5 Blower AL, Brooks A, Fenn GC, Hill A, Pearce MY, Morant S, Bardhan KD: Emergency admissions for upper gastrointestinal disease and their relation to NSAID use. Aliment Pharmacol Ther 1997;11:283–291.

6 Laine L, Connors LG, Reicin A, Hawkey CJ, Burgos-Vargas R, Schnitzer TJ, Yu Q, Bombardier C: Serious lower gastrointestinal clinical events with nonselective NSAID or coxib use. Gastroenterology 2003;124:288–292.

7 Morris AJ, Wasson LA, MacKenzie JF: Small bowel enteroscopy in undiagnosed gastrointestinal blood loss. Gut 1992;33:887–889.

8 Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB: Arachidonic acid metabolism. Annu Rev Biochem 1986;55:69–102.

9 Vane JR: Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 1971;231:232–235.

10 Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR: TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 1991;266:12866–12872.

11 Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, Seibert K: Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci USA 1994;91:3228–3232.

12 Emery P, Zeidler H, Kvien TK, Guslandi M, Naudin R, Stead H, Verburg KM, Isakson PC, Hubbard RC, Geis GS: Celecoxib versus diclofenac in long-term management of rheumatoid arthritis: randomised double-blind comparison. Lancet 1999;354:2106–2111.

13 Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS: Gastrointestinal toxicity with celecoxib vs. nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. JAMA 2000;284:1247–1255.

14 Simon LS, Weaver AL, Graham DY, Kivitz AJ, Lipsky PE, Hubbard RC, Isakson PC, Verburg KM, Yu SS, Zhao WW, Geis GS: Anti-inflammatory and upper gastrointestinal effects of celecoxib in rheumatoid arthritis: a randomized controlled trial. JAMA 1999;282:1921–1928.

15 Rostom A, Muir K, Dubé C, Jolicoeur E, Boucher M, Joyce J, Tugwell P, Wells GW: Gastrointestinal safety of cyclooxygenase-2 inhibitors: a Cochrane Collaboration systematic review. Clin Gastroenterol Hepatol 2007; 5:818–828.

16 Goldstein JL, Eisen GM, Lewis B, Gralnek IM, Zlotnick S, Fort JG: Video capsule endoscopy to prospectively assess small bowel injury with celecoxib, naproxen plus omeprazole, and placebo. Clin Gastroenterol Hepatol 2005;3:133–141.

17 Goldstein JL, Eisen GM, Lewis B, Gralnek IM, Aisenberg J, Bhadra P, Berger MF: Small bowel mucosal injury is reduced in healthy subjects treated with celecoxib compared with ibuprofen plus omeprazole, as assessed by video capsule endoscopy. Aliment Pharmacol Ther 2007;25:1211–1222.

18 Meron GD: The development of the swallowable video capsule (M2A). Gastrointest Endosc 2000;52:817–819.

19 Iddan G, Meron G, Glukhovsky A, Swain P: Wireless capsule endoscopy. Nature 2000;405: 417.

20 Sugimori S, Watanabe T, Tabuchi M, Kameda N, Machida H, Okazaki H, Tanigawa T, Yamagami H, Shiba M, Watanabe K, Tominaga K, Fujiwara Y, Oshitani N, Koike T, Higuchi K, Arakawa T: Evaluation of small bowel injury in patients with rheumatoid arthritis by capsule endoscopy: effects of anti-rheumatoid arthritis drugs. Digestion 2008;78:208–213.

21 Maiden L, Thjodleifsson B, Theodors A, Gonzalez J, Bjarnason I: A quantitative analysis of NSAID-induced small bowel pathology by capsule enteroscopy. Gastroenterology 2005; 128:1172–1178.

22 Gralnek IM, Defranchis R, Seidman E, Leighton JA, Legnani P, Lewis BS: Development of a capsule endoscopy scoring index for small bowel mucosal inflammatory change. Aliment Pharmacol Ther 2008;27:146–154.

23 Inoue E, Yamanaka H, Hara M, Tomatsu T, Kamatani N: Comparison of Disease Activity Score (DAS)28-erythrocyte sedimentation rate and DAS28-C-reactive protein threshold values. Ann Rheum Dis 2007;66:407–409.

24 Mizukami K, Murakami K, Yamauchi M, Matsunari O, Ogawa R, Nakagawa Y, Okimoto T, Kodama M, Fujioka T: Evaluation of selective cyclooxygenase-2 inhibitor-induced small bowel injury: randomized cross-over study compared with loxoprofen in healthy subjects. Dig Endosc 2013;25:288–294.

25 Chan FK, Lanas A, Scheiman J, Berger MF, Nguyen H, Goldstein JL: Celecoxib versus omeprazole and diclofenac in patients with osteoarthritis and rheumatoid arthritis (CONDOR): a randomised trial. Lancet 2010;376:173–179.

26 Hitchon CA, Alex P, Erdile LB, Frank MB, Dozmorov I, Tang Y, Wong K, Centola M, El-Gabalawy HS: A distinct multicytokine profile is associated with anti-cyclical citrullinated peptide antibodies in patients with early untreated inflammatory arthritis. J Rheumatol 2004;31:2336–2346.

27 Meyer PW, Hodkinson B, Ally M, Musenge E, Wadee AA, Fickl H, Tikly M, Anderson R: Circulating cytokine profiles and their relationships with autoantibodies, acute phase reactants, and disease activity in patients with rheumatoid arthritis. Mediators Inflamm 2010;2010:158514.

28 Alex P, Szodoray P, Knowlton N, Dozmorov IM, Turner M, Frank MB, Arthur RE, Willis L, Flinn D, Hynd RF, Carson C, Kumar A, El-Gabalawy HS, Centola M: Multiplex serum cytokine monitoring as a prognostic tool in rheumatoid arthritis. Clin Exp Rheumatol 2007;25: 584–592.

29 Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P, Group AIS: Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 2002;359:1541–1549.

30 Ito H, Takazoe M, Fukuda Y, Hibi T, Kusugami K, Andoh A, Matsumoto T, Yamamura T, Azuma J, Nishimoto N, Yoshizaki K, Shimoyama T, Kishimoto T: A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. Gastroenterology 2004;126: 989–996.

31 Watanabe T, Tanigawa T, Shiba M, Nadatani Y, Nagami Y, Sugimori S, Yamagami H, Watanabe K, Tominaga K, Fujiwara Y, Koike T, Arakawa T: Anti-tumour necrosis factor agents reduce non-steroidal anti-inflammatory drug-induced small bowel injury in rheumatoid arthritis patients. Gut DOI: 10.1136/gutjnl-2013-304713.

32 Cury DH, Costa JE, Irika K, Mijji L, Garcez A, Buchiguel C, Silva I, Sipahi A: Protective effect of octreotide and infliximab in an experimental model of indomethacin-induced inflammatory bowel disease. Dig Dis Sci 2008; 53:2516–2520.

33 Niwa Y, Nakamura M, Ohmiya N, Maeda O, Ando T, Itoh A, Hirooka Y, Goto H: Efficacy of rebamipide for diclofenac-induced small-intestinal mucosal injuries in healthy subjects: a prospective, randomized, double-blinded, placebo-controlled, cross-over study. J Gastroenterol 2008;43:270–276.

34 Fujimori S, Seo T, Gudis K, Ehara A, Kobayashi T, Mitsui K, Yonezawa M, Tanaka S, Tatsuguchi A, Sakamoto C: Prevention of nonsteroidal anti-inflammatory drug-induced small-intestinal injury by prostaglandin: a pilot randomized controlled trial evaluated by capsule endoscopy. Gastrointest Endosc 2009;69: 1339–1346.

35 Niwa Y, Nakamura M, Miyahara R, Ohmiya N, Watanabe O, Ando T, Kawashima H, Itoh A, Hirooka Y, Goto H: Geranylgeranylacetone protects against diclofenac-induced gastric and small intestinal mucosal injuries in healthy subjects: a prospective randomized placebo-controlled double-blind cross-over study. Digestion 2009;80:260–266.

36 Kuramoto T, Umegaki E, Nouda S, Narabayashi K, Kojima Y, Yoda Y, Ishida K, Kawakami K, Abe Y, Takeuchi T, Inoue T, Murano M, Tokioka S, Higuchi K: Preventive effect of irsogladine or omeprazole on nonsteroidal anti-inflammatory drug-induced esophagitis, peptic ulcers, and small intestinal lesions in humans, a prospective randomized controlled study. BMC Gastroenterol 2013; 13:85.

37 Higuchi K, Yoda Y, Amagase K, Kato S, Tokioka S, Murano M, Takeuchi K, Umegaki E: Prevention of NSAID-induced small intestinal mucosal injury: prophylactic potential of lansoprazole. J Clin Biochem Nutr 2009;45: 125–130.

38 Yoda Y, Amagase K, Kato S, Tokioka S, Murano M, Kakimoto K, Nishio H, Umegaki E, Takeuchi K, Higuchi K: Prevention by lansoprazole, a proton pump inhibitor, of indomethacin-induced small intestinal ulceration in rats through induction of heme oxygenase-1. J Physiol Pharmacol 2010;61:287–294.
39 Wallace JL, Syer S, Denou E, de Palma G, Vong L, McKnight W, Jury J, Bolla M, Bercik P, Collins SM, Verdu E, Ongini E: Proton pump inhibitors exacerbate NSAID-induced small intestinal injury by inducing dysbiosis. Gastroenterology 2011;141:1314–1322.
40 Satoh H, Amagase K, Takeuchi K: Exacerbation of nonsteroidal anti-inflammatory drug-induced small intestinal lesions by antisecretory drugs in rats: the role of intestinal motility. J Pharmacol Exp Ther 2012;343:270–277.
41 Lim YJ, Phan TM, Dial EJ, Graham DY, Lichtenberger LM: In vitro and in vivo protection against indomethacin-induced small intestinal injury by proton pump inhibitors, acid pump antagonists, or indomethacin-phosphatidylcholine. Digestion 2012;86:171–177.
42 Wallace JL: Glucocorticoid-induced gastric mucosal damage: inhibition of leukotriene, but not prostaglandin biosynthesis. Prostaglandins 1987;34:311–323.
43 Nobuhara Y, Ueki S, Takeuchi K: Influence of prednisolone on gastric alkaline response in rat stomach. A possible explanation for steroid-induced gastric lesion. Dig Dis Sci 1985;30:1166–1173.
44 Filaretova LP, Filaretov AA, Makara GB: Corticosterone increase inhibits stress-induced gastric erosions in rats. Am J Physiol 1998;274:G1024–G1030.
45 Filaretova L, Podvigina T, Bagaeva T, Makara G: Gastroprotective action of glucocorticoids during the formation and the healing of indomethacin-induced gastric erosions in rats. J Physiol Paris 2001;95:201–208.

Peyer's Patches Play a Protective Role in Nonsteroidal Anti-inflammatory Drug-induced Enteropathy in Mice

Satoshi Hiyama, MD, Hideki Iijima, MD, PhD,* Shinichiro Shinzaki, MD, PhD,* Takahiro Inoue, MD, PhD,* Eri Shiraishi, MD,* Shoichiro Kawai, MD,* Manabu Araki, MD,* Motohiko Kato, MD, PhD,* Yoshito Hayashi, MD, PhD,* Tsutomu Nishida, MD, PhD,* Hironobu Fujii, MSc,† Akira Mukai, MD,‡ Naoko Shibata, PhD,§ Shintaro Sato, PhD,§ Hiroshi Kiyono, DDS, PhD,§ Kazuyoshi Gotoh, PhD,¶ Daisuke Motooka, PhD,¶ Shota Nakamura, PhD,¶ Tetsuya Iida, PhD,¶ Masahiko Tsujii, MD, PhD,* and Tetsuo Takehara, MD, PhD**

Background: Peyer's patches (PPs) play a major role in mucosal immunity. However, their roles in nonsteroidal anti-inflammatory drug–induced enteropathy are poorly understood.

Methods: Wild-type (WT) and PP-null mice were injected with indomethacin. Twenty-four hours later, the cellular profiles and cytokine levels in the PPs, mesenteric lymph nodes (MLNs), and lamina propria (LP) of the small intestine were measured. WT and PP-null mice were given antibiotics before indomethacin treatment to evaluate enteropathy. Naive $CD4^+$ T cells were co-cultured with $CD103^+$ or $CD103^-$ dendritic cells (DCs) to analyze the interleukin (IL)-10 expression levels. Finally, WT mice adoptively transferred with $CD103^+$ or $CD103^-$ DCs were injected with indomethacin.

Results: The proportion of $CD103^+$ DCs in PPs and MLNs and IL-10-expressing $CD4^+$ T cells of PPs and the LP increased after indomethacin treatment. The PP-null mice showed greater indomethacin-induced enteropathy, fewer $CD103^+$ DCs in their MLNs, and lower proportion of IL-10-expressing $CD4^+$ T cells of their LP than WT mice, regardless of commensal bacteria. Naive splenic $CD4^+$ T cells co-cultured with $CD103^+$ DCs isolated from the MLNs of indomethacin-injected WT mice produced a higher amount of IL-10 compared with those co-cultured with $CD103^-$ DCs. Moreover, WT mice that received $CD103^+$ DCs showed milder enteropathy than those that received $CD103^-$ DCs.

Conclusions: PPs play a protective role in nonsteroidal anti-inflammatory drug–induced enteropathy, and this protection is associated with an increase in $CD103^+$ DCs and IL-10-producing $CD4^+$ T cells in the intestine, independent of the commensal bacteria.

(Inflamm Bowel Dis 2014;20:790–799)

Key Words: mucosal immunity, NSAID-induced enteropathy, $CD103^+$ DCs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are increasingly used in patients suffering from orthopedic disorders and rheumatoid arthritis.[1] The advent of video capsule endoscopy and balloon enteroscopy has revealed that 70% of chronic NSAID users show small intestinal inflammation.[2–4] The pathogenesis of NSAID-associated enteropathy is suggested to be multifactorial and includes changes in mucosal permeability, bile acid, proteolytic enzymes, and intestinal bacteria.[5] The mechanisms of NSAID enteropathy seem to be distinct from those of NSAID gastropathy,[4] but the immunological mechanisms of NSAID-induced enteropathy have not been extensively investigated.

The gut-associated lymphoid tissue (GALT) has unique and distinct functions in the active immune response to infectious microorganisms and oral tolerance toward commensal gut microbes and food antigens.[6] Peyer's patches (PPs) are aggregates of lymphoid follicles and are considered to be a major component of GALT. PPs are covered by a specific type of epithelium, the follicle-associated epithelium, which forms the interface between the luminal microenvironment and the GALT. PPs serve as an inductive site for mucosal immune responses by initiating antigen uptake via specialized cells called microfold cells that exist in the follicle-associated epithelium.[6] Antigen-sensitized immune cells exit PPs and migrate to mesenteric lymph nodes (MLNs) and then into the thoracic duct to reach the bloodstream. The migrating cells finally enter mucosal effector sites [e.g., intestinal lamina propria

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.ibdjournal.org).

