked immunosorbent assays after stimulation with the above cytokines for 30 min.

Results: Th17 cell differentiation was significantly inhibited by ibudilast.

Conclusions: Ibudilast inhibits Th17 cell differentiation and may contribute to development of new treatments for MS.

Toho J Med 1 (1): 2–6, 2015

KEYWORDS: T helper 17 cell (Th17 cell), interleukin-17 (IL-17), ibudilast, phosphodiesterase inhibitor

Multiple sclerosis (MS) is a $CD4^+$ T-cell-mediated autoimmune disease of the central nervous system (CNS)[1, 2] and is characterized pathologically by inflammatory foci in the CNS, with varying degrees of demyelination, axonal damage, and glial proliferation. CNS-infiltrating T lymphocytes (T cells) are major mediators of MS,[3] and evidence from recent genome-wide association studies indicates that T cell activation and differentiation pathways are relevant to MS pathology in humans.[4]

T helper 17 (Th17) cells are a recently identified subset of $CD4^+$ T cells.[5] These cells produce cytokine interleukin (IL)-17 and are important modulators of autoimmune response in MS. In patients with MS, IL-17 expression is increased in mononuclear cells of the blood and cerebrospinal fluid and in lesions.[6, 7] Moreover, differentiation of Th17 cells from naïve T cells (nTh) is reportedly regulated

6-11-1 Omorinishi, Ota, Tokyo 143-8541
*Corresponding Author: tel: 03(3762)4151
e-mail: kawabekiyokazu@med.toho-u.ac.jp

Received Nov. 6, 2014: Accepted Dec. 12, 2014
Toho Journal of Medicine 1 (1), Mar. 1, 2015.
ISSN 2189-1990

by signal transducer and activator of transcription 3 (STAT3).[8]

Ibudilast is used in Japan to treat bronchial asthma and cerebrovascular disorders.[9] It inhibits phosphodiesterases, which results in inhibition of leukotrienes and nitric oxidase mechanisms. The resulting anti-inflammatory effects make ibudilast an attractive candidate for MS treatment. In particular, ibudilast reportedly decreased the severity of clinical signs in an animal model of autoimmune encephalomyelitis,[10, 11] and the effects of ibudilast against MS have been investigated in several studies of humans. Specifically, ibudilast reduced atrophy rates, slowed progression of expanded disability status scale (EDSS) score,[12] and suppressed Th1 cells and associated cytokines.[11, 13] However, the effects of ibudilast on Th17 cells are not known. Thus, in the present study we examined the effects of ibudilast on Th17 cell differentiation.

Methods

Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) from healthy donors were separated using Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich Corp., St. Louis, MO, USA). PBMCs were then cultured in 24-well plates (10^6 cells/ml) in 5% CO_2 at 37°C using RPMI-1640 media supplemented with 10% fetal calf serum, 2 mM glutamine, 20 mM HEPES, 100 g/ml streptomycin, and 100 U/ml penicillin (Sigma-Aldrich).

Isolation and culture of naïve T cells and differentiation of Th17 cells

Naïve T cells ($CD4^+$ $CD45RA^+$ CD45RO-CD25-T cells) (nTh cells) were isolated using immunomagnetic cell separation with a MACS® naïve T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Isolated nTh cells were seeded at 0.25×10^6 cells/ml in 48-well plates and then stimulated using beads coated with anti-CD3 and anti-CD28 antibodies (1 bead per 20 cells; Miltenyi Biotec), IL-1β (25 ng/ml), IL-6 (10 ng/ml), IL-23 (25 ng/ml), transforming growth factor (TGF)-1β (5 ng/ml), and IL-21 (25 ng/ml) for 7 days in X-VIVO™-10 cell culture medium (Lonza Group Ltd., Basel, Switzerland).

Flow cytometry

Cultured differentiated Th17 cells were re-stimulated with phorbol myristate acetate, ionomycin, and BD GolgiStop™ (BD Biosciences, San Jose, CA, USA) for 5 h. Restimulated cells were fixed and permeabilized using a Fixation and Permeabilization Solution Kit with BD GolgiStop™ (BD Biosciences). The cells were then stained with anti-CD4-Cy5.5 and anti-IL-17A-PE (BD Biosciences) and analyzed using a FACS Caliber™ instrument (BD Biosciences).