Received for publication January 16, 2014; Accepted January 29, 2014.

From the *Department of Gastroenterology and Hepatology, and †Department of Molecular Biochemistry and Clinical Investigation, Osaka University Graduate School of Medicine, Osaka, Japan; ‡Department of Gastroenterology, Sumitomo Hospital, Osaka, Japan; §Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; and ¶Department of Infection Metagenomics, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

Supported by Grant in Aid from the Japan Society for the Promotion of Science (Grant No. 23590941).

The authors have no conflicts of interest to disclose.

Reprints: Hideki Iijima, MD, PhD, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 K1 Yamadaoka, Suita, Osaka 565-0871, Japan (e-mail: hiijima@gh.med.osaka-u.ac.jp).

DOI 10.1097/MIB.0000000000000017

Published online 19 March 2014.

(LP)].[6] In animal models, PPs play a crucial role in the induction of oral tolerance through the generation of T cells, which secrete transforming growth factor β and interleukin (IL)-10.[7,8]

Dendritic cells (DCs) are key initiators of the adaptive immune response and play a central role in regulating the balance between tolerance and immunity in the intestinal mucosa.[9] Recent progress has shown that CD103$^+$ DCs are present in the PPs and the LP, which constitutively traffic antigens to the draining MLNs.[10] CD103$^+$ DCs in the MLNs induce the expression of the gut-homing receptors, e.g., C-C chemokine receptor 9 and integrin α4β7, which communicate with T cells and promote the differentiation of naive CD4$^+$ T cell into regulatory T cells (Tregs) in vitro.[11–13] Although CD103$^+$ DCs are inherently tolerogenic in the steady state, their function during inflammation is poorly understood. In the present study, we investigated the roles of PPs in NSAID-induced enteropathy in relation to CD103$^+$ DCs.

MATERIALS AND METHODS

Mice

BALB/c, C57BL/6, and EGFP-Tg mice were purchased from Japan Clea (Tokyo, Japan) or SLC Inc. (Hamamatsu, Shizuoka, Japan). *Il-10*$^{-/-}$ mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). To develop PP-null mice, pregnant BALB/c mice were injected with 1 mg of anti-IL-7Rα antibody via the tail vein at 14.5 days after coitus, as described previously.[14] Female mice between the age of 8 to 11 weeks were used in this study, with the exception of 4-week-old female *Il-10*$^{-/-}$ mice and their counterpart wild-type (WT) mice. All mice were kept under specific pathogen-free conditions in an environmentally controlled clean room at the Institute of Experimental Animal Sciences of the Osaka University Graduate School of Medicine. The Institutional Committee on Animal Research approved all the experiments.

Initiation of Indomethacin-induced Enteropathy

Enteropathy was induced by subcutaneous injection with indomethacin (7.5 mg/kg body weight; Sigma-Aldrich, St. Louis, MO). Twenty-four hours after the injection with indomethacin, the mice were killed via deep CO_2 inhalation. The tissues of the small intestine (SI) were fixed with 4% paraformaldehyde, opened along the anti-mesenteric attachment, and examined for lesions under a stereomicroscope. The ulcer area (in square millimeters) was measured by tracing the ulcers in the SI under ×20 magnification.

Isolation of Mononuclear Cells

Mononuclear cells from the PPs and MLN were isolated by mechanical dissociation. Mononuclear cells from the small intestinal lamina propria (SI-LP) were collected as described previously.[15] Briefly, after the removal of the PPs, the LP mononuclear cells were isolated by enzymatic dissociation using collagenase (Wako, Osaka, Japan) and DNase (Sigma-Aldrich) with stirring at 37°C. The lymphocytes were obtained from the interface between the 40% and 75% layers of a discontinuous Percoll gradient (GE Healthcare, Waukesha, WI). CD4$^+$ T cells were isolated from the PPs using MACS CD4 columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA), and complementary DNA was synthesized using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Quantitative real-time reverse transcription–polymerase chain reaction (PCR) for *Il-1β* (Mm00434228-m1), *Il-6* (Mm00446190-m1), *tumor necrosis factor-α* (*Tnf-α;* Mm99999068-m1), *interferon-γ* (*Ifn-γ*; Mm99999071-m1), *Il-10* (Mm99999062-m1), *Il-17* (Mm00439619-m1), *c-Maf* (Mm02581355-S1), *Il-21* (Mm00517640-m1), *aryl hydrocarbon receptor (Ahr*; Mm00478932-m1), and *β-actin* were performed using Thunderbird Probe qPCR Mix (Toyobo Life Science, Osaka, Japan) and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). *β-actin* was used as an endogenous control. All real-time PCRs were performed in duplicate.

Flow Cytometric Analysis

The following reagents were used to stain the murine cells: Pacific Blue-labeled anti-CD4, phycoerythrin-labeled anti-B220, phycoerythrin-labeled anti-CD25 (BD Biosciences, San Jose, CA), fluorescein isothiocyanate-labeled anti-CD8a, phycoerythrin-labeled anti-F4/80 (eBioscience, San Diego, CA), perCP-Cy5.5–labeled anti-CD103, and Pacific Blue-labeled anti-CD11c (Biolegend, San Diego, CA). Intracellular IL-10 and forkhead box p3 (Foxp3) were stained using allophycocyanin-labeled anti-IL-10 or Alexa Fluor 488–labeled anti-Foxp3 with Cytofix/Cytoperm (BD Biosciences), respectively, according to the manufacturer's instructions. These samples were subjected to flow cytometric analysis using a FACS Canto II (BD Biosciences) or FACS Verse (BD Biosciences) instrument. The data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

T-cell Differentiation Assay

CD4$^+$ CD62L$^+$ naive T cells were isolated from the spleens of BALB/c mice using a naive CD4$^+$ T-cell enrichment column (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. CD103$^+$ and CD103$^-$ CD11c$^+$ DCs were sorted from the MLNs of indomethacin-treated BALB/c mice using anti-CD11c and anti-CD103 antibodies on a FACS Aria II Cell Sorter (BD Biosciences). Naive splenic CD4$^+$ T cells ($4–5 \times 10^5$) were co-cultured with 4×10^4 to 5×10^4 of the sorted CD103$^+$ or CD103$^-$ CD11c$^+$ DCs in RPMI 1640 (Sigma-Aldrich) containing 10% fetal bovine serum, a hamster anti-mouse CD3 antibody (1 μg/mL; BD Biosciences), 1 μg/mL lipopolysaccharide from *Escherichia coli* (O111:B4; Sigma-Aldrich) and recombinant human IL-2 (10 ng/mL; Invitrogen, Carlsbad, CA) in 96-well plates for 4 days. The IL-10 expression levels were then assessed by

quantitative real-time reverse transcription–PCR and flow cytometry as described above.

Adoptive Transfer of PP Mononuclear Cells

PP mononuclear cells of indomethacin-treated BALB/c mice were sorted by flow cytometric side scatter/forward scatter analysis, and 1×10^7 cells per mouse were intravenously injected to PP-null mice. At the time of the adoptive transfer, indomethacin (7.5 mg/kg body weight) was subcutaneously injected, and SI injury was evaluated 24 hours after the injection.

Adoptive Transfer of DCs

Sorted $CD103^+$ or $CD103^-$ DCs were adoptively transferred to WT mice by intravenous injection at 6×10^5 to 7×10^5 cells per mouse. Twenty-four hours after the transfer, the mononuclear cells were isolated from the MLNs and SI-LP, and the expression of IL-10 by the $CD4^+$ T cells was analyzed by flow cytometry. In a separate experiment, indomethacin (7.5 mg/kg body weight) was subcutaneously injected at the time of the adoptive transfer, and the mice were killed 24 hours after the injection to analyze the resulting SI injury.

Depletion of Commensal Bacteria

To deplete the commensal bacteria with antibiotics, the mice were treated for 2 weeks with 200 mg/L ciprofloxacin (Sigma-Aldrich), 1 g/L ampicillin (Nacalai Tesque, Kyoto, Japan), 1 g/L metronidazole (Sigma-Aldrich), and 500 mg/L vancomycin (Sigma-Aldrich), based on the protocol of Vereecke et al.[16] The presence of intestinal microflora was determined by culturing fecal samples for 48 hours using a standard agar plate culture method.

Statistical Analysis

The results are expressed as the mean ± standard error of the mean. Groups of data were compared by Student's *t* test, and differences were considered to be statistically significant when $P < 0.05$.

RESULTS

PP-null Mice Develop Severe Indomethacin-induced Enteropathy

Mice lacking PPs were generated by injecting an anti-IL-7Rα antibody during gestation (Fig., Supplemental Digital Content 1, http://links.lww.com/IBD/A434) and we investigated the role of PPs in indomethacin-induced enteropathy by using the PP-null mice. PP-null mice that did not receive indomethacin exhibited negligible intestinal injury, and there was no difference in the number of $CD4^+$, $CD8^+$, and $CD11c^+$ cells in the MLNs of the PP-null and WT mice (data not shown).

Both WT and PP-null mice showed inflammation, including punched-out and linear ulcers, primarily on the mesenteric side of the SI after subcutaneous administration of indomethacin, and the PP-null mice exhibited more severe enteropathy, including annular ulcers, compared with WT mice (Fig. 1A). The ulcer area was significantly larger in the PP-null mice than

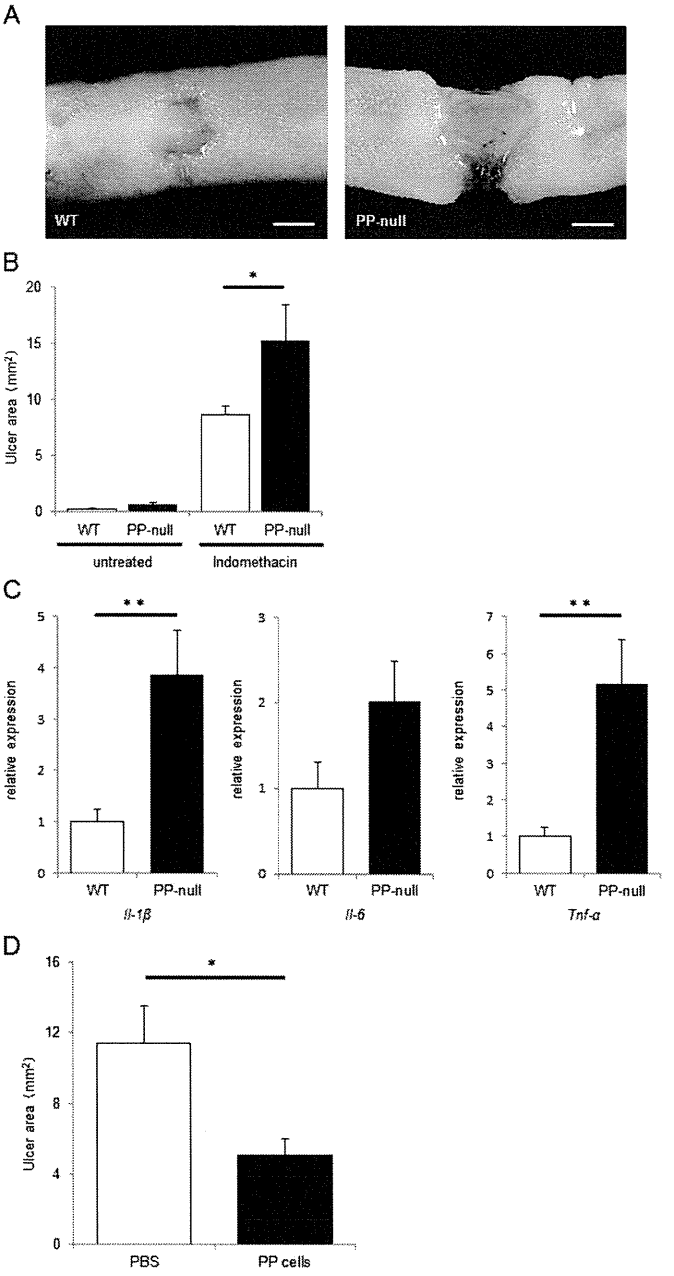


FIGURE 1. PPs played a protective role in indomethacin-induced enteropathy. A, Representative pictures of indomethacin-induced injury in WT and PP-null mice. The scale bars are 1.0 mm. B, Ulcer areas of untreated and indomethacin-treated WT and PP-null mice. C, Cytokine expression profiles of mononuclear cells isolated from SI-LP of indomethacin-treated WT and PP-null mice. D, Ulcer areas after indomethacin treatment in PP-null mice transferred with mononuclear cells isolated from PPs of WT mice or phosphate-buffered saline (PBS). The data are expressed as the mean ± standard error of 5 to 6 (A, B) or 4 (C, D) mice per group. *$P < 0.05$, **$P < 0.01$.

in the WT mice (Fig. 1B; $P < 0.05$). Proinflammatory cytokines were induced by injection of indomethacin in the mononuclear cells of SI-LP isolated from WT mice (Fig., Supplemental

Digital Content 2, http://links.lww.com/IBD/A435) and the messenger RNA (mRNA) expression levels of IL-1β and TNF-α in mononuclear cells of SI-LP isolated from the PP-null mice were significantly higher than those of WT mice (Fig. 1C; $P < 0.01$). When PP cells were adoptively transferred to the PP-null mice at the time of indomethacin injection, the mice that received PP cells showed a significantly smaller ulcer area compared with the mice that were injected with phosphate-buffered saline (Fig. 1D; $P < 0.05$). From these results, PPs are considered to have a protective function in indomethacin-induced enteropathy.

Effect of Indomethacin on the Cellular Profile and Cytokine Production of the PPs, MLNs, and LP

We next analyzed the alteration in cellular profile and cytokine production in GALT after indomethacin injection. The numbers of $CD4^+$ T cells and $CD11c^+$ DCs in the PPs of the WT mice were significantly increased after the injection with indomethacin (Fig. 2A). Significant decrement of $B220^+$ cells was observed in the PPs of indomethacin-injected WT mice (Fig. 2A). However, adoptive transfer of $B220^+$ cells did not affect the indomethacin-induced SI injury (Fig., Supplemental Digital Content 3, http://links.lww.com/IBD/A436). M2 macrophages were shown to have a suppressive function for colonic injury.[17] The numbers of $F4/80^+$ macrophages in PPs were similar before and after the injection of indomethacin (Fig. 2A). In addition, the proportion of $F4/80^+$ macrophages in SI-LP, nor their macrophage phenotype, did not differ between the control and indomethacin-injected WT mice (Fig. A and B, Supplemental Digital Content 4, http://links.lww.com/IBD/A437). These results suggested that B cells and macrophages do not play a major role in the NSAID-induced enteropathy.

The proportion of $CD103^+$ cells in the $CD11c^+$ DC population ($CD103^+$ DCs) was significantly higher in the PPs and MLNs, which are the inductive sites of GALT, but not in SI-LP, the effector site, of the indomethacin-treated WT mice than in those of the control mice (Fig. 2B). When we compared the proportion of $CD103^+$ DCs of WT and PP-null mice, the PP-null mice showed a significantly lower proportion of $CD103^+$ DCs in the MLNs than the WT mice after indomethacin injection (Fig. 2C; $P < 0.05$).

We next isolated $CD4^+$ T cells from the PPs of the WT mice and measured their cytokine expressions by quantitative real-time reverse transcription–PCR. The IL-10 mRNA levels were significantly higher ($P < 0.05$) and the IL-17 mRNA levels were significantly lower ($P < 0.05$) in the PPs of the indomethacin-treated WT mice than in those of the control mice (Fig. 3A). There was no significant difference in the expression of IFN-γ between the 2 groups. The proportion of IL-10-expressing $CD4^+$ T cells in the PPs and SI-LP was significantly higher in the indomethacin-injected WT mice than in the control WT mice (Fig. 3B, C; $P < 0.05$). In contrast, the PP-null mice showed a significantly lower proportion of IL-10-producing $CD4^+$ T cells in their SI-LP (Fig. 3D; $P < 0.05$) compared with the WT mice after indomethacin injection. To investigate whether the IL-10-expressing $CD4^+$ T cells are inducible Tregs, which are

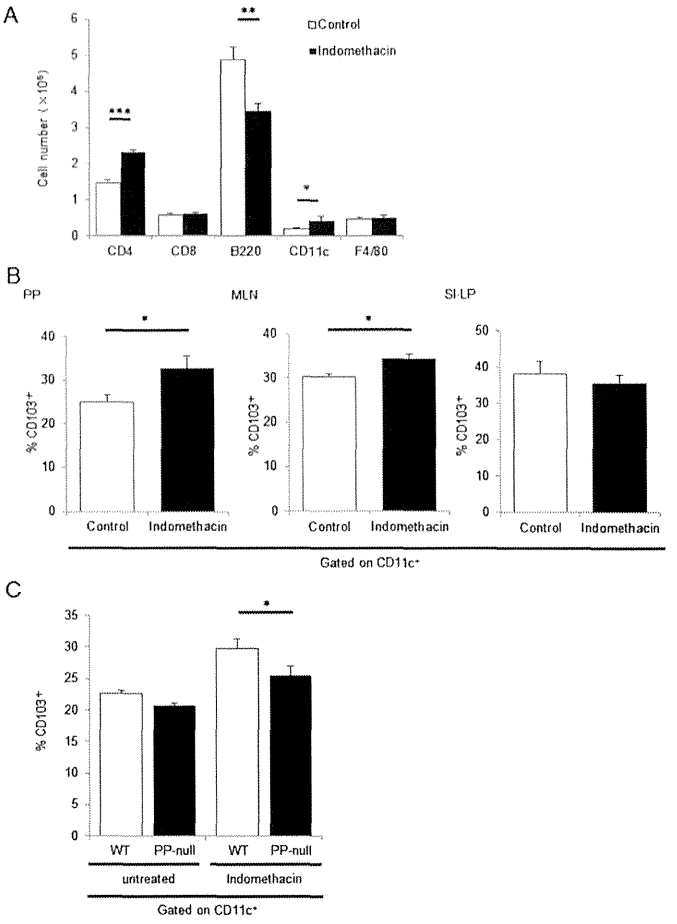


FIGURE 2. $CD103^+$ DCs were induced during indomethacin-induced enteropathy. Alterations in the number of cell populations in the PPs (A) and the proportion of $CD103^+$ DCs in the PPs, MLNs, and SI-LP (B) after indomethacin injection in WT mice. C, Proportion of $CD103^+$ DCs in the MLNs of untreated or indomethacin-treated WT and PP-null mice. The data are expressed as the mean ± standard error of 5 to 6 mice per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

characterized by the expression of Foxp3, we examined the expression of Foxp3 in the $CD4^+$ cells. The proportion of $CD4^+$ $Foxp3^+$ cells did not differ between the vehicle and indomethacin-injected group in the PPs or SI-LP in WT mice (Fig. 3E). Type 1 regulatory (Tr1) cells are regarded as the major subset of IL-10-producing $Foxp3^-$ T cells and they highly express c-Maf, IL-21, and the Ahr.[18–20] The expression levels of c-Maf, IL-21, and Ahr mRNA were significantly increased in the $CD4^+$ T cells of the PPs in the indomethacin-treated WT mice (Fig. 3F; $P < 0.05$). These results revealed that the number of IL-10-producing Tr1 cells is increased in the PPs during indomethacin-induced enteritis.

Il-10-deficient mice show severe indomethacin-induced enteropathy

To investigate whether IL-10 plays a protective role in indomethacin-induced enteropathy, 4-week-old female *Il-10*$^{-/-}$ mice,

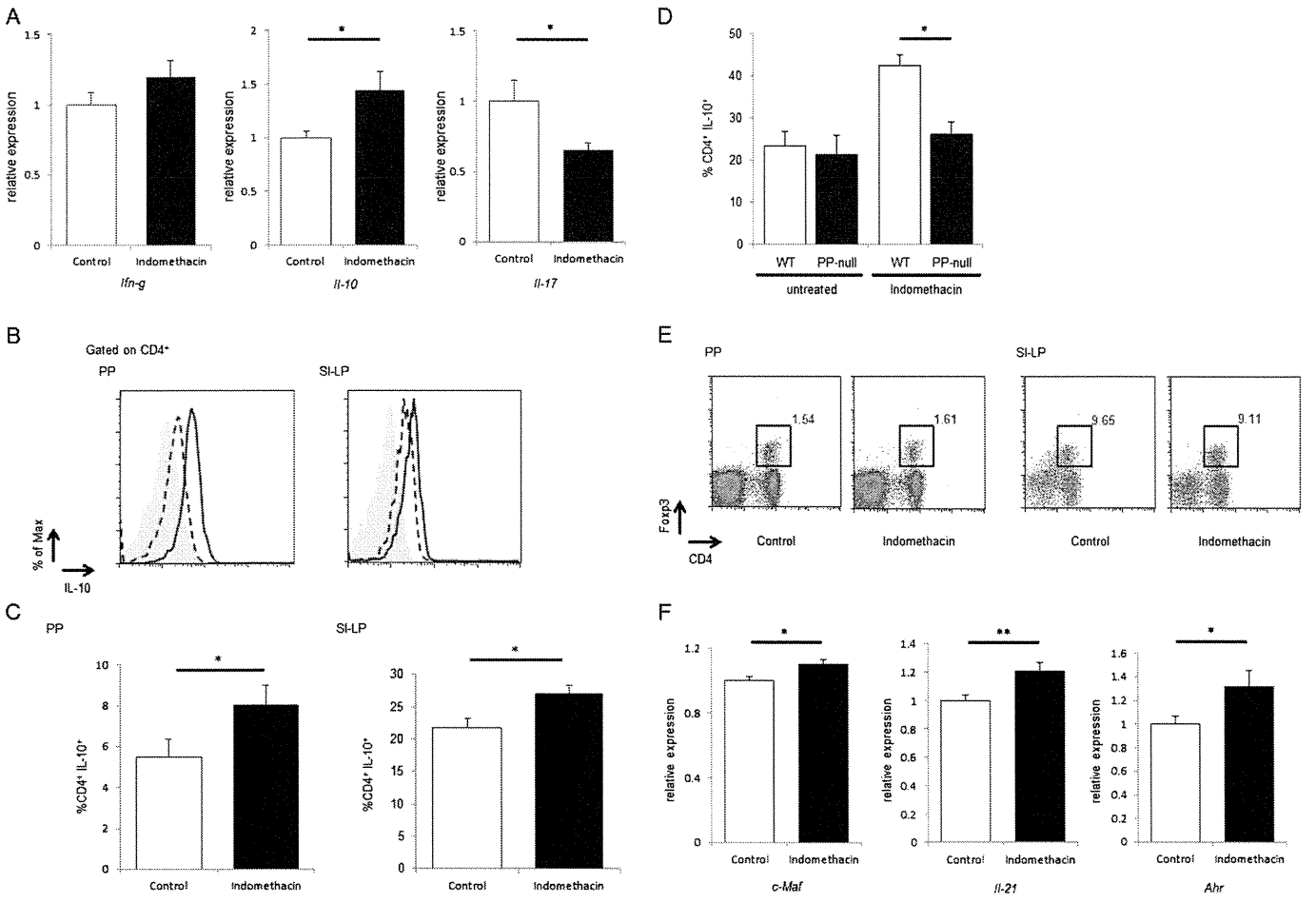


FIGURE 3. IL-10-producing CD4+ T cells were increased in both the PPs and SI-LP after indomethacin treatment. A, Cytokine expression profiles of CD4+ T cells sorted from the PPs of WT mice, as measured by quantitative real-time reverse transcription–PCR. B, Representative intracellular staining of IL-10 gated on the CD4+ cells of the PPs and SI-LP in WT mice. Solid line, indomethacin-treated mice; dotted line, control mice; shaded area, isotype control. C, The proportion of IL-10-expressing CD4+ T cells in the PPs and SI-LP in WT mice. D, The proportions of IL-10-producing CD4+ T cells in the SI-LP of untreated or indomethacin-treated WT and PP-null mice. E, Flow cytometric analysis of the CD4+ Foxp3+ regulatory T cells in the indomethacin-injected WT mice. F, Expression levels of c-Maf, IL-21 and Ahr in the CD4+ T cells from the PPs of WT mice after the indomethacin treatment. The data are expressed as the mean ± standard error of 4 to 6 mice per group (A, C, D, and F). *$P < 0.05$, **$P < 0.01$. Representative pictures of at least 4 independent experiments are shown (B and E).

which have not yet developed enteropathy at this age, and WT mice were injected with indomethacin. The ulcer area was significantly larger in the *Il-10*$^{-/-}$ mice than in the WT mice at 24 hours after the injection with indomethacin (Fig. 4; $P < 0.001$). These results indicate that IL-10 has a protective effect in NSAID-induced enteropathy.

Protection of PPs in Indomethacin-induced Enteropathy Occurs Independently of the Commensal Bacteria

We next investigated whether the protective roles of PPs in NSAID-induced enteropathy are affected by commensal bacteria, which have been reported to play a major role in this model.[21–26] To eliminate the commensal bacteria, we treated WT and PP-null mice with broad-spectrum antibiotics (ciprofloxacin, ampicillin, metronidazole, and vancomycin) for 2 weeks. The amount of commensal bacteria in the intestine was reduced below the level of detection after the antibiotic treatment (data not shown). Although antibiotic treatment showed marked reduction in the ulcer area in both WT and PP-null mice (Fig. 5A; 75% and 65% reduction, respectively), the ulcer area in the PP-null mice still remained significantly larger than that in the WT mice after the administration of antibiotics (Fig. 5A; $P < 0.05$). The PP-null mice showed a significantly lower proportion of CD103+ DCs in their MLNs (Fig. 5B; $P < 0.001$) and demonstrated a significantly lower proportion of IL-10-expressing CD4+ T cells in their SI-LP (Fig. 5C; $P < 0.001$) compared with the WT mice.

Functional Characterization of CD103+ DCs In Vitro

To investigate if the development of IL-10-expressing CD4+ T cells is influenced by CD103+ DCs, CD103+, or

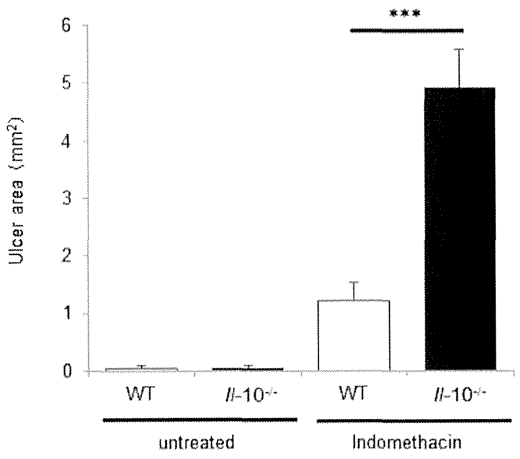


FIGURE 4. *Il-10*$^{-/-}$ mice developed more severe small bowel injury than WT mice after indomethacin injection. The data are expressed as the mean ± standard error of 3 to 5 mice. ***$P < 0.001$.

$CD103^-$, DC subsets from the MLNs of indomethacin-treated WT mice were cultured in vitro with naive $CD4^+$ T cells purified from the spleens of WT mice. The IL-10 mRNA expression levels were higher in the co-culture containing naive $CD4^+$ T cells and $CD103^+$ DCs than in the co-culture containing naive $CD4^+$ T cells and $CD103^-$ DCs (Fig. 6A; $P < 0.05$). To identify the cells that were producing IL-10, intracellular staining of IL-10 was performed. $CD4^+$ T cells produced higher levels of IL-10 when the cells were co-cultured with $CD103^+$ DCs than when they were cultured with $CD103^-$ DCs (Fig. 6B). In addition, the proportion of IL-10-producing $CD4^+$ T cells was significantly higher in the group cultured with $CD103^+$ DCs compared with that cultured with $CD103^-$ DCs ($P < 0.05$). In contrast, no difference was observed in the proportion of IL-10-expressing $CD11c^+$ cells between the 2 groups (Fig. 6C).

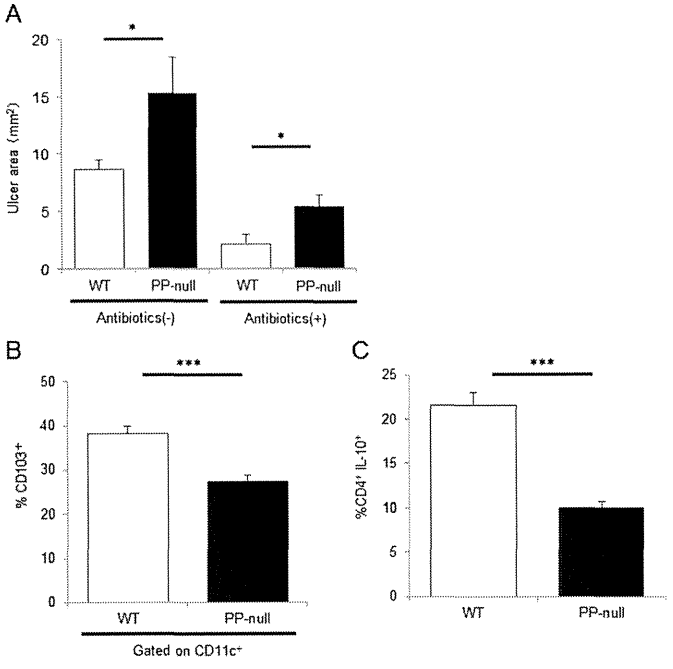


FIGURE 5. PPs played a protective role in indomethacin-induced enteropathy, and this effect was independent of the gut flora. A, Ulcer areas of WT and PP-null mice after indomethacin injection with or without antibiotics treatment. The proportion of $CD103^+$ DCs in the MLNs (B) and IL-10-producing $CD4^+$ T cells in the SI-LP (C) of indomethacin-treated WT mice and PP-null mice after antibiotics administration. The data are expressed as the mean ± standard error of 5 to 6 (A, B) or 3 (C) mice per group. *$P < 0.05$, ***$P < 0.001$.