Enzyme-linked immunosorbent assay

Activated STAT3, which is phosphorylated on Tyr705, was measured by enzyme-linked immunosorbent assay (ELISA). nTh cells were stimulated using beads coated with anti-CD3 and anti-CD28 antibodies (1 bead per 20 cells), IL-1β (25 ng/ml), IL-6 (10 ng/ml), IL-23 (25 ng/ml), TGF-1β (5 ng/ml), and IL-21 (25 ng/ml) for 30 min in the presence or absence of ibudilast. Phosphorylated STAT3 was measured using ELISAs with a PathScan® Phospho-Stat3 (Tyr705) Sandwich ELISA Kit (Cell Signaling Technology Inc., Danvers, MA, USA) according to the manufacturer's protocol. For normalization, the protein concentration of cell lysate was measured by a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the protein concentration of cell lysates after dilution was adjusted at 100 μg/ml.

Statistical methods

Statistical differences were evaluated using Dunnett's test, and a p value of less than 0.05 was considered to indicate statistical significance. Values are expressed as mean ± standard deviation (SD) of the mean.

Ethical approval

This study was approved by the Ethical Review Board of Toho University Faculty of Medicine (25073).

Results

Ibudilast inhibited Th17 cell differentiation from nTh cells

nTh cells were isolated from 5 healthy donors, stimulated with cytokines for 1 week, and differentiated into Th17 cells. Ibudilast was added to the culture medium at concentrations of 0.1–10 μM to determine if it inhibited differentiation into Th17 cells. Stimulated cells were then stained and analyzed by flow cytometry. The numbers of IL-17A-positive cells were significantly decreased in the presence of ibudilast (Fig. 1).

Ibudilast blocked STAT3 activation

STAT3 is a transcription factor that controls Th17 cell differentiation of nTh cells.[8] Thus, we hypothesized that ibudilast inhibits STAT3 activation. After isolation from 4 healthy volunteers, nTh cells were stimulated with cytokines and were differentiated into Th17 cells in the presence