Adoptive Transfer of $CD103^+$ DCs Ameliorates Indomethacin-induced Enteropathy

To assess the effect of $CD103^+$ DCs in vivo, WT mice were adoptively transferred with $CD103^+$ or $CD103^-$ DCs isolated from the MLNs of indomethacin-treated mice. $CD103^+$ DCs that were adoptively transferred from EGFP-Tg mice were shown to migrate to the MLNs (Fig., Supplemental Digital Content 5, http://links.lww.com/IBD/A438). At 24 hours after the transfer of the DCs from the indomethacin-treated mice, the mice injected with the $CD103^+$ DCs showed a higher proportion of IL-10-expressing $CD4^+$ T cells in their MLNs and SI-LP than the mice injected with $CD103^-$ DCs (Fig. 7A; $P < 0.05$). When indomethacin was injected concurrently with the adoptive transfer of either $CD103^+$ or $CD103^-$ DCs, the mice that received the $CD103^+$ DCs showed a significantly smaller ulcer area than the mice that received the $CD103^-$ DCs (Fig. 7B, C; $P < 0.05$). These results clearly indicate that $CD103^+$ DCs play a protective role in indomethacin-induced enteropathy.

DISCUSSION

In this study, we first demonstrated that PPs play protective roles in indomethacin-induced enteropathy through the induction of immune-regulatory $CD103^+$ DCs and IL-10-expressing $CD4^+$ T cells in the GALT. These protective effects were found to be independent of the commensal bacteria. NSAIDs, e.g., indomethacin, inhibit cyclooxygenase to suppress the production of prostaglandins, which promote mucus secretion, mucosal blood flow, and epithelial proliferation.[27] NSAIDs also inhibit the immunological function of prostaglandin E_2, which suppresses type 1 effector cells,[28–30] induces Tregs,[31,32] and blocks the ability of DCs to attract naive T cells.[31,32] Thus, NSAIDs induce enteropathy by creating an environment that is susceptible to noxious factors, such as bile acid or intestinal bacteria,[4] and we clearly demonstrated that PPs serve to ameliorate NSAID-induced enteropathy, especially through immune-mediated mechanisms.

In PPs, luminal antigens are taken up by DCs directly or indirectly through microfold cells. The DCs migrate to the T-cell area to present antigens to $CD4^+$ T cells and differentiate to secrete cytokines.[33] We found that the proportion of $CD103^+$ DCs was increased in both the PPs and MLNs after the injection of indomethacin. $CD103^+$ DCs in the GALT are reported to induce tolerance against commensal bacteria or food antigens by promoting naive $CD4^+$ T cell differentiation into $Foxp3^+$ Tregs

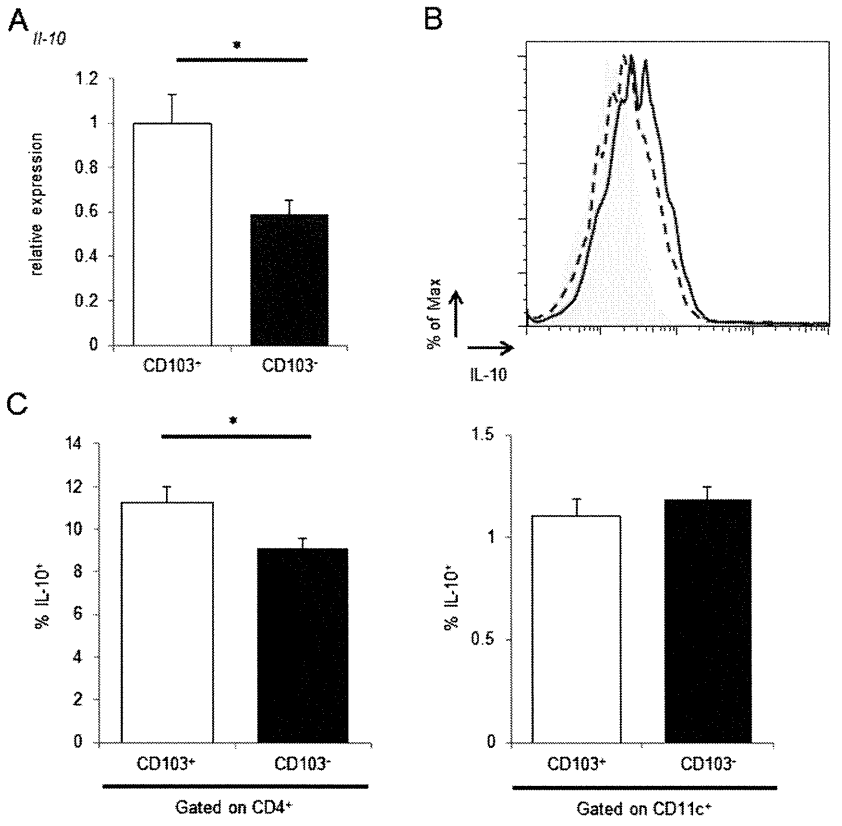


FIGURE 6. CD103$^+$ DCs induced IL-10-producing CD4$^+$ T cells in vitro. Naive splenic CD4$^+$ T cells (4–5 × 10^5) were co-cultured with CD103$^+$ or CD103$^-$ DCs isolated from the MLN (4–5 × 10^4) for 4 days. A, IL-10 mRNA expression levels in both groups were measured by quantitative real-time reverse transcription–PCR. B, Intracellular staining of IL-10 gated on CD4$^+$ cells. Solid line, the group co-cultured with CD103$^+$ DCs; dotted line, the group co-cultured with CD103$^-$ DCs; shaded area, isotype control. C, Percentage of IL-10-producing CD4$^+$ T cells and CD11c$^+$ DCs. The data are expressed as the mean ± standard error of 4 independent experiments. *$P < 0.05$.

under noninflammatory conditions.[11–13] The role of CD103$^+$ DCs in intestinal inflammatory conditions, however, has not been defined. Here, we found that CD103$^+$ DCs increased the number of CD4$^+$ T cells that express IL-10, which are classified as Tr1 cells during indomethacin-induced enteropathy. In addition, mice that received CD103$^+$ DCs developed milder intestinal injury after the indomethacin injection compared with those that received CD103$^-$ DCs. These results indicate that CD103$^+$ DCs have an ameliorative effect during indomethacin-induced enteropathy and that this effect is due to the induction of Tr1 cells. In contrast, Laffont et al[34] recently showed that CD103$^+$ DCs have an impaired ability to induce Foxp3$^+$ Treg and accelerate murine colitis in recombinase-activating gene–deficient mice that have been adoptively transferred with naive T cells. There might be a discrepancy in the effect of CD103$^+$ DCs on intestinal inflammation between the indomethacin and naive T-cell transfer models. This incongruity might be derived from differences between the acute and chronic murine models. In addition, the disease location might also be involved in these discrepancies because nearly all the IL-10-producing T cells in the colonic LP are Foxp3$^+$ Tregs, whereas most IL-10-producing T cells are Foxp3$^-$ in the SI-LP.[35] Because CD103$^+$ DCs are capable of inducing IL-10-producing T cells, CD103$^+$ DCs are considered to be targets for protection of intestinal injury and inflammation. Decrement of IL-17 mRNA expression was seen in the CD4$^+$ T cells of PPs after the indomethacin treatment. Because *Il-17A*$^{-/-}$ mice showed milder indomethacin-induced enteropathy than WT mice (Fig., Supplemental Digital Content 6, http://links.lww.com/IBD/A439), similarly to the previous report,[36] there may be another axis through IL-17-expressing CD4$^+$ T cells to explain the protective role of PPs in NSAID-induced SI injury. Better understanding of immune-mediated protective function of PPs will help to identify effective medications for mucosal injury in the SI.

Several methodologies have been reported to generate PP-null mice.[14,37–40] We used in utero anti-IL-7R antibody treatment because mice generated by this system are well characterized and are reported not to develop PPs but to develop completely intact splenic and lymphoid tissues, including MLNs and isolated lymphoid follicles.[14,41] Using PP-null mice, PPs were previously shown to be nonessential for intestinal IgA responses to orally administered soluble protein under noninflammatory conditions.[42] In an inflammatory or disease state, PP-null mice suffer from severe dextran sodium salt–induced colitis[39] and

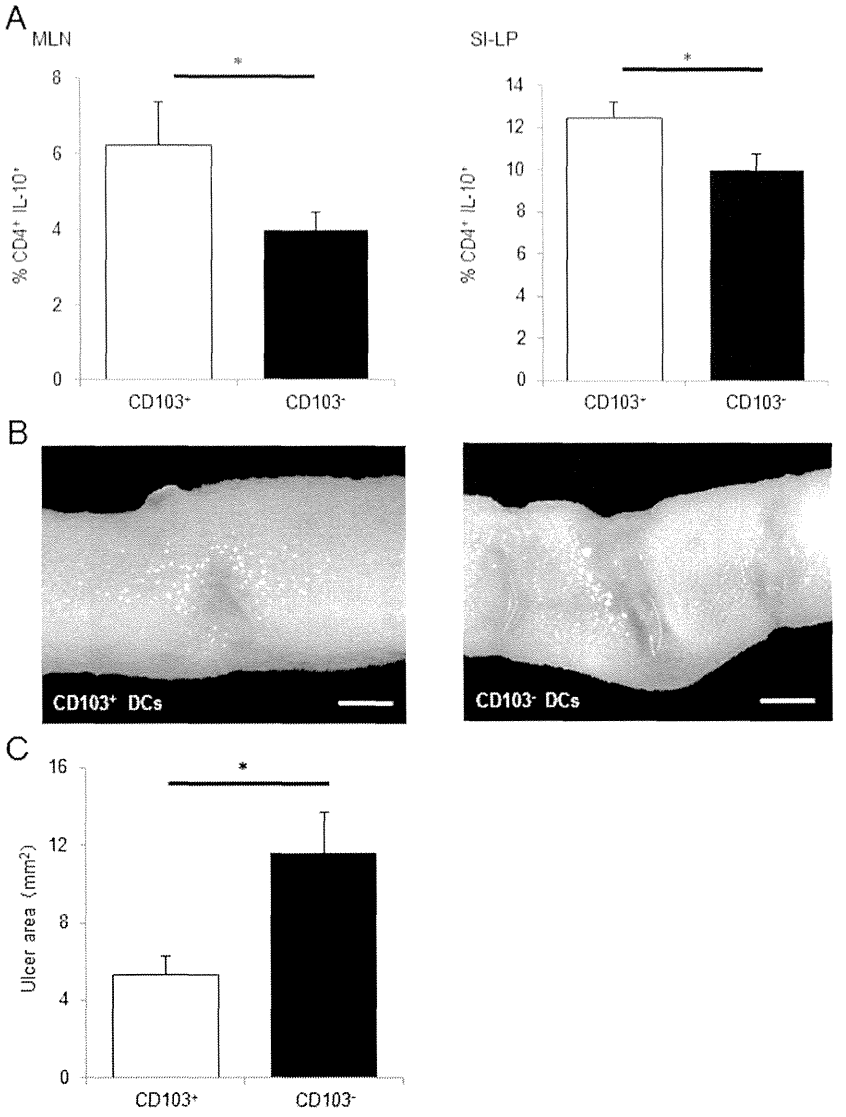


FIGURE 7. Adoptive transfer of CD103$^+$ DCs induced IL-10-producing CD4$^+$ T cells in the GALT and ameliorated indomethacin-induced enteropathy. Sorted CD103$^+$ or CD103$^-$ DCs ($6–7 \times 10^5$) were transferred to WT mice. A, The proportion of IL-10-producing CD4$^+$ T cells in the MLN and SI-LP at 24 hours after the transfer. B, Representative pictures of indomethacin-induced injury in the mice that received the CD103$^+$ DCs or CD103$^-$ DCs. The scale bars are 1.0 mm. C, Ulcer areas in mice transferred with CD103$^+$ DCs or CD103$^-$ DCs. The data are expressed as the mean ± standard error of 6 (A) or 4 (C) mice per group. *$P < 0.05$.

severe ovalbumin-induced allergic diarrhea[37] and fail to induce intestinal IgA responses against *Salmonella* species.[38] In our study, the PP-null mice also developed more severe indomethacin-induced enteropathy than the WT mice, and this enteropathy was accompanied by a lower proportion of CD103$^+$ DCs in the MLNs. These reports imply that PPs play an especially critical role under inflammatory conditions.

Kwa et al[40] reported that Th1 induction during primary infection with an enteric apicomplexan parasite was delayed in PP-null mice and attributed this result to the delayed arrival of a broad range of DCs to the MLN. We also observed a reduction in the accumulation of fluorescently labeled, orally administered ovalbumin in the MLNs of PP-null mice compared with WT mice (unpublished data). Because the DCs from PPs migrate to the MLNs,[43,44] it is possible that the lower number of the CD103$^+$ DCs that we observed in the MLNs was caused by the deficient migration of CD103$^+$ DCs from the PPs in the PP-null mice. The protective role of PPs in NSAID-induced enteropathy is presumed to not only be conferred through the induction of regulatory IL-10-expressing CD4$^+$ T cells but also through an indirect mechanism in which luminal antigens are delivered to the MLNs by CD103$^+$ DCs from the PPs. The migration of CD103$^+$ DCs to the MLNs has been reported to be enhanced after oral or systemic adjuvant administration.[45] Because CD103$^+$ DCs are also present in the human MLNs and mucosa with similar properties and functions as in mice,[13] it is important

to investigate the function of CD103$^+$ DCs in humans to evaluate future therapeutic targets of inflammatory diseases in the gut. To date, drugs that are highly effective in preventing mucosal injury in the SI are not available. To explore oral nutrition therapy or medication to induce the immune-suppressive cells, e.g. CD103$^+$ DCs, in GALT might be effective to reduce SI injury in NSAID users, and we are now working on it.

Enteric gram-negative bacteria are reported to contribute significantly to NSAID-induced intestinal damage.[21,22,25,26,46] These reports are supported by a study that demonstrated that mice lacking Toll-like receptor 4 do not develop intestinal damage when given NSAIDs,[24] and we also showed that indomethacin-induced enteropathy was improved in both WT and PP-null mice after the antibiotics treatment. However, even after the depletion of commensal bacteria, the PP-null mice showed more severe indomethacin-induced enteropathy, a significantly lower proportion of CD103$^+$ DCs in their MLNs and a significantly lower proportion of IL-10-producing CD4$^+$ T cells in their SI-LP than the WT mice. In addition, neither major difference in the intestinal microflora by metagenomic analysis (data not shown) nor difference in the Toll-like receptor 4 expression in SI-LP (Fig., Supplemental Digital Content 7, http://links.lww.com/IBD/A440) was evident between the PP-null and WT mice. These data revealed that the protective effects of PPs in indomethacin-induced enteropathy are independent of the commensal gut flora. We previously reported that opportunistic bacteria, primarily *Alcaligenes* species, inhabit the PP interior and cannot be depleted by oral antibiotic treatment.[47] The bacteria that inhabit the PPs might trigger the phenotypic changes observed during NSAID-induced enteropathy, but more studies are required to evaluate this hypothesis.

In conclusion, we demonstrated that PPs regulate NSAID-induced enteropathy through the induction of CD103$^+$ DCs and IL-10-producing CD4$^+$ T cells in the GALT, independently of the commensal bacteria in the gut. Further study may provide an alternate therapeutic strategy using an immunological mechanism to regulate NSAID-induced enteropathy.

REFERENCES

1. Sinha M, Gautam L, Shukla PK, et al. Current perspectives in NSAID-induced gastropathy. *Mediators Inflamm.* 2013;2013:258209.
2. Yamamoto H, Sekine Y, Sato Y, et al. Total enteroscopy with a nonsurgical steerable double-balloon method. *Gastrointest Endosc.* 2001;53:216–220.
3. Fujimori S, Seo T, Gudis K, et al. Diagnosis and treatment of obscure gastrointestinal bleeding using combined capsule endoscopy and double balloon endoscopy: 1-year follow-up study. *Endoscopy.* 2007;39:1053–1058.
4. Bjarnason I, Hayllar J, MacPherson AJ, et al. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology.* 1993;104:1832–1847.
5. Higuchi K, Umegaki E, Watanabe T, et al. Present status and strategy of NSAIDs-induced small bowel injury. *J Gastroenterol.* 2009;44:879–888.
6. Iijima H, Takahashi I, Kiyono H. Mucosal immune network in the gut for the control of infectious diseases. *Rev Med Virol.* 2001;11:117–133.
7. Tsuji NM, Mizumachi K, Kurisaki J. Interleukin-10-secreting Peyer's patch cells are responsible for active suppression in low-dose oral tolerance. *Immunology.* 2001;103:458–464.
8. Chen Y, Inobe J, Marks R, et al. Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature.* 1995;376:177–180.
9. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol.* 2008;8:435–446.
10. Scott CL, Aumeunier AM, Mowat AM. Intestinal CD103+ dendritic cells: master regulators of tolerance? *Trends Immunol.* 2011;32:412–419.
11. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* 2007;204:1757–1764.
12. Annacker O, Coombes JL, Malmstrom V, et al. Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med.* 2005;202:1051–1061.
13. Jaensson E, Uronen-Hansson H, Pabst O, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med.* 2008;205:2139–2149.
14. Kunisawa J, Takahashi I, Okudaira A, et al. Lack of antigen-specific immune responses in anti-IL-7 receptor alpha chain antibody-treated Peyer's patch-null mice following intestinal immunization with microencapsulated antigen. *Eur J Immunol.* 2002;32:2347–2355.
15. Aicher WK, Fujihashi K, Yamamoto M, et al. Effects of the lpr/lpr mutation on T and B cell populations in the lamina propria of the small intestine, a mucosal effector site. *Int Immunol.* 1992;4:959–968.
16. Vereecke L, Sze M, Mc Guire C, et al. Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis. *J Exp Med.* 2010;207:1513–1523.
17. Hunter MM, Wang A, Parhar KS, et al. In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. *Gastroenterology.* 2010;138:1395–1405.
18. Pot C, Jin H, Awasthi A, et al. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol.* 2009;183:797–801.
19. Spolski R, Kim HP, Zhu W, et al. IL-21 mediates suppressive effects via its induction of IL-10. *J Immunol.* 2009;182:2859–2867.
20. Apetoh L, Quintana FJ, Pot C, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol.* 2010;11:854–861.
21. Robert A, Asano T. Resistance of germfree rats to indomethacin-induced intestinal lesions. *Prostaglandins.* 1977;14:333–341.
22. Uejima M, Kinouchi T, Kataoka K, et al. Role of intestinal bacteria in ileal ulcer formation in rats treated with a nonsteroidal antiinflammatory drug. *Microbiol Immunol.* 1996;40:553–560.
23. Konaka A, Kato S, Tanaka A, et al. Roles of enterobacteria, nitric oxide and neutrophil in pathogenesis of indomethacin-induced small intestinal lesions in rats. *Pharmacol Res.* 1999;40:517–524.
24. Watanabe T, Higuchi K, Kobata A, et al. Non-steroidal anti-inflammatory drug-induced small intestinal damage is Toll-like receptor 4 dependent. *Gut.* 2008;57:181–187.
25. Endo H, Higurashi T, Hosono K, et al. Efficacy of *Lactobacillus casei* treatment on small bowel injury in chronic low-dose aspirin users: a pilot randomized controlled study. *J Gastroenterol.* 2011;46:894–905.
26. Montalto M, Gallo A, Gasbarrini A, et al. NSAID enteropathy: could probiotics prevent it? *J Gastroenterol.* 2013;48:689–697.
27. Kalinski P. Regulation of immune responses by prostaglandin E2. *J Immunol.* 2012;188:21–28.
28. Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol.* 1991;146:108–113.
29. Snijdewint FG, Kalinski P, Wierenga EA, et al. Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J Immunol.* 1993;150:5321–5329.
30. van der Pouw Kraan TC, Boeije LC, Smeenk RJ, et al. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. *J Exp Med.* 1995;181:775–779.
31. Baratelli F, Lin Y, Zhu L, et al. Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. *J Immunol.* 2005;175:1483–1490.
32. Soontrapa K, Honda T, Sakata D, et al. Prostaglandin E2-prostaglandin E receptor subtype 4 (EP4) signaling mediates UV irradiation-induced systemic immunosuppression. *Proc Natl Acad Sci U S A.* 2011;108:6668–6673.
33. Neutra MR, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol.* 2001;2:1004–1009.
34. Laffont S, Siddiqui KR, Powrie F. Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. *Eur J Immunol.* 2010;40:1877–1883.

35. Maynard CL, Harrington LE, Janowski KM, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol.* 2007;8:931–941.
36. Yamada S, Naito Y, Takagi T, et al. Reduced small-intestinal injury induced by indomethacin in interleukin-17A-deficient mice. *J Gastroenterol Hepatol.* 2011;26:398–404.
37. Takayama N, Igarashi O, Kweon MN, et al. Regulatory role of Peyer's patches for the inhibition of OVA-induced allergic diarrhea. *Clin Immunol.* 2007;123:199–208.
38. Hashizume T, Togawa A, Nochi T, et al. Peyer's patches are required for intestinal immunoglobulin A responses to *Salmonella* spp. *Infect Immun.* 2008;76:927–934.
39. Spahn TW, Herbst H, Rennert PD, et al. Induction of colitis in mice deficient of Peyer's patches and mesenteric lymph nodes is associated with increased disease severity and formation of colonic lymphoid patches. *Am J Pathol.* 2002;161:2273–2282.
40. Kwa SF, Beverley P, Smith AL. Peyer's patches are required for the induction of rapid Th1 responses in the gut and mesenteric lymph nodes during an enteric infection. *J Immunol.* 2006;176:7533–7541.
41. Yoshida H, Honda K, Shinkura R, et al. IL-7 receptor alpha+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int Immunol.* 1999;11:643–655.
42. Yamamoto M, Rennert P, McGhee JR, et al. Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. *J Immunol.* 2000;164:5184–5191.
43. Mora JR, Bono MR, Manjunath N, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature.* 2003;424:88–93.
44. Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science.* 2004;303:1662–1665.
45. Schulz O, Jaensson E, Persson EK, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med.* 2009;206:3101–3114.
46. Hagiwara M, Kataoka K, Arimochi H, et al. Role of unbalanced growth of gram-negative bacteria in ileal ulcer formation in rats treated with a non-steroidal anti-inflammatory drug. *J Med Invest.* 2004;51:43–51.
47. Obata T, Goto Y, Kunisawa J, et al. Indigenous opportunistic bacteria inhabit mammalian gut-associated lymphoid tissues and share a mucosal antibody-mediated symbiosis. *Proc Natl Acad Sci U S A.* 2010;107:7419–7424.

Send Orders for Reprints to reprints@benthamscience.net

Current Pharmaceutical Design, 2014, *20*, 879-885

Flavonoids for Allergic Diseases: Present Evidence and Future Perspective

Toshio Tanaka[a,b*]

[a]*Department of Clinical Application of Biologics, Osaka University Graduate School of Medicine, and* [b]*Department of Immunopathology, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan*

Abstract: The prevalence of allergic diseases has increased worldwide during the past two decades. Change of dietary habit is thought to be one of the environmental factors, which has caused such an increase and worsened allergic symptoms, which suggests that an appropriate dietary habit may substantially prevent the onset of allergic diseases and ameliorate allergic symptoms. Flavonoids, which are polyphenolic plant secondary metabolites ubiquitously present in vegetables, fruits and beverages, possess antioxidant and anti-allergic activities as well as immune-modulating traits. Flavonoids inhibit the activation of mast cells and basophils and therefore suppress the release of chemical mediators, synthesis of Th2 type cytokines such as interleukin (IL)-4 and IL-13, and CD40 ligand expression, They also reportedly inhibit IL-4-induced signal transduction and affect the differentiation process of naïve CD4+ T cells into effector T cell subsets. A cohort epidemiological study in Finland found a significantly low incidence of asthma in a population with a high intake of flavonoids. Moreover, various studies of flavonoids in allergic models such as asthma, atopic dermatitis, anaphylaxis and food allergy demonstrated their beneficial effects, while experience in humans is at the moment limited to respiratory allergy. Although further validation is required, it is anticipated that an appropriate intake of flavonoids may play a role in the prevention and eventually in the management of allergic diseases.

Keywords: Allergic rhinitis, allergy, asthma, complementary and alternative medicine, dietary treatment, flavonoid, prevention.

INTRODUCTION

The prevalence of allergic diseases including allergic rhinitis, asthma, atopic dermatitis and food allergy has increased worldwide during the past two decades [1, 2]. It is believed that the interaction between environmental and genetic factors causes individuals to be sensitized to environmental allergens and to suffer from these diseases [3-5]. Since foods and beverages contain both allergy-promoting and anti-allergic nutrients, it has been proposed that change of dietary habit may be one of the environmental factors responsible for the increase [6-10]. Vitamins A, C, D and E, minerals such as selenium, copper, zinc and magnesium, probiotics, and omega-3 polyunsaturated fatty acids (PUFAs) as well as polyphenols have been shown to possess anti-allergic properties. Conversely, omega-6 PUFAs are precursors for leukotriene C4, which is known to promote allergic inflammation. Flavonoids, on the other hand, which are polyphenolic plant secondary metabolites, can have powerful antioxidant and anti-allergic activities as well as immune-modulating effects [11, 12]. This review article discusses the possibility that an appropriate intake of flavonoids may play a role in the prevention and eventually in the management of allergic diseases on the basis of recent findings.

FLAVONOIDS MODULATE IGE-MEDIATED IMMUNE RESPONSE

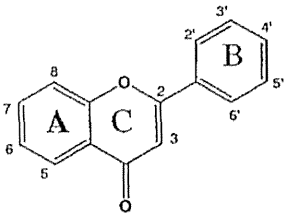
Fifteen years ago we evaluated the clinical effect of one kind of traditional vegetarian diet on 20 adult patients with atopic dermatitis. The patients were given a glass of fresh vegetable juice corresponding to 250 g of fresh vegetables including spinach, komatsuna (brassica rapa var. perviridis), cabbage, pak-choi, and garland chrysanthemum at breakfast. For lunch and dinner, brown rice porridge corresponding to 80 g of brown rice sprinkled with 5 g of kelp powder, soybean curd (tofu: wet weight approximately 200 g) and 10 g of sesame paste. Instead of water, the patients were given persimmon leaf tea (1-2 l/day). After a two-month treatment, the value of severity of dermatitis evaluated by the scoring atopic dermatitis (SCORAD) had decreased significantly from 49.9±18.6 to 27.4±16.8 in association with a reduction in the urinary secretion of 8-hydroxy-2'-deoxyguanosine, one of markers of oxidative DNA damage [13, 14]. What factor(s) caused this amelioration of dermatitis remained unknown but it was found that one of the characteristics of this vegetarian diet was a high daily intake of flavonoids.

Flavonoids comprise a large group of low-molecular-weight polyphenolic plant metabolites that are found in fruits, vegetables and beverages, and thus are common substances in the daily diet [11, 12]. Flavonoids, which share a common structure consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), are classified into 6 subclasses: flavones (including luteolin and apigenin), flavonols (fisetin, kaempferol, quercetin and myricetin) (Fig. **1**), flavanones (hesperetin, naringenin, and eriodictyol), isoflavones (daidzein and genistein), anthocyanidins (cyanidin and pelargonidin) and flavanols (catechins and proanthocyanidins) [15]. It has been shown that flavonoids exert various biological activities, that is, antioxidant, anti-inflammatory, anticarcinogenic, anti-obesity, anti-diabetic and immune-modulating effects as well as anti-allergic effects [11, 12, 16-21], and epidemiological evidence of the role of flavonoid intake against the risk of chronic diseases is promising [22, 23].