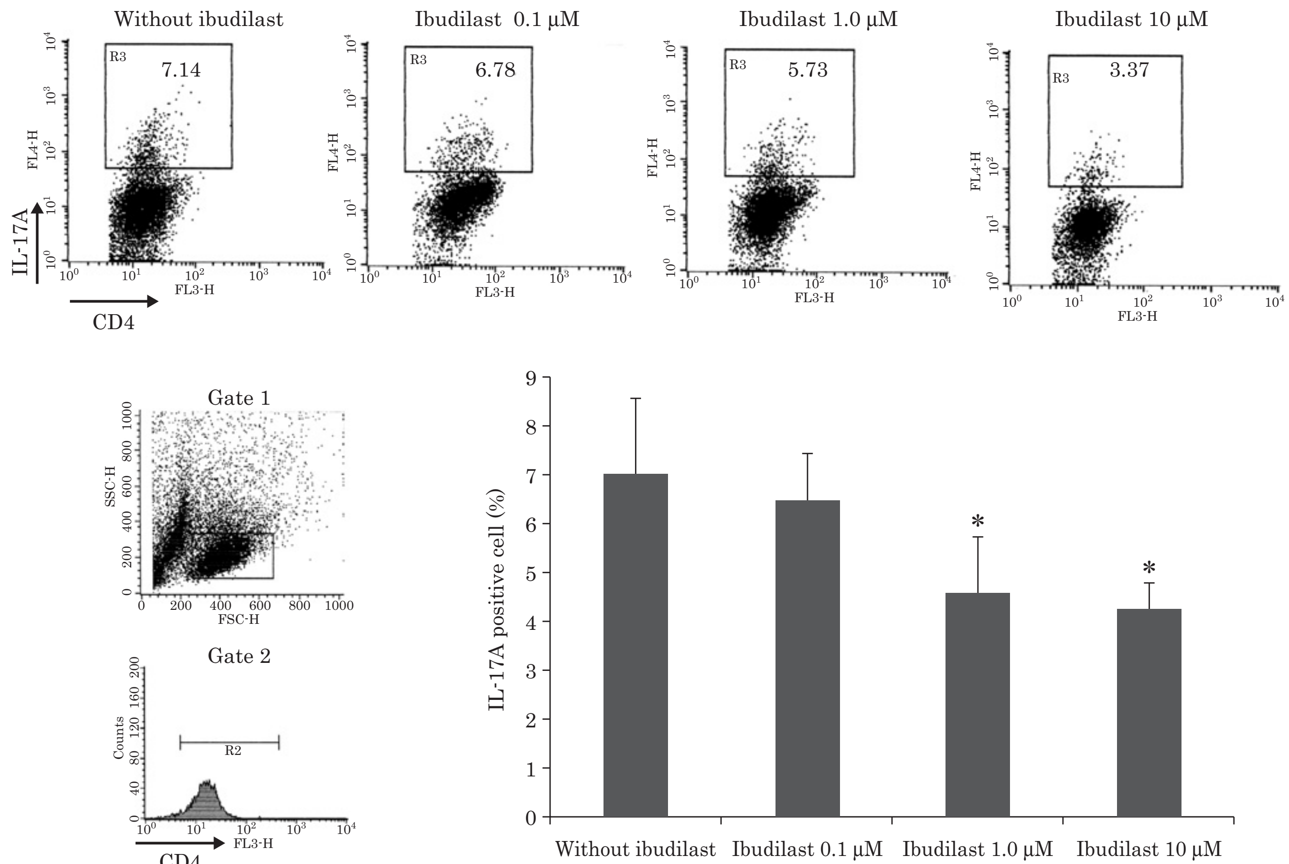

Fig. 1 Naïve helper T cells from healthy volunteers (n = 5) were treated with ibudilast and stimulated, as described in the Methods section. Stimulated cells were stained and assessed using flow cytometry. Gates 1 and 2 were set to isolate $CD4^+$ cells. The numbers of $Th17A^+$ cells was significantly lower after ibudilast treatment. $*p < 0.05$, Dunnett's test.
IL-17A: interleukin-17A, $Th17A^+$: T $helper17A^+$

or absence of ibudilast. After 30 minutes of stimulation, ELISA showed that activated STAT3 in cell lysates was significantly decreased in the presence of ibudilast (Fig. 2).

Discussion

In this study ibudilast inhibited differentiation of Th17 cells from nTh cells. Moreover, ibudilast suppressed the activity of the transcription factor STAT3, which is involved in Th17 cell differentiation.

Several previous animal studies showed that ibudilast significantly suppresses cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-1β, and IL-6.[13,14] Moreover, in human studies, 4-week treatment with ibudilast downregulated messenger ribonucleic acid (mRNA) expression of Th1 cell cytokines IFN-γ and TNF-α and upregulated expression of Th2 cell cytokines IL-4 and IL-10.[11]

Several studies reported that ibudilast is an effective treatment for MS patients. Specifically, relapse rates were reduced by ibudilast in patients with active MS.[11] In addition, a phase 2 study of ibudilast for MS patients showed significant effects on rates of newly active lesions and relapse. In radiologic studies, ibudilast appeared to have neuroprotective effects.[12] Moreover, IL-17 had neurotrophic effects and induced neurodegeneration in MS patients.[15,16]

Phosphodiesterase types 3 and 4 are predominantly expressed in immune cells, and inhibitors of these phosphodiesterase types decreased Th1 cytokine production in T cells from patients with MS.[17] The pharmacologic effects of ibudilast are also related to inhibition of phosphodiesterase type 4 and other phosphodiesterases. Treatment with the phosphodiesterase type 4 inhibitor cilomilast markedly decreased conjunctival expression of IL-6, IL-23, and IL-17 in an experimental dry eye animal model of Sjögren disease and decreased expressions of IL-17 and IL-23 in lymph nodes.[18]

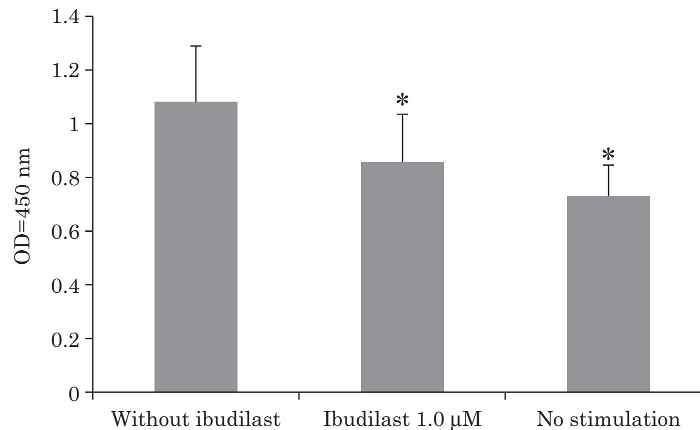

Fig. 2 After isolation from healthy volunteers (n = 4), naïve helper T cells were stimulated for 30 min as described in the Methods section. Phosphorylated STAT3 was assayed using ELISA kits. STAT3 phosphorylation was suppressed by ibudilast.
*$p < 0.05$, Dunnett's test.
STAT3: signal transducer and activator of transcription 3,
ELISA: enzyme-linked immunosorbent assay, OD: optical density

STAT3 is activated by IL-6 and IL-23 and is critical in Th17 cell differentiation.[8] In the present study ibudilast inhibited STAT3 activation, which suggests that its pharmacologic effects are related to nonselective inhibition of phosphodiesterases. In support of this hypothesis, a previous study found that the phosphodiesterase inhibitor pentoxifylline, which has anti-angiogenic activity against many cancers, suppressed phosphorylation and binding of STAT3.[19] Moreover, pentoxifylline decreased TNF-α and IL-12 mRNA expression and increased IL-4 and IL-10 mRNA expression in PBMCs of MS patients.[20]

Ibudilast is orally active, inexpensive, and well tolerated by most MS patients. Thus, the present changes in Th17 cell differentiation via STAT3 suppression are a potential pharmacologic mechanism for treatment of MS.