IgE-mediated immune responses consist of sensitization phase and effector phase. Flavonoids have been shown to possess anti-allergic properties affecting both phases. Fewtress and Gomperts first reported the inhibition of histamine secretion by flavones in rat mast cells [24], which was followed by the discovery of the inhibition of allergen-stimulated human basophils by quercetin [25, 26]. Flavonoids were also found to suppress hexosaminidase release from rat mast cells [27] and cysteinyl leukotriene synthesis through inhibition of phospholipase A_2 and 5-lipoxygenase [28, 29]. Moreover, luteolin, quercetin and baicalein were found to inhibit the synthesis of granulocyte macrophage-colony stimulating factor (GM-CSF) by human cultured mast cells in response to cross-linkage of a high-affinity IgE receptor, FcεRI [30], and it was subsequently demonstrated that these flavonoids inhibited IgE-mediated tumor necrosis factor (TNF)-α and interleukin (IL)-6

*Address correspondence to this author at the Department of Clinical Application of Biologics, Osaka University Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan; Tel: +81-6-6879-3838; Fax: +81-6-6879-3839;
E-mail: ttanak@imed3.med.osaka-u.ac.jp

1873-4286/14 $58.00+.00



Flavone (2-phenylchromen-4-one)

	3	5	6	7	8	2'	3'	4'	5'	6'
Luteolin	H	OH	H	OH	H	H	OH	OH	H	H
Apigenin	H	OH	H	OH	H	H	H	OH	H	H
Scutellarein	H	OH	OH	OH	H	H	OH	H	H	H
Baicalein	H	OH	OH	OH	H	H	H	H	H	H
Chrysin	H	OH	H	OH	H	H	H	H	H	H
Rutin	O-Rutinose	OH	H	OH	H	H	OH	OH	H	H
Isoquercitrin	O-Glc	OH	H	OH	H	H	OH	OH	H	H
Astragalin	O-Glc	OH	H	OH	H	H	H	OH	H	H
EMIQ	O-$(Glc)_{1\sim8}$	OH	H	OH	H	H	OH	OH	H	H

Flavonol (3-hydroxy-2-phenylchromen-4-one)

	3	5	6	7	8	2'	3'	4'	5'	6'
Fisetin	OH	H	H	OH	H	H	OH	OH	H	H
Kaempferol	OH	OH	H	OH	H	H	H	OH	H	H
Quercetin	OH	OH	H	OH	H	H	OH	OH	H	H
Myricetin	OH	OH	H	OH	H	H	OH	OH	OH	H

Fig. (1). Structure of basic flavonoid skeletons and representative flavones and flavonols
EMIQ; enzymatically modified isoquercitrin

production by murine mast cells [31]. In addition, we found that some kinds of flavonoids possessed the inhibitory activity of IL-4 and IL-13 synthesis by either allergen- or anti-IgE antibody-stimulated peripheral blood purified basophils. Among 45 kinds of flavones, flavonols and their related compounds examined, luteolin, apigenin and fisetin were the most powerful inhibitors and the half-maximal inhibitory concentration (IC_{50}) value of these flavonoids for inhibition of IL-4 synthesis ranged from 2.7 to 5.8 μM [32-34]. Quercetin and kaempferol, which are representative of flavonoids associated with a substantial daily intake, were found to have a moderate but substantial inhibitory effect on IL-4 synthesis with an IC_{50} value of 15.7-18.8 μM, but myricetin showed no such effect even at 30 μM. These analyses of structure-activity relationship determined the fundamental structure of flavonoids for the activity and for the maximal inhibition, the presence of OH in positions 7 and 4' is essential and additionally that in either position 3 or 5 is required (Fig. **1**). Similarly, luteolin, apigenin and fisetin also suppressed CD40 ligand expression by activated basophils, whereas myricetin did not reveal such an effect [35]. Both the interaction of CD40 ligand with CD40 and the action of IL-4 or IL-13 on B cells are required for B cells to differentiate into IgE-secreting cells [36], so that flavonoids such as luteolin, apigenin and fisetin are potential natural IgE inhibitors.

In addition to the inhibitory effect of flavonoids on IL-4 and IL-13 synthesis in FcεRI-expressing cells, kaempferol reportedly inhibits the IL-4-induced signal transducer and activator of transcription (STAT)6 activation by targeting Janus kinase (JAK)3 in hematopoietic cell lines, which thus constitutes another anti-allergic activity of flavonoids [37].

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor involved in the regulation of biological responses to planar aromatic hydrocarbons such as dioxins [38]. An AhR-based *in vitro* bioassay of the dioxin [2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)] revealed that apigenin, luteolin, baicalein, quercetin, kaempferol and myricetin had noticeable inhibitory effects on AhR activation with an EC_{70} value (equal to 70% of the maximal response to TCDD) of 1.9-5.1 μM, whereas marked AhR activation was shown by daidzein, resveratrol, naringenin and baicalein at higher concentrations [39]. It has recently been shown that AhR regulates the differentiation of naïve CD4+ T cells into effector T cell subsets [40-43], which suggests that flavonoids can modulate immune functions through their binding to AhR.

Moreover, the presence of flavonoids in an allergic source may directly modulate the allergenicity of a particular protein [44], and flavonoids are potential to alter gut microecology and, by affecting the beneficial microflora in the gut, may confer positive gut health benefits and have some influence on food allergy as well [45].

EPIDEMIOLOGICAL REPORTS ON THE RELATIONSHIP BETWEEN FLAVONOID INTAKE AND ASTHMA

Several epidemiological studies have reported that a high intake of fresh fruit and vegetables, both of which include flavonoids, may provide protection against asthma [46, 47]. A population-based case-control study of 607 cases and 864 controls in South London, UK indicated that apple consumption was negatively associated with asthma while red wine intake was negatively associated with asthma severity [48]. The authors speculated that the associations between apple and red wine consumption and asthma might indicate a protective effect of flavonoids. The subsequent study of dietary intake of catechins, flavonols and flavones by the same research group, however, did not find any significant association with asthma prevalence and severity [49]. A cohort epidemiological study of 10,054 adults in Finland concerning the association between flavonoid intake and chronic diseases found that asthma incidence was lower at higher quercetin and hesperetin intakes [50].

A longitudinal birth cohort study of maternal intake during pregnancy, in which 1,253 children participated at 5 years, reported that maternal apple intake was beneficially associated with ever-wheeze, ever-asthma and doctor-confirmed asthma in children [51]. The Irish Life-ways Cross-Generation Cohort Study reported an association between high maternal fruit and vegetable intake during

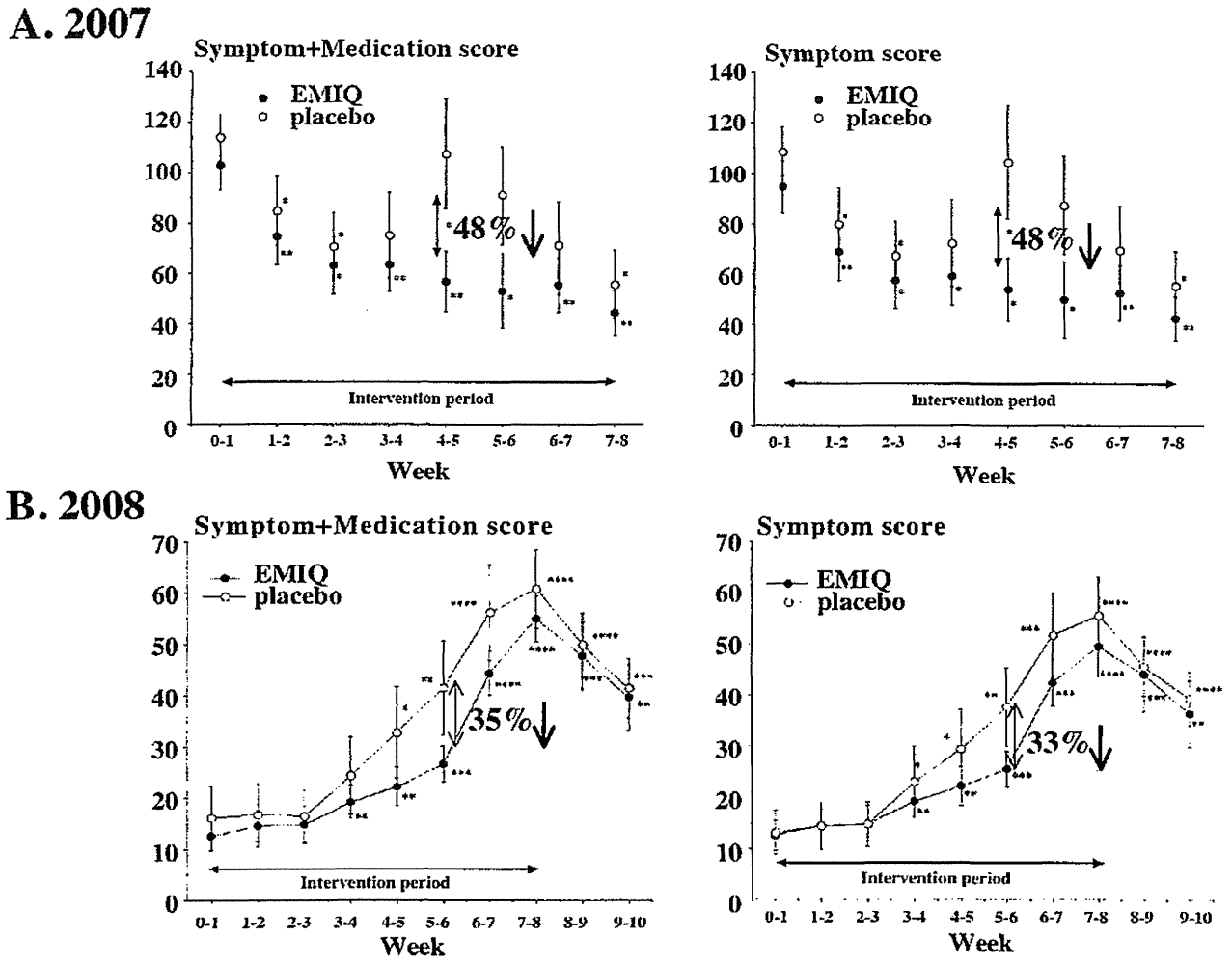


Fig. (2). Effects of enzymatically modified isoquercitrin on allergic symptoms caused by Japanese cedar pollinosis
The ameliorative effect of enzymatically modified isoquercitrin (EMIQ) on the total symptom (nasal and ocular symptoms)+medication scores and total symptom scores related to Japanese cedar pollinosis in 2007 [97] and 2008 [98].

pregnancy and reduced likelihood of asthma for 632 three-year-old children [52].

These findings mentioned here suggest that higher flavonoid intake is beneficial for asthma. However, there have been so far few reports of epidemiological studies examining direct associations between flavonoid intake and the prevalence or incidence of asthma. Recent development of databases of the flavonoid content of vegetables, fruits and beverages such as by the US Department of Agriculture (USDA) [53], the European BioActive Substances in Food Informative System (EuroFIR-BASIS) [54] and the Phenol-Explorer [55, 56] can definitely contribute to epidemiological studies aimed at clarifying the relationship between flavonoid intake and the prevalence, incidence or severity of asthma. By using the Phenol-Explorer database, the total intake of flavonoids was reported to be an average amount of 506 mg/day with 51 mg/day of flavonols and 33 mg/day of flavones in France [57], 370.2 mg/day with 24.8 mg/day of flavonols and 5.6 mg/day of flavones in Mediterranean countries and 373.7 mg/day with 29.5 mg/day of flavonols and 4.1 mg/day of flavones in non-Mediterranean countries [58].

EFFECT OF FLAVONOIDS IN ALLERGIC ANIMAL MODELS

Based on the anti-allergic and immune-modulating characteristics of flavonoids observed *in vitro*, it was anticipated that administration of flavonoids might produce beneficial effects on allergic diseases. NC/Nga mice are considered to be atopic dermatitis model mice and spontaneously develop severe eczema, scratching behaviour and serum IgE elevation with aging under nonspecific pathogen-free conditions [59]. To determine the preventive effect of flavonoids, the mice were orally given astragalin, kaempferol 3'glucoside (1.5 mg/kg) (Fig. 1) or a control diet before the onset of dermatitis. Development of dermatitis, scratching behaviour and serum IgE elevation with aging were observed in the control group whereas oral intake of astragalin markedly prevented the appearance of the skin symptoms, scratching behaviour and serum IgE elevation [60]. Moreover, astragalin had a therapeutic effect on the dermatitis [61]. It was shown with NC/Nga mice that administration of an extract from petals of *Impatiens balsamina L.*, containing kaempferol 3-rutinoside and 2-hydroxy-1,4-naphthoquinone as active ingredients [62], of apigenin [63], or of baicalein [64] suppressed skin lesions.

It was further demonstrated that in an asthmatic mouse model sensitized with ovalbumin (OVA) oral administration of luteolin, even as little as 0.1 mg/kg, led to a significant suppression of bronchial hyper-reactivity and bronchoconstriction [65]. It was also reported that a polymethoxyflavonoid nobiletin, when administered intraperitoneally to OVA-sensitized rats at a dose of 1.5 or 5 mg/kg, could reduce OVA-induced increases in eosinophils and eotaxin expression [66]. Subsequent investigations found that numerous

flavonoids such as quercetin, isoquercitrin, rutin, 3-*O*-methyl-quercetin 5,7,3',4'-*O*-tetraacetate, narirutin, apigenin, luteolin, sulfuretin, hesperdin, fisetin, kaempferol, acacetin, silibinin, naringin, limonene, chrysin, genistein, and skullcapflavone II produced improvement in asthmatic models [67-88]. Moreover quercetin effectively quelled peanut-induced anaphylactic reactions in peanut-sensitized rats. [89].

HUMAN INTERVENTION STUDIES USING FLAVONOIDS

These findings regarding the *in vitro* and *in vivo* anti-allergic properties of flavonoids support the idea that an appropriate intake of flavonoids may ameliorate allergic symptoms and/or prevent the onset of allergic diseases in humans [34, 90-93]. Indeed, the results of previous several clinical studies using flavonoid extracts indicate that flavonoids have beneficial effects on symptoms related to allergic rhinitis [94-98]. The extracts examined were *Perilla frutescens* (rosmarinic acid as a major flavonoid), apple polyphenols (procyanidins, or apple condensed tannin, catechin, epicatechin, phlorizin, and chlorogenic acid), hop water extract (quercetin and kaempferol glycosides), and tomato extract (naringenin chalcone). However, the direct effect of flavonoids on allergic symptoms remained unknown.

Enzymatically modified isoquercitrin (EMIQ) is a quercetin glycoside that consists of isoquercitrin and its maltooligosaccharides (Fig. **1**), and is manufactured from rutin through an enzymatic modification, which markedly enhances the bioavailability [99]. This flavonoid is approved as a food additive in Japan. Clinical trials to evaluate efficacy of EMIQ for Japanese cedar pollinosis were performed in 2007 and 2008 [100, 101]. In a parallel-group, double-blind, placebo-controlled design, patients with Japanese cedar pollinosis were randomly assigned to the EMIQ group or the placebo group. The EMIQ capsule contained 50 mg EMIQ, while the placebo capsule looked identical to the EMIQ capsule. The 2007 study began after the pollen had dispersed and thus examined the therapeutic effect of EMIQ, while the 2008 study began 3 weeks before the first day of pollen dispersion to evaluate the preventive effect of EMIQ on symptoms of pollinosis. The daily intake for these studies was 100 mg EMIQ for 8 weeks. The respective total symptom (nasal and ocular symptoms)+medication scores and total symptom scores of the two studies are shown in (Fig. **2**). The scores for the EMIQ groups in the 2007 and 2008 trials were optimally lower by 48% and 33-35%, respectively, compared with the scores for the placebo groups, indicating substantial ameliorative effect of EMIQ. In addition, a randomized clinical trial of silymarin demonstrated its statistically effective role in alleviating the severity of allergic rhinitis symptoms [102]. A randomized, double-blind, placebo-controlled study of pycnogenol [103], a proprietary mixture of water-soluble bioflavonoids extracted from French maritime pine, which contains proanthocyanidines, showed its ameliorative effect on seasonal allergic rhinitis symptoms.

Pycnogenol was also effective for asthma. The first study was performed to evaluate its effect on asthma in a randomized, double-blinded, placebo-controlled, crossover design in which 26 patients with varying asthma severity were enrolled [104]. The patients were randomly assigned to receive either 1 mg/lb/day (maximum 200 mg/day) pycnogenol or a placebo for 4 weeks and then crossed over to the alternate regimen for the next 4 weeks. 22 patients who completed the study responded favorably to pycnogenol and pycnogenol treatment led to a significant reduction in serum leukotrienes compared with the response to the placebo. Subsequently, a randomized, placebo-controlled, double-blind study involving 60 asthmatic patients, aged 6-18 years old was performed to evaluate the effect of pycnogenol on mild-to-moderate asthma [105]. Compared with the placebo group, the pycnogenol group showed significantly greater improvement in lung function and asthmatic symptoms in association with a significant reduction in urinary leukotrienes at 3 months, which resulted in a reduction in or discontinuation of the use of rescue inhalers in the pycnogenol group. Another recent study, which evaluated for six months the efficacy of pycnogenol for improving allergic asthma management of patients, also showed a favorable result [106]. In this study, a daily dosage of 100 mg of pycnogenol proved to be effective for better control of signs and symptoms of allergic asthma and could reduce the need for medication.

CONCLUSION

Allergy has become a common disease worldwide because of its increasing rate of prevalence [1, 2]. Dietary changes may contribute to this increase as one of environmental factors [6-10], and recent studies have shown that flavonoids, which have antioxidant and anti-allergic activities as well as immune-modulating properties, exert their potent beneficial effect on allergic rhinitis as well as asthma. Furthermore, recent development of databases of the flavonoid content of foods and beverages [53-56] can definitely make a contribution to epidemiological studies aimed at clarifying the direct association between flavonoid intake and the prevalence, incidence or severity of allergic diseases as well as to the development of the dietary menu for allergic diseases. Whether an appropriate intake of flavonoids can in fact constitute a dietary management for the prevention and amelioration of allergic diseases is thus an important issue for future studies. To achieve this goal, further well-designed, adequately powered trials are needed to determine the value of this dietary management [107, 108]. Moreover, some issues need to be addressed such as the modulating potency of the different flavonoids, the characterization of the bioavailability, the safety of long term administration, the administration route of flavonoids and the precise effect on cells on different anatomic sites.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

The author thanks Dr. Mari Kawai, Dr. Toru Hirano, Dr. Shinji Higa, Ms. Mihoko Koyanagi, Ms. Tomoko Kai, Mr. Ryosuke Shimizu, Mr. Hisashi Tanaka, Dr. Masamitsu Moriwaki, Dr. Yukio Suzuki, and Dr. Satoshi Ogino as collaborators for the clinical studies.

ABBREVIATIONS

PUFA	=	Polyunsaturated fatty acid
SCORAD	=	Scoring atopic dermatitis
GM-CSF	=	Granulocyte macrophage-colony stimulating factor
TNF	=	Tumor necrosis factor
IL	=	Interleukin
IC_{50}	=	Half-maximal inhibitory concentration
STAT	=	Signal transducer and activator of transcription
JAK	=	Janus kinase
AhR	=	Aryl hydrocarbon receptor
TCDD	=	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
USDA	=	US Department of Agriculture
EuroFIR-BASIS	=	European BioActive Substances in Food Informative System
OVA	=	Ovalbumin
EMIQ	=	Enzymatically modified isoquercitrin

REFERENCES

[1] Holgate ST. The epidemic of allergy and asthma. Nature 1999; 402(6760 Suppl): B2-4.

[2] Eder W, Ege MJ. von Mutius E. The asthma epidemic. N Engl J Med 2006; 355: 2226-35.
[3] Nolte H, Backer V, Porsbjerg C. Environmental factors as a cause for the increase in allergic disease. Ann Allergy Asthma Immunol 2001; 87(6 Suppl 3): 7-11.
[4] Ho SM. Environmental epigenetics of asthma: an update. J Allergy Clin Immunol 2010; 126: 453-65.
[5] Kauffmann F, Demenais F. Gene-environment interactions in asthma and allergic diseases: Challenges and perspectives. J Allergy Clin Immunol 2012; 130: 1229-40.
[6] McKeever TM, Britton J. Diet and asthma. Am J Respir Crit Care Med 2004; 170: 725-9.
[7] Devereux G, Seaton A. Diet as a risk factor for atopy and asthma. J Allergy Clin Immunol 2005; 115: 1109-17.
[8] Raviv S, Smith LJ. Diet and asthma. Curr Opin Pulm Med 2010; 16: 71-6.
[9] Nurmatov U; Devereux G, Sheikh A. Nutrients and foods for the primary prevention of asthma and allergy: systemic review and meta-analysis. J Allergy Clin Immunol 2011; 127: 724-33.
[10] Allan K, Devereux G. Diet and asthma: nutrition implications from prevention to treatment. J Am Diet Assoc 2011; 111: 258-68.
[11] Hollman PC, Katan MB. Health effects and bioavailability of dietary flavonols. Free Rad Res 1999; 31 Suppl: S75-80.
[12] Middleton EJ, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000; 52: 673-751.
[13] Tanaka T, Kouda K, Kotani M, *et al.* Vegetarian diet ameliorates symptoms of atopic dermatitis through reduction of the number of peripheral eosinophils and of PGE2 synthesis by monocytes. J Physiol Anthropol Appl Human Sci 2001; 20: 353-61.
[14] Kouda K, Tanaka T, Kouda M, *et al.* Low-energy diet in atopic dermatitis patients: clinical findings and DNA damage. J Physiol Anthropol Appl Human Sci 2000; 19: 225-8.
[15] Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004; 79: 727-47.
[16] Williams CA, Grayer RJ. Anthocyanins and other flavonoids. Nat Prod Rep 2004; 21: 539-73.
[17] Chirumbolo S. The role of quercetin, flavonols and flavones in modulating inflammatory cell function. Inflamm Allergy Drug Targets 2010; 9: 263-85.
[18] Visioli F, De La Lastra CA, Andres-Lacueva C, *et al.* Polyphenols and human health: a prospectus. Crit Rev Food Sci Nutr 2011; 51: 524-46.
[19] Calderon-Montano JM, Burgos-Moron E, Perez-Guerrero C, Lopez-Lazaro M. A review on the dietary flavonoid kaempferol. Mini Rev Med Chem 2011; 11: 298-344.
[20] Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL. The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem Pharmacol 2012; 83: 6-15.
[21] Magrone T, Jirillo E. Influence of polyphenols on allergic immune reactions: mechanisms of action. Proc Nutr Soc 2012; 71: 316-21.
[22] Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr 2005; 81(1 Suppl): 317S-25S.
[23] Hooper L, Kroon PA, Rimm, EB, *et al.* Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. Am J Clin Nutr 2008; 88: 38-50.
[24] Fewtrell CM, Gomperts BD. Effect of flavone inhibitors on transport ATPases on histamine secretion from rat mast cells. Nature 1997; 265: 635-6.
[25] Middleton EJ, Drzewiecki G, Krishnarao D. Quercetin: an inhibitor of antigen-induced human basophil histamine release. J Immunol 1981; 127: 546-50.
[26] Middleton EJ, Kandaswami C. Effects of flavonoids on immune and inflammatory cell functions. Biochem Pharmacol 1992; 43: 1167-79.
[27] Cheong H, Ryu SY, Oak MH, Cheon SH, Yoo GS, Kim KM. Studies of structure activity relationship of flavonoids for the anti-allergic actions. Arch Pharm Res 1998; 21: 478-80.
[28] Lee TP, Matteliano ML, Middleton EJ. Effect of quercetin on human polymorphonuclear leukocyte lysosomal enzyme release and phospholipid metabolism. Life Sci 1982; 31: 2765-74.
[29] Yoshimoto T, Furukawa M, Yamamoto S, Horie T, Watanabe-Kohno S. Flavonoids: potent inhibitors of arachidonate 5-lipoxygenase. Biochem Biophys Res Commun 1983; 116: 612-8.
[30] Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. Clin Exp Allergy 2000; 30: 501-8.
[31] Kimata M, Inagaki N, Nagai H. Effects of luteolin and other flavonoids on IgE-mediated allergic reactions. Plant Med 2000; 66: 25-9.
[32] Higa S, Hirano T, Kotani M, *et al.* Fisetin, a flavonol, inhibits TH2-type cytokine production by activated human basophils. J Allergy Clin Immunol 2003; 111: 1299-306.
[33] Hirano T, Higa S, Arimitsu J, *et al.* Flavonoids such as luteolin, fisetin and apigenin are inhibitors of interleukin-4 and interleukin-13 production by activated human basophils. Int Arch Allergy Immunol 2004; 134: 135-40.
[34] Kawai M, Hirano T, Higa S, *et al.* Flavonoids and related compounds as anti-allergic substances. Allergol Int 2007; 56: 113-23.
[35] Hirano T, Arimitsu J, Higa S, *et al.* Luteolin, a flavonoid, inhibits CD40 ligand expression by activated human basophils. Int Arch Allergy Immunol 2006; 140: 150-6.
[36] Yanagihara Y. Regulatory mechanisms of human IgE synthesis. Allegol Int 2003; 52: 1-12.
[37] Cortes JR, Perez-G M, Rivas MD, Zamorano J. Kaempferol inhibits IL-4-induced STAT6 activation by specifically targeting JAK3. J Immunol 2007; 179: 3881-7.
[38] Connor KT, Aylward LL. Human response to dioxin: aryl hydrocarbon receptor (AhR) molecular structure, function, and dose-response data for enzyme induction indicate an impaired human AhR. J Toxicol Environ Health B Crit Rev 2006; 9: 147-71.
[39] Amakura Y, Tsutsumi T, Sasaki K, Nakamura M, Yoshida T, Maitani T. Influence of food polyphenols on aryl hydrocarbon receptor-signaling pathway estimated by *in vitro* bioassay. Phytochemistry 2008; 69: 3117-30.
[40] Quintana FJ, Basso AS, Iglesias AH, *et al.* Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature 2008; 453: 65-71.
[41] Veldhoen M, Hirota K, Westendorf AM, *et al.* The aryl hydrocarbon receptor links Th17-cell-mediated autoimmunity to environmental toxins. Nature 2008; 453: 106-9.
[42] Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. Proc Natl Acad Sci USA 2008; 105: 9721-6.
[43] Marshall NB, Kerkvliet NI. Dioxin and immune regulation: emerging role of aryl hydrocarbon receptor in the generation of regulatory T cells. Ann NY Acad Sci 2010; 1183: 25-37.
[44] Chung S-Y, Champagne ET. Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. Food Chem 2009; 115: 1345-9.
[45] Parker SG, Stevenson DE, Skinner MA. The potential influence of fruit polyphenols on colonic microflora and human gut health. Int J Food Microbiol 2008; 124: 295-8.
[46] La Vecchia C, Decarli A, Pagano R. Vegetable consumption and risk of chronic disease. Epidemiology 1998; 9: 208-10.
[47] Butland BK, Strachan DP, Anderson HR. Fresh fruit intake and asthma symptoms in young British adults: confounding or effect modification by smoking? Eur Respir J 1999; 13: 744-50.
[48] Shaheen SO, Sterne JA, Thompson RL, Songhurst CE, Margetts BM, Burney PG. Dietary antioxidants and asthma in adults: population-based case-control study. Am J Respir Crit Care Med 2001; 164(10 Pt 1): 1823-8.
[49] Garcia V, Arts IC, Sterne JA, Thompson RL, Shaheen SO. Dietary intake of flavonoids and asthma in adults. Eur Respir J 2005; 26: 449-52.
[50] Knekt P, Kumpulainen J, Jarvinen R, *et al.* Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 2002; 76: 560-8.
[51] Willers SM, Devereux G, Craig LC, *et al.* Maternal food consumption during pregnancy and asthma, respiratory and atopic symptoms in 5-year-old children. Thorax 2007; 62: 773-9.
[52] Fitzsimon N, Fallon UB, O'Mahony D, *et al.* Lifeways Cross Generation Cohort Study Steering Group. Mothers' dietary patterns during pregnancy and the risk of asthma symptoms in children at 3 years. Irish Med J 2007; 100: suppl 27-32.
[53] USDA database for the flavonoid content of selective foods. Release 3. Prepared by Seema Bhagwat, David B. Haytowitz and Joanne M. Holden. U.S. Department of Agriculture. September 2011. Available from http://www.ars.usda.gov/nutrientdata

[54] Black L, Kiely M, Kroon P, Plumb J, Gry J. Development of Euro-FIR-BASIS – a composition and biological effects database for plant-based bioactive compounds. Nutr Bull 2008; 33: 58-61. Available from http://ebasis.eurofir.org.

[55] Neveu V, Perez-Jimenez J, Vos F, *et al.* Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. Database (Oxford) 2010; 2010: bap024.

[56] Rothwell JA, Urpi-Sarda M, Boto-Ordonez M, *et al.* Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. Database (Oxford) 2012; 2012:bas031. Available from http://www.phenol-explorer.eu

[57] Perez-Jimenez J, Fezeu L, Touvier M, *et al.* Dietary intake of 337 polyphenols in French adults. Am J Clin Nutr 2011; 93: 1220-8.

[58] Zamora-Ros R, Knaze V, Lujan-Barroso L, *et al.* Differences in dietary intakes, food sources and determinants of total flavonoids between Mediterranean and non-Mediterranean countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Br J Nutr 2012 Sep14: 1-10. [Epub ahead of print]

[59] Matsuda H, Watanabe N, Geba, GP, *et al.* Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 1997; 9: 461-6.

[60] Kotani M, Matsumoto M, Fujita A, *et al.* Persimmon leaf extract and astragalin inhibit development of dermatitis and IgE elevation in NC/Nga mice. J Allergy Clin Immunol 2000; 106(1 Pt 1): 159-66.

[61] Matsumoto M, Kotani M, Fujita A, *et al.* Oral administration of persimmon leaf extract ameliorates skin symptoms and transepidermal water loss in atopic dermatitis model mice, NC/Nga. Brit J Dermatol 2002; 146: 221-7.

[62] Oku H, Ishiguro K. Antipruritic and antidermatitic effects of extract and compounds of Impatiens balsamina L. in atopic dermatitis model NC mice. Phytother Res 2001; 15: 506-10.

[63] Yano S, Umeda D, Yamashita S, Yamada K, Tachibana H. Dietary apigenin attenuates the development of atopic dermatitis-like skin lesions in NC/Nga mice. J Nutr Biochem 2009; 20: 876-81.