We would like to thank Professor Yasuo Iwasaki, Department of Neurology, Toho University Omori Medical Center for the critical suggestion and support in this study.

Conflicts of interest (COI): The authors have no COIs to report.

References

1) Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol. 2005; 23: 683-747.
2) Frohman EM, Racke MK, Raine CS. Multiple sclerosis--the plaque and its pathogenesis. N Engl J Med. 2006; 354: 942-55.
3) Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Clin Exp Immunol. 2010; 162: 1-11.
4) Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, et al; International Multiple Sclerosis Genetics Consortium; Wellcome Trust Case Control Consortium 2. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011; 476: 214-9.
5) Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005; 6: 1123-32.
6) Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol. 2008; 172: 146-55.
7) Carlson T, Kroenke M, Rao P, Lane TE, Segal B. The Th17-ELR^+ CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. J Exp Med. 2008; 205: 811-23.
8) Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem. 2007; 282: 9358-63.
9) Armstead WM, Mirro R, Leffler CW, Busija DW. The role of prostanoids in the mediation of responses to KC-404, a novel cerebrovasodilator. J Pharmacol Exp Ther. 1988; 244: 138-43.
10) Fujimoto T, Sakoda S, Fujimura H, Yanagihara T. Ibudilast, a phosphodiesterase inhibitor, ameliorates experimental autoimmune encephalomyelitis in Dark August rats. J Neuroimmunol. 1999; 95: 35-42.
11) Feng J, Misu T, Fujihara K, Sakoda S, Nakatsuji Y, Fukaura H, et al. Ibudilast, a nonselective phosphodiesterase inhibitor, regulates Th1/Th2 balance and NKT cell subset in multiple sclerosis. Mult Scler. 2004; 10: 494-8.
12) Barkhof F, Hulst HE, Drulovic J, Uitdehaag BM, Matsuda K, Landin R; MN166-001 Investigators. Ibudilast in relapsing-remitting multiple sclerosis: a neuroprotectant? Neurology. 2010;

74: 1033-40.

13) Suzumura A, Ito A, Mizuno T. Phosphodiesterase inhibitors suppress IL-12 production with microglia and T helper 1 development. Mult Scler. 2003; 9: 574-8.

14) Mizuno T, Kurotani T, Komatsu Y, Kawanokuchi J, Kato H, Mitsuma N, et al. Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. Neuropharmacology. 2004; 46: 404-11.

15) Kawanokuchi J, Shimizu K, Nitta A, Yamada K, Mizuno T, Takeuchi H, et al. Production and functions of IL-17 in microglia. J Neuroimmunol. 2008; 194: 54-61.

16) Kostic M, Dzopalic T, Zivanovic S, Zivkovic N, Cvetanovic A, Stojanovic I, et al. IL-17 and glutamate excitotoxicity in the pathogenesis of multiple sclerosis. Scand J Immunol. 2014; 79: 181-6.

17) Ekholm D, Hemmer B, Gao G, Vergelli M, Martin R, Manganiello V. Differential expression of cyclic nucleotide phosphodiesterase 3 and 4 activities in human T cell clones specific for myelin basic protein. J Immunol. 1997; 159: 1520-9.

18) Sadrai Z, Stevenson W, Okanobo A, Chen Y, Dohlman TH, Hua J, et al. PDE4 inhibition suppresses IL-17-associated immunity in dry eye disease. Invest Ophthalmol Vis Sci. 2012; 53: 3584-91.

19) Kamran MZ, Gude RP. Pentoxifylline inhibits melanoma tumor growth and angiogenesis by targeting STAT3 signaling pathway. Biomed Pharmacother. 2013; 67: 399-405.

20) Rieckmann P, Weber F, Günther A, Martin S, Bitsch A, Broocks A, et al. Pentoxifylline, a phosphodiesterase inhibitor, induces immune deviation in patients with multiple sclerosis. J Neuroimmunol. 1996; 64: 193-200.