[64] Yun MY, Yang JH, Kim DK, *et al.* Therapeutic effects of Baicalein on atopic dermatitis-like skin lesions of NC/Nga mice induced by dermatophagoides pteronyssinus. Int Immunopharmacol 2010; 10: 1142-8.

[65] Das M, Ram A, Ghosh B. Luteolin alleviates bronchoconstriction and airway hyperreactivity in ovalbumin sensitized mice. Inflamm Res 2003; 52: 101-6.

[66] Wu YQ, Zhou CH, Tao J, Li SN. Antagonistic effects of nobiletin, a polymethoxyflavonoid, on eosinophilic airway inflammation of asthmatic rats and relevant mechanisms. Life Sci 2006; 78: 2689-96.

[67] Rogerio AP, Kanashiro A, Fontanari C, *et al.* Anti-inflammatory activity of quercetin and isoquercitrin in experimental murine allergic asthma. Inflamm Res 2007; 56: 402-8.

[68] Jung CH, Lee JY, Cho CH, Kim CJ. Anti-asthmatic action of quercetin and rutin in conscious guinea-pigs challenged with aerosolized ovalbumin. Arch Pharm Res 2007; 30: 1599-607.

[69] Jiang JS, Chien HC, Chen CM, Lin CN, Ko WC. Potent suppressive effects of 3-O-methylquercetin 5,7,3',4'-O-tetraacetate on ovalbumin-induced airway hyperresponsiveness. Planta Med 2007; 73: 1156-62.

[70] Funaguchi N, Ohno Y, La BL, *et al.* Narirutin inhibits airway inflammation in an allergic mouse model. Clin Exp Pharmacol Physiol 2007; 34: 766-70.

[71] Park HJ, Lee CM, Jung ID, *et al.* Quercetin regulates Th1/Th2 balance in a murine model of asthma. Int Immunopharmacol 2009; 9: 261-7.

[72] Choi JR, Lee CM, Jung ID, *et al.* Apigenin protects ovalbumin-induced asthma through the regulation of GATA-3 gene. Int Immunopharmacol 2009; 9(7-8): 918-24.

[73] Li RR, Pang LL, Du Q, Shi Y, Dai WJ, Yin KS. Apigenin inhibits allergen-induced airway inflammation and switches immune response in a murine model of asthma. Immunopharmacol Immunotoxicol 2010; 32: 364-70.

[74] Leemans J, Cambier C, Chandler T, *et al.* Prophylactic effects of omega-3 polyunsaturated fatty acids and luteolin on airway hyperresponsiveness and inflammation in cats with experimentally-induced asthma. Vet J 2010; 184: 111-4.

[75] Song MY, Jeong GS, Lee HS, *et al.* Sulfuretin attenuates allergic airway inflammation in mice. Biochem Biophys Res Commun 2010; 400: 83-8.

[76] Kim SH, Kim BK, Lee YC. Antiasthmatic effects of hesperdin, a potential Th2 cytokine antagonist, in a mouse model of allergic asthma. Mediators Inflamm 2011; 2011: 485402.

[77] Wu MY, Hung SK, Fu SL. Immunosuppressive effects of fisetin in ovalbumin-induced asthma through inhibition of NF-kB activity. J Agric Food Chem 2011; 59: 10496-504.

[78] Gong JH, Shin D, Han SY, Kim JL, Kang YH. Kaempferol suppresses eosinophil infiltration and airway inflammation in airway epithelial cells and in mice with allergic asthma. J Nutr 2012; 142: 47-56.

[79] Huang WC, Liou CJ. Dietary acacetin reduces airway hyperresponsiveness and eosinophil infiltration by modulating eotaxin-1 and Th2 cytokines in a mouse model of asthma. Evid Based Complement Alternat Med 2012; 2012: 910520.

[80] Choi YH, Jin GY, Guo HS, *et al.* Silibinin attenuates allergic airway inflammation in mice. Biochem Biophys Res Commun 2012; 427: 450-5.

[81] Luo YL, Zhang CC, Li PB, *et al.* Naringin attenuates enhanced cough, airway hyperresponsiveness and airway inflammation in a guinea pig model of chronic bronchitis induced by cigarette smoke. Int Immunopharmacol 2012; 13: 301-7.

[82] Hirota R, Nakamura H, Bhatti SA, *et al.* Limonene inhalation reduces allergic airway inflammation in Dermatophagoides farinae-treated mice. Inhal Toxicol 2012; 24: 373-81.

[83] Du Q, Gu X, Cai J, Huang M, Su M. Chrysin attenuates allergic airway inflammation by modulating the transcription factors T-bet and GATA-3 in mice. Mol Med Report 2012; 6: 100-4.

[84] Gao F, Wei D, Bian T, *et al.* Genistein attenuated allergic airway inflammation by modulating the transcription factors T-bet, GATA-3 and STAT-6 in a murine model of asthma. Pharmacology 2012; 89(3-4): 229-36.

[85] Jang HY, Ahn KS, Park MJ, Kwon OK, Lee HK, Oh SR. Skullcapflavone II inhibits ovalbumin-induced airway inflammation in a mouse model of asthma. Int Immunopharmacol 2012; 12: 666-74.

[86] Goh FY, Upton N, Guan S, *et al.* Fisetin, a bioactive flavonol, attenuates allergic airway inflammation through negative regulation of NF-kB. Eur J Pharmacol 2012; 679(1-3): 109-16.

[87] Toledo AC, Sakoda CP, Perini A, *et al.* Flavonone treatment reverses airway inflammation and remodeling in an asthma murine model. Br J Pharmacol 2013; 168: 1736-49.

[88] Joskova M, Sadlonova V, Nosalova G, Novakova E, Franova S. Polyphenols and their components in experimental allergic asthma. Adv Exp Med Biol 2013; 756: 91-8.

[89] Shishebor F, Behroo L, Ghafouriyan Broujerdnia M, Namjoyan F, Latifi SM. Quercetin effectively quells peanut-induced anaphylactic reactions in the peanut sensitized rats. Iran J Allergy Asthma Immunol 2010; 9: 27-34.

[90] Tanaka T, Higa S, Hirano T, *et al.* Flavonoids as potential anti-allergic substances. Curr Med Chem-Anti-Inflammatory & Anti-Allergy Agents 2003; 2: 57-65.

[91] Tanaka T, Hirano T, Kawai M, *et al.* Flavonoids, natural inhibitors of basophil activation. In: Paul K. Vellis, Ed. Basophil Granulocytes. New York: Nova Science Publishers, Inc. 2011; pp. 61-72.

[92] Tanaka T. The effect of flavonoids on allergic diseases. Anti-Inflammatory & Anti-Allergy Agents Med Chem 2011; 10: 374-81.

[93] Tanaka T. Efficacy of flavonoids for patients with Japanese cedar pollinosis. In: Jose C. Jimenez-Lopez Ed. Current Insight in Pollen Allergens. Croatia: Intech-Open Access Company. 2012; pp. 103-122.

[94] Takano H, Osakabe N, Sanbongi C, *et al.* Extract of Perilla frutescens enriched for rosmarinic acid, a polyphenolic phytochemical, inhibits seasonal allergic rhinoconjunctivitis in humans. Exp Biol Med (Maywood) 2004; 229: 247-54.

[95] Kishi K, Saito M, Saito T, *et al.* Clinical efficacy of apple polyphenol for treating cedar pollinosis. Biosci Biotechnol Biochem 2005; 69: 829-32.

[96] Enomoto T, Nagasako-Akazome Y, Kanda T, Ikeda M, Dake T. Clinical effects of apple polyphenols on persistent allergic rhinitis: a randomized double-blind placebo-controlled parallel arm study. J Investig Allergol Clin Immunol 2006; 16: 283-9.

[97] Segawa S, Takata Y, Wakita Y, *et al.* Clinical effects of a hop water extract on Japanese cedar pollinosis during the pollen season:

a double-blind, placebo-controlled trial. Biosci Biotechnol Biochem 2007; 71: 1955-62.

[98] Yoshimura M, Enomoto T, Dake Y, *et al.* An evaluation of the clinical efficacy of tomato extract for perennial allergic rhinitis. Allergol Int 2007; 56: 225-30.

[99] Salim EI, Kaneko M, Wanibuchi H, Morimura K, Fukushima S. Lack of carcinogenicity of enzymatically modified isoquercitrin in F344/DuCrj rats. Food Chem Toxicol 2004; 42: 1949-69.

[100] Kawai M, Hirano T, Arimitsu J, *et al.* Enzymatically modified isoquercitrin, a flavonoid, on symptoms of Japanese cedar pollinosis: a randomized double-blind placebo-controlled trial. Int Arch Allergy Immunol 2009; 149: 359-68.

[101] Hirano T, Kawai M, Arimitsu J, *et al.* Preventative effect of a flavonoid, enzymatically modified isoquercitrin on ocular symptoms of Japanese cedar pollinosis. Allergol Int 2009; 58: 373-82.

[102] Bakhshaee M, Jabbari F, Hoseini S, *et al.* Effect of silymarin in the treatment of allergic rhinitis. Otolaryngol Head Neck Surg 2011; 145: 904-9.

[103] Wilson D, Evans M, Guthrie N, *et al.* A randomized, double-blind, placebo-controlled exploratory study to evaluate the potential of pycnogenol for improving allergic rhinitis symptoms. Phytother Res 2010; 24: 1115-9.

[104] Hosseini S, Pishnamazi S, Sadrzadeh SM, Farid F, Farid R, Watson RR. Pycnogenol® in the management of asthma. J Med Food 2001; 4: 201-9.

[105] Lau BH, Riesen SK, Truong KP, Lau EW, Rohdewald P, Barreta RA. Pycnogenol as an adjunct in the management of childhood asthma. J Asthma. 2004; 41: 825-32.

[106] Belcaro G, Luzzi R, Cesinaro Di Rocco P, *et al.* Pycnogenol improvements in asthma management. Panminerva Med 2011; 53(3 Suppl 1): 57-64.

[107] Schoonees A, Visser J, Musekiwa A, Volmink J. Pycnogenol® (extract of French maritime pine bark) for the treatment of chronic disorders® for the treatment of chronic disorders. Cochrane Database Syst Rev 2012 April 18; 4: CD008294.

[108] Singh A, Holvoet A, Mercenier A. Dietary polyphenols in the prevention and treatment of allergic diseases. Clin Exp Allergy 2011; 41: 1346-59.

Received: March 19, 2013 **Accepted: May 15, 2013**

Arthritis Care & Research
Vol. 66, No. 3, March 2014, pp 344–354
DOI 10.1002/acr.22110

ORIGINAL ARTICLE

Phase III Study of the Efficacy and Safety of Subcutaneous Versus Intravenous Tocilizumab Monotherapy in Patients With Rheumatoid Arthritis

ATSUSHI OGATA,[1] KAZUHIDE TANIMURA,[2] TOYOHIKO SUGIMOTO,[3] HIROSHI INOUE,[4] YUKITOMO URATA,[5] TSUKASA MATSUBARA,[6] MASAKAZU KONDO,[7] YUKITAKA UEKI,[8] MITSUHIRO IWAHASHI,[9] SHIGETO TOHMA,[10] SHUJI OHTA,[11] YUKIHIKO SAEKI,[12] TOSHIO TANAKA,[1] AND THE MUSASHI STUDY INVESTIGATORS

Objective. To evaluate the efficacious noninferiority of subcutaneous tocilizumab injection (TCZ-SC) monotherapy to intravenous TCZ infusion (TCZ-IV) monotherapy in Japanese patients with rheumatoid arthritis (RA) with an inadequate response to synthetic and/or biologic disease-modifying antirheumatic drugs (DMARDs).

Methods. This study had a double-blind, parallel-group, double-dummy, comparative phase III design. Patients were randomized to receive TCZ-SC 162 mg every 2 weeks or TCZ-IV 8 mg/kg every 4 weeks; no DMARDs were allowed during the study. The primary end point was to evaluate the noninferiority of TCZ-SC to TCZ-IV regarding the American College of Rheumatology criteria for 20% improvement in disease activity (ACR20) response rates at week 24 using an 18% noninferiority margin. Additional efficacy, safety, pharmacokinetic, and immunogenicity parameters were assessed.

Results. At week 24, ACR20 response was achieved in 79.2% (95% confidence interval [95% CI] 72.9, 85.5) of the TCZ-SC group and in 88.5% (95% CI 83.4, 93.5) of the TCZ-IV group; the weighted difference was −9.4% (95% CI −17.6, −1.2), confirming the noninferiority of TCZ-SC to TCZ-IV. Remission rates of the Disease Activity Score in 28 joints using the erythrocyte sedimentation rate and the Clinical Disease Activity Index at week 24 were 49.7% and 16.4% in the TCZ-SC group and 62.2% and 23.1% in the TCZ-IV group, respectively. Serum trough TCZ concentrations were similar between the groups over time. Incidences of all adverse events and serious adverse events were 89.0% and 7.5% in the TCZ-SC group and 90.8% and 5.8% in the TCZ-IV group, respectively. Anti-TCZ antibodies were detected in 3.5% of the TCZ-SC group; no serious hypersensitivity was reported in these patients.

Conclusion. TCZ-SC monotherapy demonstrated comparable efficacy and safety to TCZ-IV monotherapy. TCZ-SC could provide additional treatment options for patients with RA.

INTRODUCTION

Tocilizumab (TCZ) is a humanized monoclonal antibody directed against the interleukin-6 (IL-6) receptor that is approved for the treatment of patients with rheumatoid arthritis (RA), polyarticular-course and systemic juvenile idiopathic arthritis, and Castleman's disease by intravenous (IV) administration. Multiple phase III trials of TCZ, in combination with synthetic disease-modifying antirheumatic drugs (DMARDs) or as monotherapy, demonstrated an improvement of clinical symptoms and prevention of joint destruction (1–7).

ClinicalTrials.jp identifier: JapicCTI-101117.
Supported by Chugai Pharmaceutical Co., Ltd.
[1]Atsushi Ogata, MD, PhD, Toshio Tanaka, MD, PhD: Osaka University, Osaka, Japan; [2]Kazuhide Tanimura, MD: Hokkaido Medical Center for Rheumatic Diseases, Hokkaido, Japan; [3]Toyohiko Sugimoto, MD: National Hospital Organization, Shimoshizu Hospital, Chiba, Japan; [4]Hiroshi Inoue, MD: Inoue Hospital, Gunma, Japan; [5]Yukitomo Urata, MD: Seihoku Central Hospital, United Municipalities of Tsugaru, Aomori, Japan; [6]Tsukasa Matsubara, MD: Matsubara Mayflower Hospital, Hyogo, Japan; [7]Masakazu Kondo, MD: Kondo Clinic of Rheumatology and Orthopedic Surgery, Fukuoka, Japan; [8]Yukitaka Ueki, MD: Sasebo Chuo Hospital, Nagasaki, Japan; [9]Mitsuhiro Iwahashi, MD: