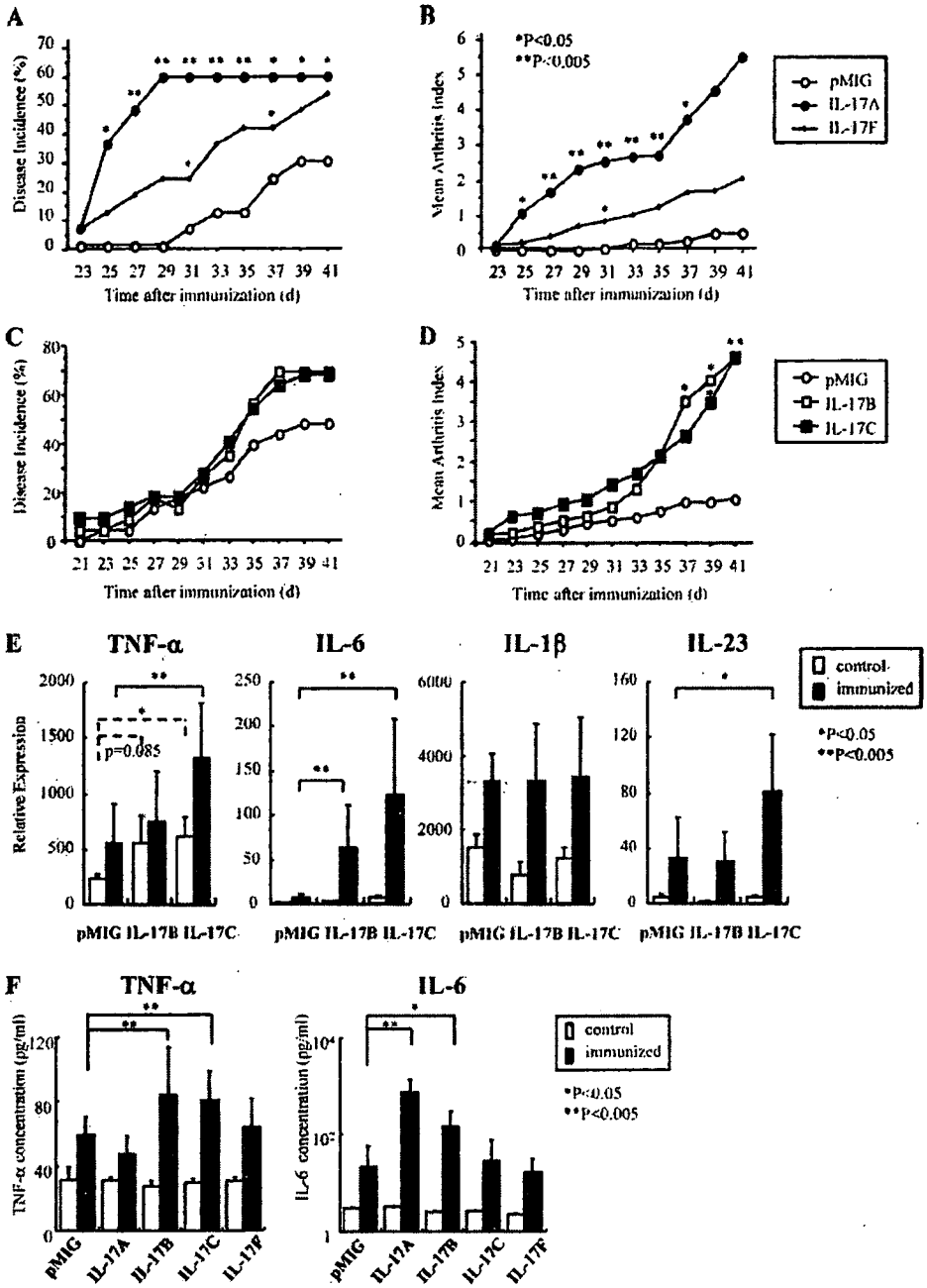

FIGURE 5. Incidence of CIA and arthritis scores in IL-17 family BM chimeric mice. Incidence of CIA and arthritis scores in IL-17A and IL-17F BM chimeric mice (*A* and *B*), and in IL-17B and IL-17C BM chimeric mice (*C* and *D*). Mice were immunized with BCII 8 wk after the BM transplantation. Values are the mean of experiments for IL-17A and IL-17F BM chimeric mice (n = 20 per group) and experiments for IL-17B and IL-17C BM chimeric mice (n = 30 per group). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$. *E*, The mRNA expression of inflammatory cytokines in the spleen of BM chimeric mice of IL-17B and IL-17C, which were immunized with BCII (■; n = 15 per group) or nonimmunized controls (□; n = 6 per group). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$. *F*, The secreted TNF-α and IL-6 levels in the serum of IL-17 family BM chimeric mice that were immunized with BCII (■; n = 15) or nonimmunized controls (□; n = 6). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$.

We next examined the alterations of inflammatory cytokine production in these BM chimeric mice. Interestingly, nonimmunized IL-17C BM chimeric mice showed increased mRNA expression of TNF-α in the spleen compared with controls (Fig. 5*E*). Moreover, in the spleen of BCII-immunized IL-17C BM chimeric mice, the mRNA expressions of TNF-α, IL-6, and IL-23 were up-regulated. In contrast, BCII-immunized IL-17B BM chimeric mice showed increased mRNA expression of IL-6 in the spleen compared with controls (Fig. 5*E*). When we examined the concentrations of TNF-α and IL-6 protein in the sera of IL-17 family BM chimeric mice, the BCII-immunized IL-17B and IL-17C BM chimeric mice showed increased TNF-α concentration in the sera. And the BCII-immunized IL-17A and IL-17B BM chimeric mice showed increased IL-6 production in the sera (Fig. 5*F*). These results suggested that IL-17B and IL-17C enhanced inflammation in this mouse model of arthritis by increased inflammatory cytokine production.

Neutralization of IL-17B significantly suppressed the progression of arthritis

As shown in Fig. 5, we found that IL-17B exacerbated the progression of CIA as well as IL-17A with the method of retrovirus-mediated BM chimeric mice. Regarding IL-17A, neutralizing Abs against IL-17A have been previously shown to be effective in the treatment of CIA (8). We examined the effect of IL-17B blockade in CIA mice. CIA mice were systemically treated with polyclonal anti-mouse IL-17B Abs immediately after the first signs of arthritis. Neutralization of IL-17B significantly suppressed the progression of CIA compared with the controls (Fig. 6*A*). Moreover, histological analysis revealed significant reduction of cell infiltration

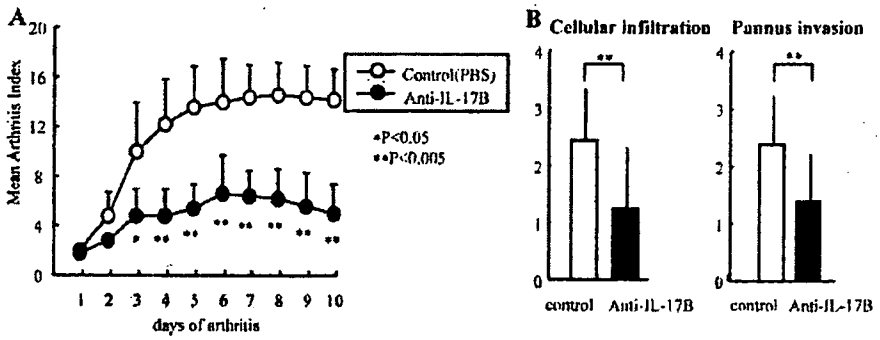

FIGURE 6. Effect of anti-IL-17B Ab treatment in CIA mice. *A*, CIA mice received i.p. injection of anti-mouse IL-17B Abs after the first clinical signs of arthritis (arthritis score between 1 and 2). As a control, PBS was injected. The arthritis score was shown. *B*, Histological score of the inflammatory joints of CIA mice treated with anti-IL-17B Abs was evaluated at 10 days after the onset of arthritis. Cellular infiltration and pannus invasion were graded in all four paws of the mice. Values are the mean of arthritis scores for anti-IL-17B Ab-treated mice and control mice (n = 5 per group). Significance of differences between control and anti-IL-17B Ab-treated mice was shown.

and pannus invasion in the anti-IL-17B Ab-treated mice (Fig. 6*B*). These results indicated that IL-17B was associated with the progression of arthritis in CIA mice.

Discussion

RA is considered to be an autoimmune disease, and is characterized by sustained inflammation of the joints and destruction of cartilage and bone. Several inflammatory cytokines are known to mediate the pathogenesis of arthritis, and TNF-α and IL-6 are the most important cytokines in the pathogenesis of RA. IL-17A, IL-17B, IL-17C, and IL-17F have the capacity to induce TNF-α production in PECs in vitro. In vivo, the mRNA expression of TNF-α was spontaneously increased in the spleen of IL-17C BM chimeric mice. Moreover, TNF-α productions in the sera of BCII-immunized IL-17B and IL-17C BM chimeric mice were up-regulated. Although IL-17A induced TNF-α production in PECs, IL-17A BM chimeric mice did not show up-regulated production of TNF-α. This result is consistent with previous observation in THP-1 cell line that IL-17B and IL-17C stimulated the release of TNF-α, whereas IL-17A has only a weak effect on TNF-α (17). In contrast to IL-17B and IL-17C, IL-17A may not be directly associated with TNF-α production in vivo. Moreover, the mRNA expression in the spleen and serum concentration of IL-6 were significantly up-regulated in IL-17B BM chimeric mice that were immunized with BCII. These results showed the close association of IL-17B and IL-17C with TNF-α and IL-6 in vivo and clearly suggested the importance of IL-17B and IL-17C in the pathogenesis of RA.

To date, the cell sources of IL-17B and IL-17C have not been identified. In this study, we showed that IL-17B was expressed in the inflammatory cartilage of CIA mice, whereas IL-17C was expressed in a broad range of cells, i.e., $CD4^+$ T cells, $CD11b^+$ MHC class II^+ macrophages, and $CD11c^+$ MHC class II^+ dendritic cells. IL-17A and IL-17F were expressed in $CD4^+$ T cells, as expected. These results suggested that $CD4^+$ T cells are involved in the expression of IL-17 family members, especially IL-17A, IL-17C, and IL-17F, at the inflammatory site. Although we did not detect a unique cell source of IL-17C, the arthritis-promoting effect of IL-17C-transduced $CD4^+$ T cells suggests the importance of IL-17C expressed in $CD4^+$ T cells.

In our in vivo analysis, we observed arthritis-promoting effects of the IL-17 family members. As shown in Fig. 3, the transfer of mIL-17A-, mIL-17B-, mIL-17C-, and mIL-17F-transduced $CD4^+$ T cells evidently exacerbated arthritis as assessed by the arthritis score. This effect was also confirmed in the CIA of the mIL-17A, mIL-17B, mIL-17C, and mIL-17F BM chimeric mice. The arthritis-promoting effect of IL-17A was previously reported in a study using adenovirus vector (5, 40). In contrast to IL-17A, which hastened the onset of arthritis, IL-17B and IL-17C did not affect the onset of arthritis evidently. This fact suggests that IL-17B and IL-17C affect arthritis rather in the effector phase. To our knowledge, this is the first observation of an in vivo arthritis-promoting effect of IL-17B and IL-17C.

Blockade of IL-17A has recently been shown to be effective in the treatment of CIA (8). In the present study, we have demonstrated the therapeutic potential of IL-17B blockade after the onset of CIA. Because blockade of TNF-α or IL-1β is not always effective in RA patients, blockade of additional cytokine might be a useful therapeutic option. Therefore, our data strongly suggest that IL-17B as well as IL-17A could be an important target for the treatment of inflammatory arthritis.

In a recent study, the combination of IL-6 and TGF-β was reported to strongly induce IL-17A production in Th17 cells (41). Moreover, it was recently recognized that IL-23 contributes to the expansion of autoreactive IL-17A-producing T cells and promotes chronic inflammation dominated by IL-17A, IL-6, IL-8, and TNF-α (14, 42). Thus, IL-17B and IL-17C may exacerbate arthritis via IL-6- and IL-23-mediated promotion of IL-17A production. However, the possibility that IL-17B and IL-17C exert a cooperative proinflammatory response together with IL-17A and IL-17F in arthritis by regulating the release of cytokines such as IL-6, IL-1β, and IL-23 still remains to be examined.

IL-17F has the highest homology with IL-17A and, like IL-17A, is produced by activated T cells. IL-17F appears to have an effect similar to that of IL-17A on cartilage proteoglycan release and inhibition of new cartilage matrix synthesis (11). Although IL-17F is thought to contribute to the pathology of inflammatory disorders such as RA, the in vivo effect of IL-17F on arthritis was not elucidated. In this study, we found that transduction of BM-expressed IL-17F resulted in both an earlier onset and a subsequent aggravation of arthritis.

We also found that the mRNA expression of all IL-17 family and IL-17R genes examined (mIL-17A, mIL-17B, mIL-17C, mIL-17F, mIL-17R, and mIL-17Rh1) was elevated in the arthritic paws of CIA mice compared with the paws of the control mice. The receptor for IL-17A is IL-17R (also named IL-17AR), which is extensively expressed in various tissues or cells tested, in contrast to the exclusive expression of IL-17A in activated T cells. Recently, IL-17R signaling has been suggested to play a crucial role in driving the synovial expression of proinflammatory and catabolic mediators, such as IL-1, IL-6, matrix metalloproteinase

(MMP)-3, MMP-9, and MMP-13, in streptococcal cell wall-induced arthritis (43). IL-17R-deficient (IL-17R$^{-/-}$) mice that were locally injected five times with streptococcal cell wall fragments into the knee joints showed a significant reduction of joint thickness and cartilage damage that was accompanied by reduced synovial expression of IL-1, IL-6, and the MMPs 3, 9, and 13 compared with arthritic wild-type mice. Therefore, these results indicate the critical role of IL-17R signaling during progression from an acute, macrophage-driven joint inflammation to a chronic, cartilage-destructive, T cell-mediated synovitis. There are four additional receptor-like molecules that share homology to IL-17R, i.e., IL-17Rh1 (also named IL-17RB or IL-17BR), IL-17RL (also named IL-17RC), IL-17RD, and IL-17RE. IL-17Rh1 was shown to bind to IL-17B, but with higher affinity to IL-17E (11, 12).

Although IL-17A transgenic mice have been reported to be embryonic lethal (39), we established BM-overexpressing mice that constitutively expressed IL-17A. The adequate control of the expression level was critically important. In our experiment, the serum concentration of IL-17A was elevated to ~600 pg/ml in IL-17A BM chimeric mice. This serum concentration of IL-17A was similar to those in patients with inflammatory diseases such as RA, inflammatory bowel diseases, familial Mediterranean fever, and the acute stage of Kawasaki disease (3, 44–46). Therefore, our BM chimeric mice approach may be useful to elucidate the physiological role of inflammatory cytokines that show lethal phenotypes in the conventional gene-transgenic technique.

In conclusion, we found that IL-17 family genes were up-regulated in association with their receptors in CIA. Each of the IL-17 family members clearly exacerbated the progression of CIA with the method of retrovirus-mediated BM chimeric mice. IL-17B and IL-17C have the capacity to exacerbate inflammatory arthritis in association with increased TNF-α and IL-6 productions from macrophages. Moreover, neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in CIA mice. Therefore, our results suggest that not only IL-17A, but also the IL-17 family members IL-17B, IL-17C, and IL-17F play an important role in the pathogenesis of inflammatory arthritis and should be a new therapeutic target of arthritis.

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Disclosures

The authors have no financial conflict of interest.

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Lipopolysaccharide-Induced Up-Regulation of Triggering Receptor Expressed on Myeloid Cells-1 Expression on Macrophages Is Regulated by Endogenous Prostaglandin E_2[1]

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Triggering receptor expressed on myeloid cells-1 (TREM-1) is a recently identified cell surface molecule that is expressed by neutrophils and monocytes. TREM-1 expression is modulated by various ligands for TLRs in vitro and in vivo. However, the influence of PGE_2, a potential mediator of inflammation, on TREM-1 expression has not been elucidated. In this study, we examined the effects of PGE_2 on LPS-induced TREM-1 expression by resident murine peritoneal macrophages (RPM) and human PBMC. PGE_2 significantly induced murine TREM-1 (mTREM-1) expression by RPM. Up-regulation of TREM-1 expression was specific to PGE_2 among arachidonic acid metabolites, while ligands for chemoattractant receptor-homologous molecule expressed on Th2 cells and the thomboxane-like prostanoid receptor failed to induce mTREM-1 expression. PGE_2 also increased expression of the soluble form of TREM-1 by PBMC. LPS-induced TREM-1 expression was regulated by endogenous PGE_2 especially in late phase (>2 h after stimulation), because cyclooxygenase-1 and -2 inhibitors abolished this effect at that points. A synthetic EP4 agonist and 8-Br-cAMP also enhanced mTREM-1 expression by RPM. Furthermore, protein kinase A, PI3K, and p38 MAPK inhibitors prevented PGE_2-induced mTREM-1 expression by RPM. Activation of TREM-1 expressed on PGE_2-pretreated PBMC by an agonistic TREM-1 mAb significantly enhanced the production of IL-8 and TNF-α. These findings indicate that LPS-induced TREM-1 expression on macrophages is mediated, at least partly, by endogenous PGE_2 followed by EP4 and cAMP, protein kinase A, p38 MAPK, and PI3K-mediated signaling. Regulation of TREM-1 and the soluble form of TREM-1 expression by PGE_2 may modulate the inflammatory response to microbial pathogens. *The Journal of Immunology,* **2007, 178: 1144–1150.**

Triggering receptor expressed on myeloid cells-1 (TREM-1)[3] is a recently discovered cell surface molecule that has been identified on neutrophils and monocytes (1, 2). The soluble form of TREM-1 (sTREM-1) is detected in bronchoalveolar lavage fluid from patients with microbial infection and has been demonstrated to act as an inhibitor of TREM-1 (3–6). TREM-1 is a 30-kDa glycoprotein belonging to the Ig superfamily and its expression is up-regulated by various ligands for TLRs (7–9). Activation of TREM-1 expressed on neutrophils and monocytes by an agonistic mAb has been shown to stimulate the expression of various proinflammatory cytokines, chemokines, and cell surface molecules (1, 7–9). Furthermore, LPS causes synergistic enhancement of cytokine production by monocytes in response to the agonistic mAb, indicating that TREM-1 amplifies inflammatory responses initiated by TLRs (1, 7–9). Although the natural ligands for TREM-1 have not been identified, its essential role in acute inflammatory responses has been demonstrated in murine models of septic shock, because blocking of TREM-1 by a sTREM-1 improves the survival of mice with bacterial sepsis (6, 9). Thus, activation of TREM-1 may play a crucial role in the inflammatory response to microbes.

PGs are multipotent mediators that modulate a number of pathophysiological responses. PGs are produced by metabolism of arachidonic acid through activation of cyclooxygenase (COX). COX has two isoforms, which are COX-1 and COX-2 (10). COX-1 is constitutively expressed, whereas COX-2 is expressed at low level by most normal resting cells. COX-2 expression is induced by various TLR ligands (11, 12) and release of PGs is significantly increased in various animal models of endotoxemia or sepsis (13, 14). In particular, PGE_2 has been shown to function as a mediator of sepsis-induced immunosuppression, an inhibitor of proinflammatory cytokine production by macrophages, and an inducer of IL-10 production (15). In contrast, PGE_2 has several detrimental effects in sepsis, including vasodilation and increased vascular permeability (16). Several previous studies have shown that COX inhibitors can improve the survival of mice after burn infection or administration of a lethal dose of LPS (17–19). These findings indicate that PGs play an important role in microbial inflammation, including sepsis or endotoxemia. However, the precise mechanisms by which PGs (particularly PGE_2) have a regulatory effect on microbial inflammation have not been determined.

Although TREM-1 is clearly induced by LPS, little is known regarding the biological influence of PGE_2 on TREM-1 during

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[3] Abbreviations used in this paper: TREM, triggering receptor expressed on myeloid cells; mTREM, murine TREM; hTREM, human TREM; sTREM, soluble form of TREM; hsTREM, human sTREM; PKA, protein kinase A; COX, cyclooxygenase; RPM, resident peritoneal macrophage; I-BOP, 1*S*-[1α,2α(*Z*),3β(1*E*,3*S*),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; TP, thromboxane-like prostanoid; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; EP, E-series of prostaglandin.

Table I. *Oligonucleotide primers and probes used for real-time PCR*

Target Gene	Type[a]	Primer or Probe (5′–3′)	Description (mer)
mTREM-1	F	CCAGAAGGCTTGGCAGAGACT	22
	B	ACTTCCCCATGTGGACTTCACT	22
mGAPDH	F	TGCAGTGGCAAAGTGGAGATT	21
	B	ATTTGCCGTGAGTGGAGTCAT	21
hTREM-1	F	GCCTTGTGCCCACTCTATACCA	22
	B	TGGAGACATCGGCAGTTGAC	20
	P	(FAM)CAGAACTGTGACCCAAGCTCCACCCA(TAMRA)	26
hsTREM-1	F	CCTCCCAAGGAGCCTCACA	19
	B	ACACCGGAACCCCTTGGT	18
	P	(FAM)CTGTTCGATCGCATCCGCTTGGT(TAMRA)	23

[a] F, forward primer; B, backward primer; and P, TaqMan Probe.

microbial inflammation. Therefore, we conducted this study to investigate the biological effects of PGE_2 on the expression and action of TREM-1.

Materials and Methods

Reagents

DI-004 (an EP1 agonist), AE1–259-01 (an EP2 agonist), AE-248 (an EP3 agonist), and AE1-329 (an EP4 agonist) were provided by Ono Pharmaceuticals. A monoclonal rat anti-mouse TREM-1 Ab and a monoclonal mouse anti-human TREM-1 Ab, as well as control mouse IgG1 and a polyclonal anti-actin Ab, were obtained from R&D Systems and Santa Cruz Biotechnology, respectively. HRP-conjugated rabbit anti-mouse IgG and HRP-conjugated rabbit anti-rat IgG were purchased from DakoCytomation. Specific ELISAs for human TNF-α and human IL-8 were obtained from BioSource International. 8-Bromoadenosine 3′, 5′ cyclic monophosphate (8-Br-cAMP), LPS, the MEK (MAPKK) inhibitor PD98059, the p38 MAPK inhibitor SB203580, and the PI3K inhibitor LY294002 were purchased from Sigma-Aldrich, while the protein kinase A (PKA) inhibitor H-89 was obtained from Seikagaku. PGD_2, PGE_2, 1*S*-[1α, 2α(*Z*),3β(1*E*,3*S*),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid (I-BOP), a COX-1 inhibitor (SC560), a COX-2 inhibitor (NS398), and a PGE_2 EIA kit were obtained from Cayman Chemical.

Cell culture

Resident peritoneal macrophages (RPM) were isolated from male ICR mice (6–8 wk old) as reported elsewhere (15). Heparinized peripheral blood was obtained from healthy volunteers and human PBMC were isolated by density-gradient centrifugation with Ficoll-Paque. After washing with PBS, the RPM or PBMC were suspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% heat-inactivated FCS (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies) for culture under a 5% CO_2 atmosphere at 37°C.

RPM (1×10^6 cells) or PBMC (2×10^6 cells) were incubated for the indicated periods with or without various concentrations of PGs (PGD_2, PGE_2, I-BOP), 8-Br-cAMP, LPS, or E-series of prostaglandin (EP EP1–4) agonists. Then the expression of murine TREM-1 (mTREM-1), human TREM-1 (hTREM-1), and soluble hTREM-1 (hsTREM-1) was investigated.

RPM were incubated in the presence or absence of SC560 and/or NS398 for 1 h to inhibit endogenous COX activity, and then the cells were incubated in the presence of LPS for the indicated periods. To block protein kinase activity, RPM were incubated in the presence or absence of various inhibitors such as SB203580, PD98059, LY294002, or H89 for 30 min, after which the cells were incubated with LPS or PGE_2 for the indicated periods.

At the termination of incubation, cells and culture supernatants were obtained by centrifugation. Total RNA and protein were isolated by using RLT lysis buffer (Qiagen). Samples of cell lysate and culture medium were stored at −80°C until use.

Quantitative real-time PCR

Total RNA was extracted from cell lysates using an RNeasy Mini kit (Qiagen). The RNA was treated with DNase I (Qiagen) and cDNA was synthesized from 2 μg of random-primed total RNA in a volume of 20 μl using Omniscript reverse transcriptase (Qiagen). mTREM-1, GAPDH, and COX-2 were assessed by quantitative real-time PCR (SYBR) using specific oligonucleotide primers. hTREM-1, hsTREM-1, and rRNA were assessed by quantitative real-time PCR (TaqMan) using specific oligonucleotide primers and probe. hsTREM-1 was identified as a splice variant of hTREM-1 with a 193-base deletion (exon 3) from bases 471 to 663 (GenBank accession no. AF287008). To avoid amplification of hsTREM-1 mRNA, the forward primer for hTREM-1 was designed to fit exon 3 (the deletion site). To amplify only hsTREM-1, the backward primer was designed to hybridize to the 3′ end of exon 2 as well as the 5′ end of exon 4. These primers could specifically amplify hTREM-1 and hsTREM-1, respectively. The sequences of the primers and probes are listed in Table I. The rRNA primers and probe were purchased from Applied Biosystems. The SYBR PCR was performed in duplicate using a 25-μl reaction mixture containing 1 μl of cDNA, QuantiTect SYBR Green PCR (Qiagen), and 300 nM each of the sense and antisense primers. The PCR mixture was incubated for 15 min at 95°C, and then amplification was performed for 45 cycles, consisting of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. TaqMan PCR was performed in duplicate with a 25-μl reaction mixture containing 1 μl of cDNA, 12.5 μl of QuantiTect Probe PCR (Qiagen), 400 nM each of the sense and antisense primers, and 200 nM of the probe. The PCR mixture was incubated for 15 min at 95°C to activate HotStarTaq DNA polymerase. Subsequently, amplification was performed for 45 cycles, consisting of denaturation at 94°C for 15 s and combined annealing extension at 59°C for 1 min. During the extension step, the ABI Prism 7700 Sequence Detection System monitored PCR amplification in real time by quantitative analysis of the emitted fluorescence. The amount of each sample mRNA was evaluated relative to the control sample, which was assigned a value of 1 arbitrary unit.

Western blot analysis

Culture medium or RPM (1×10^6 cells) was dissolved in sample buffer (350 mM Tris (pH 6.8), 10% SDS, 30% glycerol, 600 mM DTT, and 0.05% bromphenol blue), loaded onto 10% SDS-PAGE gel, and run at 20 mA for 1.5 h. Proteins in the supernatant were transferred to a polyvinylidene difluoride membrane (Roche Diagnostics) for 1.5 h at 200 mA by semidry blotting. The membrane was then blocked with 5% skim milk in PBS containing 0.05% Tween 20 for 1 h at 37°C, washed with PBS containing 0.1% Tween 20, and incubated overnight at 4°C with a monoclonal anti-human TREM-1 Ab (1 μg/ml). The blots were washed four times with TBS and incubated for 30 min with HRP-conjugated rabbit anti-mouse IgG. Immunoreactive bands were developed using a chemiluminescent substrate (ECL plus; Amersham Biosciences).

Assay of cytokine and chemokine production

Flat-bottom plates were precoated with 5 μg/ml of a monoclonal anti-human TREM-1 Ab or an isotype-matched control Ab (mouse IgG1) overnight at 4°C. After washing with PBS, PBMC (1×10^5 cells) were preincubated with or without PGE_2 (1 μM) for 5 h. Then the PBMC were added to the Ab-coated wells, and briefly spun in a centrifuge at 1200 rpm to bind TREM-1. After incubation for 24 h, culture medium was obtained by centrifugation and stored at −20°C until the levels of TNF-α and IL-8 in the supernatant were determined by specific ELISAs.

Assay of PGE_2 production

Concentration of PGE_2 in the culture supernatant was determined by using a PGE_2 EIA kit according to the manufacturer's instructions.

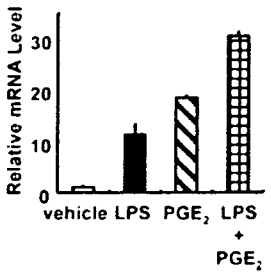

FIGURE 1. PGE_2 and LPS up-regulate mTREM-1 expression by RPM. RPM were incubated with or without PGE_2 (1 μM) for 1 h, and then were cultured in the presence or absence of LPS (100 ng/ml). The mTREM-1 mRNA level was determined by quantitative real-time PCR using murine GAPDH as the internal control. The relative level of mTREM-1 mRNA was evaluated by comparison with that in vehicle (EtOH)-treated RPM, which was defined as 1 arbitrary unit. Data are expressed as the mean ± SD of triplicate determinations.

Statistical analysis

Results are expressed as the mean ± SD. Statistical analysis was performed using the paired Student t test and $p < 0.05$ was considered to indicate significance.

Results

PGE_2 induces TREM-1 expression by RPM

PGE_2 is a mediator with a wide variety of biological effects in the process of microbial inflammation. To determine whether PGE_2 could influence the expression and action of TREM-1 in macrophages, RPM were pretreated with PGE_2 at a concentration of 1 μM for 1 h and then the cells were subsequently incubated in the presence or absence of LPS (100 ng/ml) for 1 h. Expression of mTREM-1 was determined by quantitative real-time PCR. LPS significantly increased expression of the *TREM-1* gene (Fig. 1), as previously reported. PGE_2 also caused significant induction of TREM-1 expression and the magnitude of gene expression was significantly higher in PGE_2-treated cells than in LPS-treated cells. Furthermore, a combination of PGE_2 and LPS caused additive enhancement of mTREM-1 expression by RPM.

To investigate the time course of PGE_2-induced expression of mTREM-1, RPM were incubated with 1 μM PGE_2 for the indicated periods. Induction of gene expression occurred quite rapidly and was observed as early as 1 h after stimulation, following declined for 12 h (Fig. 2*A*). RPM were incubated with varying concentrations of PGE_2 for 1 h to determine whether physiological levels of PGE_2 enhanced mTREM-1 expression. It was shown that PGE_2 increased mTREM-1 expression in a concentration-dependent manner. PGE_2 at a concentration as low as 10^{-10} M significantly induced mTREM-1 expression and maximal expression occurred after stimulation with 10^{-6}–10^{-7} M PGE_2 (Fig. 2*B*).

It has been demonstrated that monocytes and macrophages express various receptors for arachidonic acid metabolites, which are referred to EP, thromboxane-like prostanoid (TP), and CRTH2 (20). Therefore, we investigated the effects of specific ligands for these receptors on mTREM-1 expression by RPM. The cells were incubated for 1 h with PGE_2 (an EP receptor ligand), I-BOP (a TP receptor ligand), or PGD_2 (a CRTH2 ligand), and TREM-1 expression was evaluated by quantitative real-time PCR. PGE_2 induced TREM-1 expression, while neither I-BOP nor PGD_2 up-regulated mTREM-1 expression, indicating that PGE_2 was a specific inducer of mTREM-1 expression among these PGs (Fig. 2*C*).

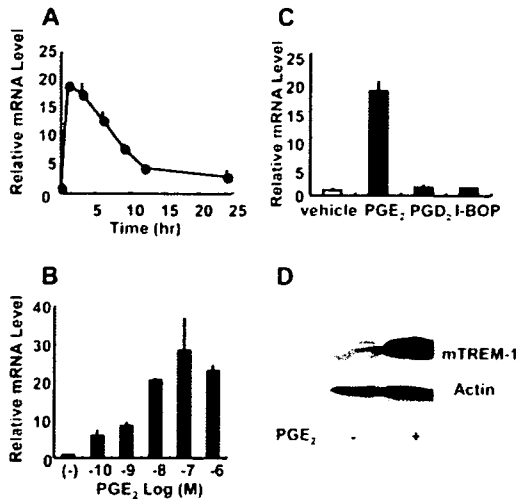

FIGURE 2. PGE_2 induces mTREM-1 expression in a time- or concentration-dependent manner. *A*, RPM were incubated with PGE_2 (1 μM) for the indicated periods and the mTREM-1 mRNA level was determined by quantitative real-time PCR. *B*, RPM were cultured with or without various concentrations of PGE_2 for 1 h and the mTREM-1 mRNA level was determined by quantitative real-time PCR. *C*, RPM were incubated with PGE_2 (1 μM), PGD_2 (1 μM), or I-BOP (0.2 μM) for 1 h and the mTREM-1 mRNA level was determined by quantitative real-time PCR. *D*, RPM were cultured with or without PGE_2 (1 μM) for 5 h, and expression of mTREM-1 protein and actin was determined by Western blot analysis. Data are expressed as the mean ± SD of triplicate determinations.

Western blot analysis using a specific anti-mouse TREM-1 mAb was performed to evaluate mTREM-1 protein expression by RPM after incubation with or without PGE_2 for 5 h. mTREM-1 protein was detected faintly when RPM were incubated with the vehicle alone, whereas increased expression of mTREM-1 was clearly seen when RPM were incubated with PGE_2 (10^{-6} M) for 5 h (Fig. 2*D*).

Endogenous PGE_2 induces TREM-1 expression by RPM

It has been demonstrated that LPS induces TREM-1 expression as well as the release of PGE_2 by macrophages (7–9, 21). To evaluate the possible influence of endogenous PGE_2 on LPS-induced mTREM-1 expression, RPM were stimulated with LPS (100 ng/ml) for the indicated periods, after which PGE_2 production and *mTREM-1* gene expression were determined by EIA and quantitative real-time PCR, respectively. PGE_2 synthesis gradually increased up to 4 h, and then the maximum level was maintained

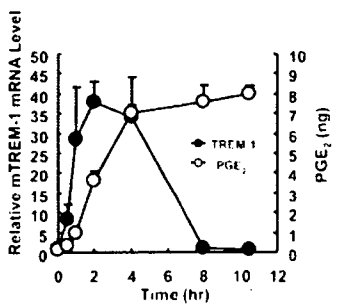

FIGURE 3. LPS-induced PGE_2 production up-regulates mTREM-1 expression by RPM. RPM were stimulated with LPS (100 ng/ml) for the indicated periods. Then the mTREM-1 mRNA level was determined by quantitative real-time PCR, and PGE_2 synthesis was determined by using a PGE_2 EIA kit. Data are expressed as the mean ± SD of triplicate determinations.

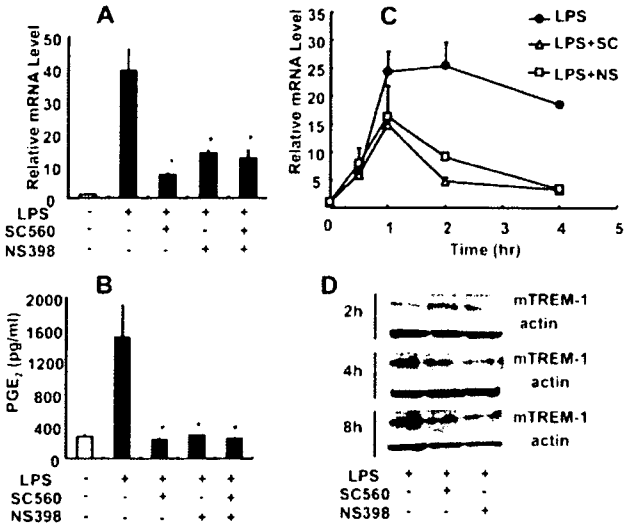

FIGURE 4. Effect of COX inhibitors on LPS-induced mTREM-1 expression by RPM. RPM were pretreated with or without SC560 and/or NS398 for 1 h, and were subsequently incubated in the presence or absence of LPS (100 ng/ml) for the indicated periods. *A*, The mTREM-1 mRNA level at 2 h after LPS stimulation was determined by quantitative real-time PCR. *B*, PGE_2 synthesis at 2 h after LPS stimulation was determined by a PGE_2 EIA kit. *C*, Time course of mTREM-1 mRNA expression was determined by quantitative real-time PCR. *D*, mTREM-1 protein and actin was determined by Western blot analysis. Data are expressed as the mean ± SD of triplicate determinations. *, $p < 0.01$, vs LPS-stimulated RPM by Student's unpaired *t* test.

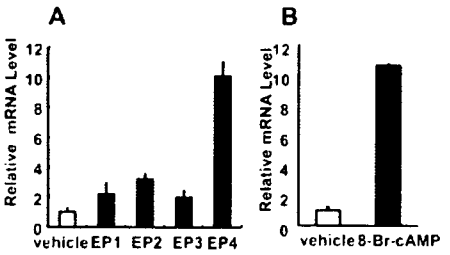

FIGURE 5. Enhancement of mTREM-1 expression by an EP4 agonist and cAMP analog. *A*, RPM were incubated with or without agonists for EP1 to EP4 agonists (1 μM) for 1 h and the mTREM-1 mRNA level was determined by quantitative real-time PCR. *B*, RPM were incubated with or without 8-Br-cAMP (0.5 mM) for 1 h. Data are expressed as the mean ± SD of triplicate determinations.

until 11 h after stimulation (Fig. 3). In contrast, gene expression of mTREM-1 occurred quite rapidly and was seen as early as 0.5 h after stimulation when PGE_2 production was not detected. Maximum induction of mTREM-1 was observed at 2–4 h after stimulation and gene expression returned to the basal level by 8 h. These findings indicated that PGE_2 synthesis did not precede the induction of *mTREM-1* gene expression.

mTREM-1 expression was significantly induced by a physiological concentration of PGE_2. Therefore, the regulatory roles of PGE_2 on TREM-1 expression was investigated. Because PGE_2 synthesis is regulated by COX-1 and COX-2, RPM were incubated for 1 h in the presence or absence of SC560 (a selective COX-1 inhibitor) or NS398 (a selective COX-2 inhibitor), and then the cells were stimulated with LPS for 2 h. mTREM-1 expression and PGE_2 synthesis was determined by quantitative real-time PCR and EIA, respectively. Both inhibitors for COX-1 and COX-2 partially, but significantly, inhibited LPS-induced expression of mTREM-1 (Fig. 4*A*). When the effects of COX inhibitors on PGE_2 synthesis by LPS-stimulated RPM were investigated, these inhibitors also suppressed PGE_2 synthesis (Fig. 4*B*). Vehicle (DMSO) did not affect on LPS-induced PGE_2 synthesis and mTREM-1 expression (data not shown).

To investigate the effect of PGE_2 on LPS-induced mTREM-1 mRNA expression at early time points, RPM were stimulated with LPS in the presence or absence of COX inhibitors for the indicated periods. Both COX-1 and COX-2 inhibitors failed to inhibit mTREM-1 expression at 0.5 h after LPS stimulation, whereas mTREM-1 expression at 1 h was partially inhibited, and that at 2 and 4 h was significantly abolished by COX inhibitors (Fig. 4*C*). These findings indicated that the effect of PGE_2 on LPS-induced mTREM-1 expression was predominant at late time points (>2 h after stimulation) but not at early time points (0.5 and 1 h after stimulation).

After RPM were incubated with LPS in the presence or absence of COX inhibitors for various times, the expression of mTREM-1 protein was determined by Western blot analysis. Both inhibitors reduced LPS-induced TREM-1 expression at 4 and 8 h but not at 2 h after stimulation (Fig. 4*D*). Actin as the internal control was similarly detected in all samples. These findings indicated that LPS-induced expression of mTREM-1 on RPM was at least partly promoted by endogenous PGE_2.

PGE_2-induced TREM-1 expression is mediated by the EP4 receptor and cAMP

It has been shown that the biological functions of PGE_2 are mediated by four specific receptors, which are coupled to G-protein and are referred to as EP1 to EP4 (20). To determine which EP receptors mediated PGE_2-induced mTREM-1 expression, RPM were incubated with four synthetic agonists specific for each of the EP receptors (each at a concentration of 1 μM), and then mTREM-1 expression was evaluated by quantitative real-time PCR. The EP4 agonist significantly up-regulated TREM-1 expression in RPM, whereas the EP1, EP2, and EP3 agonists failed to enhance TREM-1 expression (Fig. 5*A*).

Activation of the EP4 receptor enhances intracellular accumulation of cAMP via adenylate cyclase. Therefore, we examined the influence of 8-Br-cAMP (a stable cAMP analog) on mTREM-1 expression by RPM. Treatment of RPM with 8-Br-cAMP at a concentration of 5×10^{-4} M for 1 h significantly enhanced expression of the *mTREM-1* gene by RPM (Fig. 5*B*). EP4 agonist and 8-Br-cAMP also induced TREM-1 mRNA expression in J774.1 and PBMC (data not shown). These finding clearly suggested that PGE_2-induced TREM-1 expression on RPM was related to EP4 receptor- and cAMP-mediated signaling.

Blocking of PKA, p38 MAPK, and PI3K inhibits PGE_2-induced mTREM-1 expression

Intracellular cAMP is a major regulator of PKA (22) and cAMP also activates the PI3K-, p38 MAPK-, and ERK-signaling pathways (23–25). Therefore, we investigated the signaling pathways involved in PGE_2-induced expression of TREM-1 by using synthetic inhibitors of these kinases. A PKA inhibitor (H89), a p38 MAPK inhibitor (SB203580), and a PI3K inhibitor (LY294002) significantly suppressed PGE_2-induced TREM-1 expression, whereas a MAPKK inhibitor (PD98059) failed to influence TREM-1 expression (Fig. 6). Inhibitory effects of these inhibitors were observed in a dose- or time-dependent manner (data not shown). These results suggested that PGE_2-induced TREM-1 expression was mediated via the PKA, PI3K, and p38 MAPK pathways.

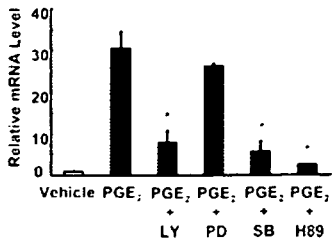

FIGURE 6. Inhibition of PKA, p38 MAPK, or PI3K suppresses PGE_2-induced mTREM-1 expression by RPM. RPM were pretreated with or without LY294002 (20 μM), PD98059 (30 μM), SB203580 (20 μM) H-89 (20 μM) for 30 min, and then were incubated with PGE_2 (1 μM) for 1 h. The mTREM-1 mRNA level was determined by quantitative real-time PCR. Data are expressed as the mean ± SD of triplicate determinations. *, $p < 0.01$ vs PGE_2-stimulated RPM by Student's unpaired t test.

Activation of TREM-1 significantly enhances cytokine production by PGE_2-treated PBMC

An agonistic anti-TREM-1 mAb has been shown to stimulate the production of proinflammatory cytokines by monocytes (1, 7, 9). It was difficult to transfer PGE_2-treated RPM to Ab-coated wells, because the cells tightly adhere to the culture dishes. Therefore, PBMC were used to determine whether TREM-1 could enhance cytokine production by PGE_2-treated monocytes. Cells were incubated in the presence or absence of PGE_2 (10^{-6} M) for 5 h, and then harvested for incubation in agonistic anti-TREM-1 mAb-coated wells for 24 h. Then the levels of TNF-α and IL-8 in the culture supernatant were determined by specific ELISAs. The agonistic anti-TREM-1 mAb caused a significant increase of TNF-α production by PGE_2-pretreated PBMC (Fig. 7*A*). Production of TNF-α by PGE_2-treated cells was 6-fold higher than that by untreated cells. The agonistic anti-TREM-1 mAb also increased IL-8 production by PGE_2-pretreated PBMC and the magnitude of this enhancement was 4.6-fold (Fig. 7*B*). These results indicated that

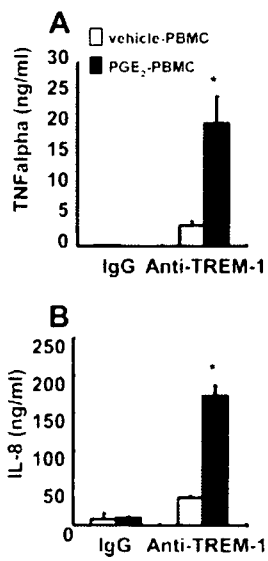

FIGURE 7. An agonistic anti-TREM-1 mAb enhances the production of proinflammatory cytokines by PGE_2-pretreated PBMC. PBMC were incubated with or without PGE_2 for 5 h, and then the cells were incubated in the presence or absence of the agonistic anti-TREM-1 mAb (5 μg/ml) or an isotype control Ab (5 μg/ml) for 24 h. Production of TNF-α (*A*) and IL-8 (*B*) was determined by specific ELISAs. Data are expressed as the mean ± SD of triplicate determinations. *, $p < 0.01$ vs vehicle-stimulated PBMC by Student's unpaired t test.

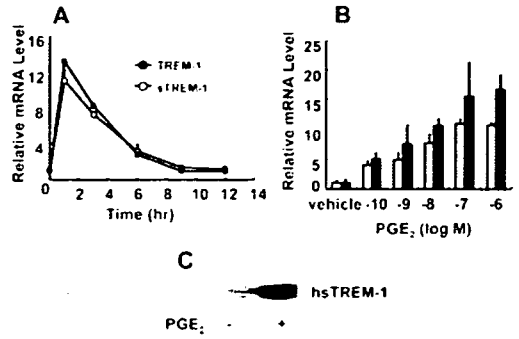

FIGURE 8. PGE_2 induces hTREM-1 and hsTREM-1 expression by PBMC in a time- and concentration-dependent manner. *A*, PBMC were incubated with PGE_2 (1 μM) for the indicated periods. Expression of the *hTREM-1* gene (○) and the *hsTREM-1* gene (●) was determined by real-time quantitative PCR. *B*, PBMC were cultured with or without various concentrations of PGE_2 for 1 h. Then the hTREM-1 (□) and hsTREM-1 (■) mRNA levels were determined by real-time quantitative PCR. *C*, PBMC were incubated with or without PGE_2 (1 μM) for 5 h and hsTREM-1 protein in the same volume of the supernatant (20 μl) was determined by Western blot analysis. Data are expressed as the mean ± SD of triplicate determinations.

TREM-1 induced by PGE_2 was functional and enhanced the production of proinflammatory cytokines by PBMC.

PGE_2 induces hTREM-1 and hsTREM-1 expression by PBMC

It has been demonstrated that human monocytes are capable of expressing sTREM-1 as well as the cell surface form (3–5). Although sTREM-1 has also been identified in mice, the precise structure and function of soluble mTREM-1 are not yet known. Therefore, we investigated the expression of hTREM-1 and hsTREM-1 by PGE_2-treated PBMC to evaluate which type was predominantly expressed. After PBMC were incubated with PGE_2, *hTREM-1* and *hsTREM-1* gene expression were separately determined by quantitative real-time PCR.

Induction of both *hTREM-1* and *hsTRM-1* gene expression occurred quite rapidly and was seen as early as 1 h after stimulation, a subsequent declined until 9 h (Fig. 8*A*). To determine whether physiological concentrations of PGE_2 could induce the expression of hTREM-1 and hsTREM-1, PBMC were incubated with various concentrations of PGE_2 for 1 h and gene expression was determined. It was found that PGE_2 promoted both hTREM-1 and hsTREM-1 expression in a concentration-dependent manner, with maximal expression occurring at 10^{-6} or 10^{-7} M (Fig. 8*B*).

Western blot analysis using a specific anti-human TREM-1 mAb was performed to detect sTREM-1 protein. PBMC were incubated with or without PGE_2 for 5 h, and then the sTREM-1 protein level in the culture supernatant was determined by Western blotting. sTREM-1 was detected at very low levels when PBMC were incubated with the vehicle alone, whereas sTREM-1 expression was increased when PBMC were incubated with PGE_2 for 5 h (Fig. 8*C*). Taken together, these findings showed that PGE_2 up-regulated the expression of hTREM-1 as well as hsTREM-1 by monocytes.

Discussion

The present study provided evidence that PGE_2 up-regulates mTREM-1 expression by RPM, as well as hTREM-1 and hsTREM-1 expression by human PBMC. LPS-induced TREM-1 expression is at least partly regulated by endogenous PGE_2, because COX inhibitors significantly reduced TREM-1 expression. PGE_2-induced TREM-1 expression was mediated by EP4, cAMP, and various kinases such as PKA, PI3K, and p38 MAPK. PGE_2-induced TREM-1 was functional,

because agonistic anti-TREM-1 mAb promoted a significant increase in the production of TNF-α and IL-8.

It is known that TREM-1 is specifically up-regulated by microbial products such as LPS, lipoteichoic acid (LTA), or zymosan (1, 7, 9, 26). However, the present study provided the first demonstration that PGE_2 could induce TREM-1 expression by both RPM and PBMC. Induction of TREM-1 expression by PGE_2 was also observed in a human monocyte cell line (U937) and a murine macrophage cell line (J774.1) (data not shown), indicating that PGE_2 is an inducer of TREM-1 expression by both monocytes and macrophages. PGE_2 was a specific regulator of mTREM-1 expression, because specific ligands for the CRTH2 and TP receptors (which are expressed on macrophages) failed to induce mTREM-1 expression. Biological effect of endogenous PGE_2 on TREM-1 expression was predominant in the late phase of LPS-induced TREM-1 expression. This is based on the findings that COX inhibitors abrogated mTREM-1 expression after 2 h and also reduced mTREM-1 protein expression after 4–8 h following LPS stimulation.

In the present study, both COX-1 and COX-2 inhibitors suppressed PGE_2 synthesis and TREM-1 induction. It has been documented that LPS promoted PGE_2 production through the induction and activation of the COX-2, but not COX-1 (21, 27). However, Rouzer et al. (21) also reported that SC560 (COX-1 inhibitor) inhibits PG synthesis through inhibition of COX-2 as well as COX-1 in LPS-stimulated RPM. Because this cross-inhibition was not observed in other cells, it appeared to be specific in RPM. TNF-α is also an inducer of PGE_2 synthesis, but a previous study demonstrated that TNF-α had a limited effect on TREM-1 expression (7, 28). The reasons for this difference are not known, but it might be related to different mechanisms of action on monocytes and macrophages.

EP4, one of the receptors for PGE_2, increases intracellular cAMP levels via activation of adenylate cyclase and promotes activation of the PKA, PI3K, p38 MAPK, and MAPKK pathways (22–25). The present study demonstrated that a specific EP4 agonist and 8-Br-cAMP both enhanced *mTREM-1* gene expression, while inhibitors of PKA, p38 MAPK, and PI3K blocked the PGE_2-induced increase of mTREM-1 expression. These findings suggested that PGE_2-induced up-regulation of mTREM-1 expression was mediated by the binding of PGE_2 to EP4, which was followed by accumulation of cAMP and activation of various kinases, including PKA, p38 MAPK, and PI3K. This is consistent with the findings of previous studies demonstrating that PGE_2 potentially activate various kinases such as PKA, PI3K, and p38 MAPK independently (29, 30). COX inhibitors failed to completely suppress LPS-induced up-regulation of mTREM-1 expression by RPM, and these inhibitors abolished TREM-1 expression only in the late phase, but not early phase of LPS stimulation. These indicate that other pathways might also be involved in the induction of TREM-1 expression by LPS. Knapp et al. (28) recently demonstrated that a PI3K-dependent pathway played a central role, while MAPK also played a limited role, in LPS-induced up-regulation of TREM-1 expression by monocytes. Several signaling pathways might be involved in LPS-induced TREM-1 expression, and the endogenous PGE_2-mediated pathway seems to be one of the mechanisms of LPS-induced TREM-1 expression on monocytes and macrophages.

TLR and TREM-1 cooperate to induce an inflammatory response, because activation of TREM-1 causes a marked increment in the production of proinflammatory cytokines by macrophages when LPS is used as the costimulus (1, 8, 9). TREM-1 activates a downstream signaling pathway through DAP12, which involves tyrosine phosphorylation, activation of mitogen-activated protein kinases, and mobilization of Ca^{2+}. In contrast, TLRs directly recognize certain microbial products and components, such as LPS, LTA, and bacterial DNA. MyD88, IRAK, TRAF6, and IKK are essentially involved in the TLR-signaling pathway. These kinases can potentially induce the production of proinflammatory cytokines via the activation of NF-κB (31). Natural ligands for TREM-1 remain to be identified. If specific ligands for TREM-1 are located at the foci of microbe-induced inflammation, interactions between TREM-1 and TLRs can synergistically induce inflammatory responses. In this case, cooperation between TLRs and TREM-1 could occur at several levels during the process of LPS-induced inflammation. The present study showed that an LPS-induced increase in the production of PGE_2 promoted TREM-1 expression, and activation of TREM-1 on PGE_2-treated PBMC enhanced the production of proinflammatory cytokines. Based on these findings, we hypothesized that PGE_2-induced up-regulation of TREM-1 expression may play an important role in enhancing the TLR-mediated response of macrophages to LPS stimulation.

Several line of evidence indicated that decoy receptors can modulate inflammatory responses by blocking the action by agonists (32, 33). sTREM-1 is a natural decoy receptor that could potentially inhibit TREM-1-mediated activation of cells through competition with natural ligand(s) for receptor binding. Synthetic sTREM-1 has been shown to inhibit LPS-induced cytokine production by monocytes in vitro (6). Furthermore, a recombinant sTREM-1 fusion protein and synthetic soluble TREM-1 have been shown to protect mice against lethal LPS challenge or bacterial sepsis by suppressing inflammatory cytokine production (6, 9). In contrast, it has been demonstrated that PGE_2 can suppress the production of various cytokines (such as TNF-α, IL-8, MCP-1, IFN-γ-inducible protein-10, and MIP-1β) by LPS-stimulated macrophages through EP2- and/or EP4-mediated pathways (34, 35). PGE_2 also induces the production of IL-10, which can have an anti-inflammatory effect (36). Present study demonstrated that PGE_2 induced the release of sTREM-1 by PBMC. Therefore, PGE_2 might suppress inflammation not only by inhibiting the production of proinflammatory cytokines, but also by inducing expression of the decoy receptor sTREM-1 and increasing the production of IL-10. However, activation of TREM-1 on PGE_2-treated PBMC enhanced the production of proinflammatory cytokines, indicating that PGE_2 may exert bidirectional effects on monocytes and macrophages to modulate inflammation through altering the expression of TREM-1 and sTREM-1.

Blocking of PGs has been shown to increase LPS-induced cytokine production both in vitro and in vivo (36–39). This is consistent with the previous finding that PGE_2 and EP4 agonists attenuated LPS-induced cytokine production in mice (40). However, a number of studies have provided evidence that COX inhibitors can improve survival after the onset of endotoxic shock and COX-deficient mice are resistant to endotoxin-induced inflammation and death (17, 19, 41). Thus, the precise pathophysiological role of PGE_2 in microbe infection still remains undefined. Further investigations should be directed toward the in vivo effects of PGE_2-induced TREM-1 and sTREM-1 in sepsis models.

Increased expression of TREM-1 has been observed at sites of inflammation caused by microbial pathogens (9). However, we recently demonstrated that monosodium urate monohydrate (MSU) crystals induced mTREM-1 expression in monocytes and macrophages in vitro and in vivo (42), indicating that TREM-1 might be involved in the development of acute gouty arthritis. We also observed that MSU crystal-induced mTREM-1 expression is regulated, at least in part, by endogenous produced PGE_2 (our unpublished data). These findings suggest the possibility that PGE_2 might enhance TREM-1 expression in nonmicrobial inflammatory diseases including acute gouty arthritis. If a natural TREM-1 ligand is also induced in nonmicrobial inflammation, it could enhance inflammatory responses by activating PGE_2-induced

TREM-1. Furthermore, nonmicrobial products such as heat shock protein 60, which are induced in various inflammatory diseases, have been shown to stimulate TLRs (43). Thus, it is presumed that activation of PGE_2-induced TREM-1 and TLRs by specific ligands might cooperatively increase the inflammatory responses in patients with nonmicrobial inflammatory diseases.

The present study provided a first evidence that PGE_2 induces the expression of both TREM-1 and sTREM-1 by macrophages. This finding sheds new light on the role of PGE_2 as a regulator of the inflammatory response to microbial infection. Further investigations should be directed toward the assessment of pathophysiological roles of TREM-1 and sTREM-1 in various inflammatory diseases. Such studies may help to elucidate the precise role of PGE_2-induced TREM-1 expression in inflammation and could possibly provide evidence leading to new strategies for the treatment of inflammatory diseases.

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Enhanced Efficacy of Regulatory T Cell Transfer Against Increasing Resistance, by Elevated Foxp3 Expression Induced in Arthritic Murine Hosts

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Objective. **To investigate the efficacy of type II collagen–reactive Foxp3-expressing T cell transfer in suppressing collagen-induced arthritis (CIA) in relation to disease progression.**

Methods. **CD3-activated CD4 T cells were retrovirally transduced with the Foxp3 gene, and their in vitro suppressive activity on T cell proliferation was assessed for correlation with Foxp3 levels. To suppress CIA, Foxp3-transduced T cells generated with type II collagen– or ovalbumin (OVA)–pulsed dendritic cells (DCs), which were fractionated by Foxp3 levels, were adoptively transferred to mice at various time points.**

Results. **The in vitro suppressive activity of Foxp3-transduced cells correlated positively with Foxp3 levels. Type II collagen–reactive, but not OVA-reactive, Foxp3-transduced cells significantly suppressed CIA when they were transferred before immunization, and this suppression was accompanied by decreased anti–type II collagen antibody production. Larger cell numbers were required to suppress CIA when transfer occurred 20 days after immunization, indicating that hosts became resistant to suppression. Transfer of 1×10^5 $Foxp3^{low}$ cells had only a marginal effect on CIA suppression in immunized hosts, while transfer of $Foxp3^{high}$ cells at smaller doses significantly suppressed CIA. Transfer of 1×10^5 $Foxp3^{high}$ cells after establishment of arthritis attenuated disease progression but did not reverse joint swelling.**

Conclusion. **Resistance to Foxp3-transduced T cells proceeded as CIA progressed, suggesting that late-stage aggressive arthritis is more resistant to regulatory T cell transfer. An elevated expression level of Foxp3 in type II collagen–specific T cells improved their suppressive function in CIA. Thus, transfer of T cells expressing high levels of Foxp3 could be a strategy to overcome the induced resistance to regulatory T cell therapy.**

Recent studies have added to the accumulated evidence that CD4+,CD25+ naturally arising regulatory T cells (Treg) play a crucial role in the maintenance of peripheral self tolerance (1,2). Foxp3, a transcription regulator belonging to the forkhead/winged helix transcription factor family (3), is expressed exclusively in T cells with regulatory activities, including naturally arising CD4+,CD25+ Treg (4–6) and some adaptive Treg subsets (7–9). Foxp3 is considered as a master gene characterizing phenotypes and functions of Treg. Retrovirus-mediated ectopic Foxp3 expression confers CD4+,CD25− non-Treg characteristics similar to those of CD4+,CD25+ Treg, such as expression of CD25, CTLA-4, CD103, and glucocorticoid-induced tumor necrosis factor receptor (GITR) (4). The suppressive activities induced in these cells are independent of soluble factors such as interleukin-10 (IL-10) or transforming growth factor β (4,10).

The involvement of CD4+,CD25+ Treg in auto-

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immune diseases in humans and animals has been intensively investigated over the past several years. Analyses of clinical samples demonstrated that patients with multiple sclerosis (11), autoimmune polyglandular syndromes (12), juvenile idiopathic arthritis (13,14), or rheumatoid arthritis (RA) (15–17) had an abnormal number and/or abnormal regulatory function of CD4+,CD25+ T cells. These facts brought forth the idea that supplementation of functionally intact Treg may reverse the activation of autoreactive T cells in patients with these diseases. The development of autoimmunity induced by transfer of CD25-depleted CD4 T cells to athymic nude mice as well as by neonatal thymectomy of normal mice was successfully prevented by adoptive transfer of polyclonal CD4+,CD25+ T cells (18,19).

Similarly, adoptive transfer of naturally arising CD4+,CD25+ Treg as well as Foxp3-transduced T cells was also successful in suppressing autoimmunity in various murine models of lymphopenia, which offer a favorable environment in which the transferred lymphocytes have room to expand. These models included experimental autoimmune encephalomyelitis, autoimmune diabetes, and autoimmune colitis (4,20–23). In some studies, antigen-specific CD4+,CD25+ cells were transferred, while in other studies, nonspecific T cells were transferred. Generally, larger numbers of polyclonal T cells were required to suppress the same disease (i.e., autoimmune diabetes) (21,22). Thus, antigen specificity is a factor promoting the in vivo effects of Treg, probably because they are recruited and/or expand at the sites of local inflammation.

A previous study demonstrated that in vivo depletion of CD25+ cells prior to initiation of collagen-induced arthritis (CIA), a model of RA, worsened arthritis. This finding suggested that CD4+,CD25+ Treg have an inhibitory role in disease development (24). As shown in other animal models of lymphopenia, transfer of non–antigen-specific Treg CD4+,CD25+ T cells inhibited CIA development when host mice received a lethal dose of total body irradiation followed by rescue with syngeneic bone marrow transplantation (25). If larger numbers of CD4+,CD25+ T cells were transferred, a suppressive effect was also observed, without induction of lymphopenia. However, transferring a large number of non–antigen-specific Treg may lead to systemic immune suppression.

The aim of this study was to determine whether antigen-specific Treg transfer efficiently suppresses arthritis in immunocompetent hosts, and whether susceptibility to suppression by Treg changes during the disease course.

MATERIALS AND METHODS

Mice. Male DBA/1J mice were purchased from the Oriental Yeast Company (Tokyo, Japan). The mice were housed in the animal facility under specific pathogen–free conditions at the Research Center for Allergy and Immunology, RIKEN.

Monoclonal antibodies (mAb) and flow cytometry. Fluorescein isothiocyanate–conjugated anti-mouse CD3e (145-2C11 antibody), phycoerythrin (PE)–conjugated anti-mouse CD4 (L3T4 antibody), biotinylated anti-mouse CD25 (mAb 7D4; IgM), and allophycocyanin (APC)–conjugated streptavidin were purchased from BD PharMingen (San Diego, CA). APC-conjugated anti-mouse CD11c (N418) antibody was purchased from eBioscience (San Diego, CA). Single-cell suspensions were incubated with fluorescent or biotinylated mAb; incubation with biotinylated mAb was followed by incubation with PE-conjugated streptavidin. Data were acquired on a FACSCalibur and were analyzed using CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, CA).

Preparation of retroviral construct and supernatants. Two Foxp3-containing vectors were prepared for these experiments. The first vector consisted of an MIGR1 vector with a mouse Foxp3 complementary DNA insert (MIGR1-Foxp3) (4). The second vector contained the *Eco* RI–*Bgl* II fragment from MIGR1-Foxp3, which was ligated into an *Eco* RI–*Bam* HI–cleaved pMCs-IG vector (Foxp3-pMCs-IG) (26). MIGR1 and pMCs-IG encode green fluorescent protein (GFP) under the control of an internal ribosomal entry site. Retroviruses were prepared by introducing the empty vectors or the vectors with Foxp3 into the Plat-E packaging cell line (26,27).

Dendritic cell (DC) preparation. Bone marrow cells were collected from 6–7-week-old male DBA/1J mice. The bone marrow cells were cultured in RPMI 1640 medium containing 20 ng/ml granulocyte-macrophage colony-stimulating factor (PeproTech, London, UK), 10% fetal bovine serum, 2 μM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics for 7 days. The cells were then cultured in the presence of 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) with 50 μg/ml bovine type II collagen (Collagen Research Center, Tokyo, Japan), ovalbumin (OVA; Sigma-Aldrich), or LPS alone for a further 24 hours. These cells were used as mature DCs. A majority of the cells harvested by this method were CD11c positive.

Priming of CD4 T cells with antigen-pulsed DCs, and retroviral infection. DBA/1J mice were immunized twice (1-week interval) with 200 mg of either bovine type II collagen or OVA in Freund's complete adjuvant (CFA; Difco, Detroit, MI), in the hind footpads. CD4 T cells were isolated from the draining lymph nodes of immunized mice or from the splenocytes of naive mice, using magnetic-activated cell sorting microbead-coupled mAb and magnetic cell separation columns (Miltenyi Biotec, Auburn, CA). The cells were cultured with type II collagen–pulsed DCs, OVA-pulsed DCs, or DCs treated with anti-mouse CD3e mAb (145-2C11; BD PharMingen) in the presence of 100 units/ml recombinant human

IL-2 (Shionogi Pharmaceuticals, Osaka, Japan) for 24 hours. The activated CD4 T cells were mixed with an equal volume of retroviral supernatant and Polybrene (6 μg/ml; Sigma-Aldrich), centrifuged at 1,750*g* at 32°C for 1 hour, and incubated for a further 7 hours at 37°C in a 5% CO_2 atmosphere. The cells were then cultured in complete medium supplemented with 100 units/ml IL-2. After 2 or 3 days, live GFP-positive fractions of the infected cells were isolated using a BD FACSAria Cell Sorter (BD Biosciences Immunocytometry Systems). To assess antigen-specific priming of CD4 T cells, 1×10^5 CD4 T cells were cultured with antigen-pulsed DCs or unpulsed DCs at various ratios, in 96-well plates. ^{3}H-thymidine uptake was measured after 3 days. For immunoblot analyses and suppression assays, activated CD4+,CD25+ T cells were prepared by culturing CD4+,CD25+ T cells with DCs in the presence of anti-CD3 mAb (0.5 μg/ml) and IL-2 (100 units/ml) for 3 days.

Immunoblot analyses. Whole cell lysates were prepared using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and a fraction (10 μg) was loaded to each lane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis/immunoblot analyses were performed using anti-Foxp3 rabbit antibody (28) as primary antibody and horseradish peroxidase–conjugated anti-rabbit antibody as secondary antibody (Santa Cruz Biotechnology), with detection accomplished using the enhanced chemiluminescence technique (Amersham Biosciences, Uppsala, Sweden). The measurement of band intensity was performed using ImageJ software (29).

Suppression assay. To assess the suppressive activities of regulatory T cells, CD4+,CD25− responder T cells (5×10^4/well) and irradiated antigen-presenting cells (whole splenocytes; 1×10^5/well) were cultured in a 96-well plate with Treg at various ratios, in the presence of anti-CD3 mAb (0.5 μg/ml). ^{3}H-thymidine (1 μCi/well) uptake was measured after 3 days.

Induction of CIA and clinical assessment of arthritis. Eight-week-old male DBA1/J mice were immunized intradermally at the tail base with 200 μg of bovine type II collagen in CFA (Difco). The mice received booster immunizations in the same manner, 21 days after the primary immunization. After the booster immunization (day 0), the disease severity in each limb was recorded using the following scoring system: 0 = normal, 1 = mild swelling in 1 joint, 2 = mild swelling in ≥2 joints, 3 = severe swelling in the paw or digits, and 4 = severe swelling in entire paw and digits.

Detection of bovine type II collagen–specific antibodies. Bovine type II collagen–specific antibodies in mouse serum were measured by enzyme-linked immunosorbent assay. Microtiter plates were coated with 2 μg/ml bovine type II collagen, which was denatured by boiling in phosphate buffered saline (PBS). The plates were washed with 0.05% Tween 20 in PBS (PBST), blocked with 2% bovine serum albumin (BSA) in PBS, and incubated with diluted mouse serum (1:1,000 ratio). Positive reactions were detected by incubation with rabbit anti-mouse IgG1, IgG2a, or IgG2b conjugated to horseradish peroxidase (Zymed, Burlingame, CA) in PBST with 2% BSA. The final reaction was visualized using the TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD). Optical density values were measured at 450 nm. Bovine type II collagen–specific antibody units were determined using a reference serum created from pooled sera from arthritic or nonarthritic mice. A 1:40 dilution of serum from arthritic mice was assigned a value of 1,000 units/ml.

Histologic assessment of arthritis. Mice with CIA were killed 14 days after receiving the booster immunization. The knee joints were removed, fixed in formalin, and decalcified in 10% EDTA. The samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistical analysis. Experimental groups were analyzed by fitting a repeated-measures model with a random intercept (30), using the linear mixed effects function in the "nlme" (31) package of R, version 2.4.1 (32). Full models were compared with models without the treatment term. *P* values less than 0.05 were considered significant.

RESULTS

Suppression of T cell proliferation in vitro by Foxp3-transduced cells. To investigate whether adoptive transfer of Foxp3-expressing T cells can suppress CIA in nonlymphopenic animals, we generated CD4 Treg by retroviral Foxp3 gene transfer. Splenic CD4 T cells from naive mice were mixed with mature DCs at a 10:1 ratio, in the presence of anti-CD3 mAb and IL-2. Stimulated T cells were infected with retroviral vectors including both GFP and Foxp3 genes (Foxp3-pMCs-IG) or GFP gene alone (pMCs-IG). The percentage of GFP-expressing cells was initially 30–50% (Figure 1A) but gradually decreased after 4 days in Foxp3-pMCs-IG–infected cells but not in pMCsIg–infected cells (data not shown), which indicates that Foxp3 expression repressed cell growth. The Foxp3-expressing cells up-regulated expression of CD25, GITR, and intracellular CTLA-4, which correlated with the GFP expression level (data not shown). Foxp3 was expressed at a higher level in Foxp3-transduced cells than in freshly isolated or anti-CD3–activated CD4+,CD25+ T cells from naive mice, based on Foxp3:β-actin intensity ratios of 1.2, 0.7, and 1.0, respectively (Figure 1B). In vitro suppressive activity was enhanced in activated cells compared with fresh cells (Figure 1C). CD3-primed Foxp3-transduced T cells significantly suppressed the proliferation of responder T cells, with efficiency that was remarkably higher than that of freshly isolated CD4+,CD25+ T cells and was comparable with that of activated CD4+,CD25+ T cells (Figure 1D).

Suppression of CIA by adoptive transfer of antigen-reactive Foxp3-transduced T cells. CD4 T cells isolated from the lymph nodes of type II collagen–immunized mice were activated in vitro with type II collagen–pulsed DCs and transfected with retroviruses. Because proliferating cells are susceptible to integration of retroviral genomes, reactivity to type II collagen of the transduced cells should be enriched. The enriched

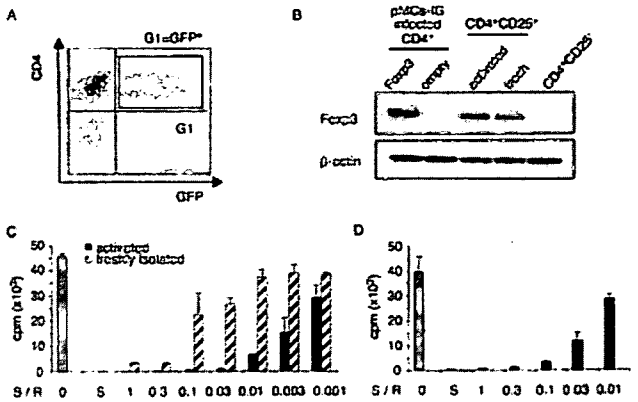


Figure 1. Foxp3 expression and suppressive activity of regulatory T cells. A, Splenic CD4 T cells from naive mice were stimulated with dendritic cells (DCs) and anti-CD3 monoclonal antibodies (mAb) and infected with Foxp3-pMCs-IG. The green fluorescent protein (GFP)–expressing fraction at 48 hours after infection is shown. B, CD4 T cells were activated and infected with Foxp3-pMCs-IG or pMCs-IG. After 48 hours, GFP-expressing fractions were isolated in order to prepare whole cell lysates for immunodetection of Foxp3 and β-actin. Whole cell lysates were also prepared from freshly isolated and activated CD4+,CD25+ T cells as well as CD4+,CD25− T cells. Activated CD4+,CD25+ T cells were prepared by culturing with DCs treated with anti-mouse CD3ε mAb in the presence of 100 units/ml interleukin-1 for 72 hours. C, Freshly isolated and activated CD4+,CD25+ T cells (suppressors; S) were cultured with CD4+,CD25− responder T cells (responders; R) in the presence of irradiated splenocytes and anti-CD3 mAb. Proliferation of responder T cells at the indicated S:R ratios was assessed by measuring ^{3}H-thymidine uptake. Values are the mean and SD and are representative of 5 individual experiments. D, Foxp3-transduced CD4 T cells were cultured with responder T cells for assessment of suppressive activity, in the same manner. Values are the mean and SD and are representative of 3 individual experiments.

specificity was confirmed by antigen-specific proliferation of CD4 T cells from lymph nodes of type II collagen–immunized mice reacting with type II collagen–pulsed DCs compared with antigen-unpulsed DCs (Figure 2A).

To evaluate whether Foxp3-transduced T cells effectively suppressed CIA, either 1×10^5 or 5×10^4 cells were adoptively transferred to the mice 1 day prior to the primary type II collagen immunization for initiation of CIA. Although arthritis should eventually develop after a single immunization, the mice received booster immunizations after 21 days, to synchronize the onset of joint swelling. Both doses of adoptively transferred cells were equally effective for lowering the arthritis scores of treated animals (Figure 2B). This indicated that transfer using this timing was effective in achieving suppression of arthritis, but that the amounts of transferred cells reached optimal levels in this setting. CD4 T cells from lymph nodes of OVA-immunized mice were activated with OVA-pulsed DCs (Figure 2C) and transfected with Foxp3-expressing retroviruses. The generated OVA-reactive Foxp3-transduced T cells exhibited suppressive activity in vitro against CD3-activated T cell proliferation, which was approximately comparable with that of CD3-primed Foxp3-transduced T cells. Transfer of OVA-reactive Foxp3-transduced T cells prepared in

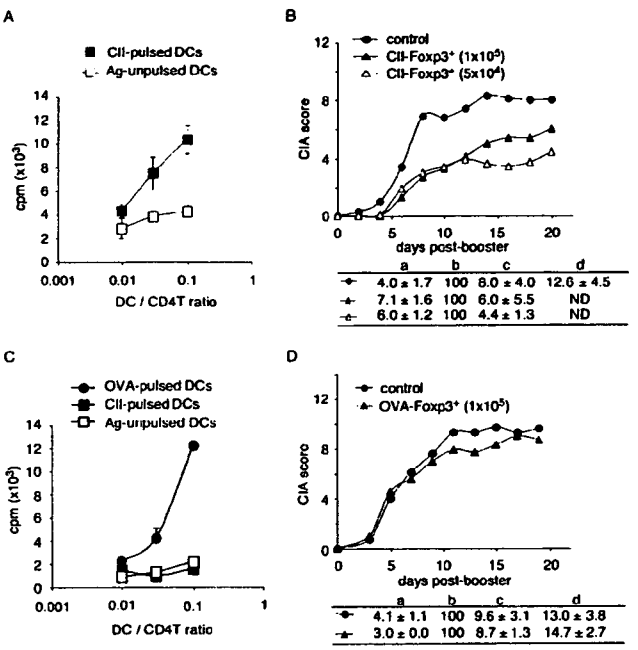


Figure 2. Suppression of collagen-induced arthritis (CIA) by type II collagen (CII)–primed Foxp3-transduced T cells transferred before the primary CII immunization. A and C, CD4 T cells derived from CII-immunized mice (A) or ovalbumin (OVA)–immunized mice (C) were mixed with either CII-pulsed dendritic cells (DCs), OVA-pulsed DCs, or antigen (Ag)–unpulsed DCs at the indicated ratios. Proliferation was measured by ^{3}H-thymidine uptake after 3 days. Values are the mean ± SD results from triplicate cultures. B and D, Naive DBA/1 mice received 1×10^5 (n = 7) or 5×10^4 (n = 7) Foxp3-transduced T cells primed by CII-pulsed DCs (CII-Foxp3+) (B) or 1×10^5 Foxp3-transduced T cells primed by OVA-pulsed DCs (OVA-Foxp3+; n = 7) (D) intravenously, 1 day prior to the primary immunization with CII. A group of mice received no T cell transfer (control; n = 15 in B, n = 7 in D). A booster immunization was administered 21 days after the primary immunization. B, Differences between the control group and the groups receiving CII-primed Foxp3+ (both doses) were significant ($P < 0.01$ by repeated-measures analysis of variance). Data below the figures represent the mean ± SD day of onset (a), the frequency (%) of arthritis induced (b), the mean ± SD maximum score (c), and the mean ± SD day on which the maximum score was reached for each mouse (d) among the mice in each group and are representative of 2 individual experiments. ND = not determined.

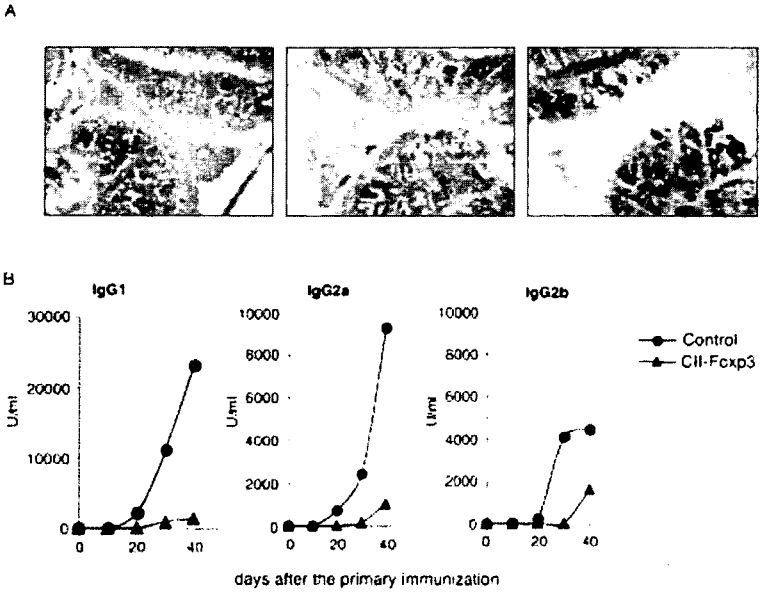
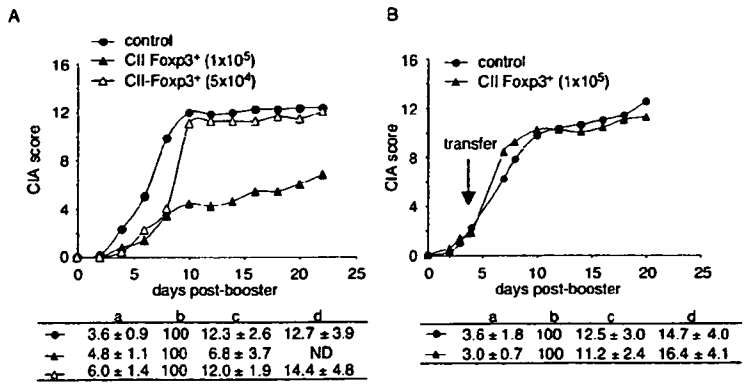


Figure 3. Suppression of joint inflammation and bovine type II collagen (CII)–specific antibody production by Foxp3-transduced T cell transfer. **A,** Histopathologic examination was performed 14 days after booster immunization to compare the knee joints of control mice with collagen-induced arthritis (CIA) (middle panel) and those of CIA mice that received transfer of 5×10^4 Foxp3-transduced T cells 1 day prior to the primary immunization (right panel) with the knee joints of normal naive mice (left panel). **B,** Mice received 1×10^5 Foxp3-transduced T cells primed with CII-pulsed dendritic cells (CII-Foxp3; n = 4) intravenously, 1 day prior to the primary immunization. A group of control mice (n = 4) received no T cell transfer. Serum was collected from each mouse on the day of the primary immunization and 10, 20, 30, and 40 days after the immunization. Unit values for each IgG subclass were measured using enzyme-linked immunosorbent assay. Plots represent the mean values for the 4 samples. Statistically significant differences, by repeated-measures analysis of variance, were observed between control and CII-Foxp3+ groups in the IgG1 and IgG2a subclasses ($P < 0.01$) and the IgG2b subclass ($P < 0.05$).

the same manner failed to suppress CIA (Figure 2D), suggesting that antigen specificity of Foxp3-transduced T cells is crucial.

Histopathologic assessment comparing the inflammation-related findings in control CIA mice, such as infiltration of mononuclear cells in the hypertrophic synovial tissue and destruction of joint cartilage (Figure 3A, middle panel), with those in normal mice (Figure 3A, left panel) demonstrated that these findings were suppressed in CIA mice that received transfer of type II collagen–reactive Foxp3-transduced T cells (Figure 3, right panel). Furthermore, the production of type II collagen–specific antibodies was suppressed for each of the IgG1, IgG2a, and IgG2b subclasses (Figure 3B).

We next examined whether adoptive transfer is effective when it is performed after immunization of the hosts. The same numbers of type II collagen–reactive Foxp3-transduced T cells as were used in the above-described experiments were transferred to the mice 20 days after the primary immunization, and booster immunization was performed on the following day. Transfer of 1×10^5 type II collagen–reactive Foxp3-transduced T cells significantly suppressed CIA, while transfer of 5×10^4 cells did not (Figure 4A). It should be noted that both approaches were effective in preventing arthritis in naive hosts (Figure 2B). Further, type II collagen–reactive Foxp3-transduced T cells were transferred intravenously 4 days after booster immunization, the time point at which joint swelling became apparent in most of the mice. At that time point, even 1×10^5 cells had no effect on CIA progression (Figure 4B). Thus, type II collagen–immunized mice became progressively more resistant to suppression by Treg.



	a	b	c	d
●	3.6 ± 0.9	100	12.3 ± 2.6	12.7 ± 3.9
▲	4.8 ± 1.1	100	6.8 ± 3.7	ND
○	6.0 ± 1.4	100	12.0 ± 1.9	14.4 ± 4.8

	a	b	c	d
●	3.6 ± 1.8	100	12.5 ± 3.0	14.7 ± 4.0
▲	3.0 ± 0.7	100	11.2 ± 2.4	16.4 ± 4.1

Figure 4. Induced resistance to Foxp3-transduced T cells after type II collagen (CII) immunization. Mice received transfer of 1×10^5 (n = 5) or 5×10^4 (n = 5) Foxp3-transduced T cells primed with CII-pulsed dendritic cells (CII-Foxp3+) intravenously, 20 days after the primary CII immunization (A) or 4 days after booster (B). A group of control mice received no T cell transfer (n = 9 in A; n = 10 in B). A, The difference between the control group and the group receiving Foxp3+ at a dose of 1×10^5 was significant ($P < 0.01$ by repeated-measures analysis of variance). Data below the figures represent the mean ± SD day of onset (a), the frequency (%) of arthritis induced (b), the mean ± SD maximum score (c), and the mean ± SD day on which the maximum score was reached for each mouse (d) among the mice in each group and are representative of 2 individual experiments. CIA = collagen-induced arthritis; ND = not determined.

Correlation of suppressive activity and expression levels of transduced Foxp3. As described previously, activated naturally arising Treg become more suppressive and express Foxp3 at higher levels than those expressed by freshly isolated cells. This fact led us to examine whether high-level Foxp3 expressers among Foxp3-transduced T cells exert stronger antiarthritic effects. Using a previously performed technique (4), we isolated $Foxp3^{high}$ and $Foxp3^{low}$ fractions based on their GFP expression level (Figure 5A, left). Immunoblot analyses of their cell lysates showed that Foxp3 expression in the fractionated cells correlated well with GFP expression. The low GFP fraction expressed Foxp3 at a level comparable with that expressed by naturally arising CD4+,CD25+ T cells (Figure 5A, right). Consistent with previous results (4), the $Foxp3^{high}$ fraction suppressed in vitro proliferation of CD4+,CD25− T cells more efficiently than did the $Foxp3^{low}$ fraction (Figure 5B).

The differential suppressive activity of the $Foxp3^{high}$ and $Foxp3^{low}$ cells may derive from differences in their activation levels. Theoretically, highly activated cells may enter the cell cycle more efficiently, thus making themselves more susceptible to retroviral integration. Actually, GFP expression levels of the T cells infected with empty vector (pMCs-IG) correlated positively with their surface CD69 levels (data not shown). To examine directly whether the Foxp3 expression level controls suppressive activity, we additionally used Foxp3-MIGR1, a retrovirus vector that was less efficient at protein expression, joined to the Foxp3 gene. The CD4 T cells activated with anti-CD3 mAb and DCs were divided for transfection with either Foxp3-pMCs-IG or Foxp3-MIGR1 retrovirus vector. Foxp3-transduced T cells generated by Foxp3-pMCs-IG expressed higher levels of Foxp3 (Figure 5C) and had higher suppressive activity (Figure 5B). Thus, the Foxp3 expression level regulated suppressive activity of Foxp3-transduced Treg.

Suppression of CIA by $Foxp3^{high}$ T cells versus $Foxp3^{low}$ T cells. For treatment of CIA, type II collagen–specific $Foxp3^{high}$ and $Foxp3^{low}$ T cell populations were generated from type II collagen–primed T cells. First, 1×10^5 cells from each population were transferred to mice 1 day prior to the primary type II collagen immunization. The 2 treatments suppressed arthritis equally, as shown by the arthritis score and measurement of joint swelling (Figure 6A). Transfer of the same number of OVA-reactive $Foxp3^{high}$ as well as $Foxp3^{low}$ T cells did not affect CIA development (Figure 6B). We next transferred $Foxp3^{high}$ or $Foxp3^{low}$ T cells to the hosts 20 days after the immunization (Figure 6C). When these mice were subjected to booster immunization for development of overt arthritis, $Foxp3^{low}$ T cells exerted only a marginal suppressive effect, demonstrating again that

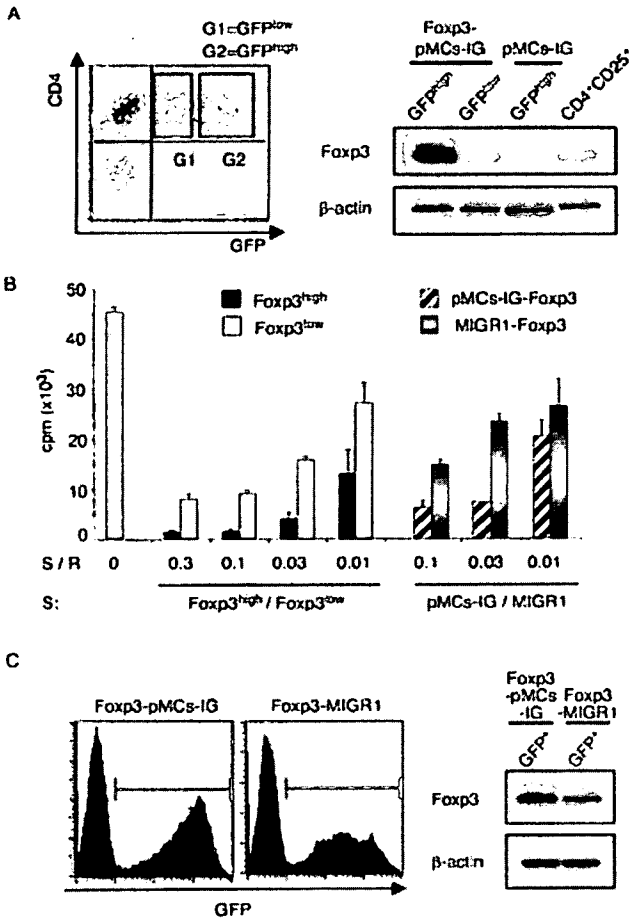


Figure 5. Dependence of Foxp3 expression level on suppressive activity of Foxp3-transduced T cells. A, CD4 T cells were activated and infected with Foxp3-pMCs-IG retroviruses as described in Figure 1. Panels represent the gates for isolating green fluorescent protein $(GFP)^{high}$ and GFP^{low} fractions (left), and immunoblots show Foxp3 or β-actin expression in GFP^{high} and GFP^{low} fractions of T cells infected with Foxp3-pMCs-IG, control pMCs-IG–infected cells, and naturally arising CD4+,CD25+ T cells (right). B, GFP^{high} or GFP^{low} fractions of T cells were infected with Foxp3-pMCs-IG viruses and isolated 48 hours after infection. Likewise, 2 T cell fractions were separately infected with Foxp3-pMCs-IG or Foxp3-MIGR1 retroviruses, and whole GFP-positive fractions were isolated. These suppressors (S) were cultured with CD4+,CD25− responder T cells (R) with irradiated splenocytes and anti-CD3 monoclonal antibody (0.5 μg/ml) for 72 hours. Proliferation of responders was assessed by ^{3}H-thymidine uptake. Bars show the mean and SD. C, CD4 T cells were activated as described in Figure 1 and infected with either Foxp3-pMCs-IG or Foxp3-MIGR1 retrovirus. Histograms (left) show GFP expression of the infected cells at 48 hours after infection. The gates on the histograms indicate Foxp3-expressing fractions, which were then sorted for detection by immunoblotting (right).

immunized hosts became resistant to suppression by Treg. In contrast, $Foxp3^{high}$ T cells overcame the resistance and suppressed CIA efficiently. A titration study demonstrated that as few as 1×10^4 $Foxp3^{high}$ T cells significantly suppressed CIA (Figure 6D). Because this number of unfractionated Foxp3-transduced cells had no effect, $Foxp3^{high}$ T cells had higher suppressive activity in vivo as well as in vitro.

Transfer of the same number of $Foxp3^{high}$ or $Foxp3^{low}$ fractions into knee joints at the same time did not suppress arthritis as efficiently as did systemic transfer. The effect was observed even in forelimbs (data not shown). This implies that the site of function for the Treg is not local synovial tissue. Finally, type II collagen–specific $Foxp3^{high}$ T cells (1×10^5 cells) were transferred intravenously 4 days after the booster immunization. The treatment attenuated progression of arthritis slightly but did not reverse the joint swelling (Figure 6E). Thus, arthritic mice gained further resistance to inhibition by Foxp3-expressing T cells.

DISCUSSION

The recent introduction of anticytokine therapies, including treatment with anti-TNFα agents, has improved the clinical outcome of RA that is refractory to conventional treatments (33). However, no current treatment targets pathogenic immune reactions to specific antigens. This often leads to the generalized immune suppression that is responsible for undesirable infections. We addressed this issue by generating antigen-specific Treg via transfer of the Foxp3 gene. Adoptive transfer of these cells to nonlymphopenic animals effectively suppressed CIA. In addition, we found that in vitro and in vivo suppressive activities of the genetically manipulated cells correlate well with the expression level of the Foxp3 gene. This allowed us to demonstrate that CIA becomes increasingly resistant to suppression by Treg during the disease course.

Activation of naturally arising CD4+,CD25+ T cells augmented their in vitro suppressive function. This is consistent with previous observations that stimulation with anti-CD3 and IL-2 (21,34) or antigen-pulsed DCs (22,35) remarkably enhanced the suppressive activity of CD4+,CD25+ Treg.

In vivo adoptive transfer experiments revealed that CD4+,CD25+ T cells specific to disease-relevant antigens were highly effective for disease suppression (21,22), which presumably was closely related to in vivo activation of the Treg. We observed that type II collagen–reactive Foxp3-transduced T cells suppressed CIA at much lower cell numbers compared with the number of non–antigen-specific CD4+,CD25+ Treg used in a previous study (25). The suppressed production of anti–bovine type II collagen antibodies (Figure 3B), which was not detected in the latter study, supports

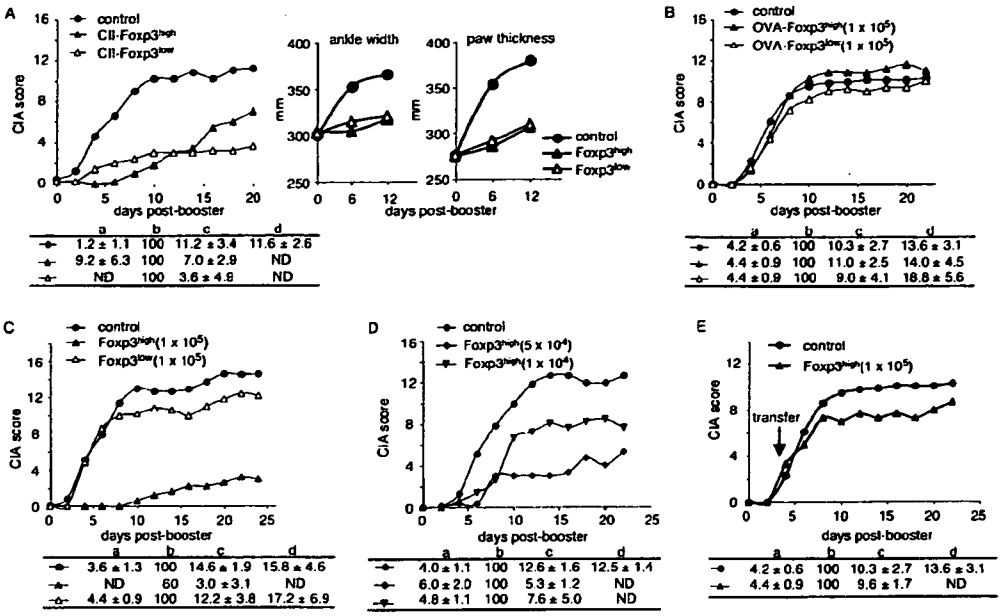


Figure 6. Collagen-induced arthritis (CIA) suppression by type II collagen (CII)–primed Foxp3^{high}/Foxp3^{low} fractions of Foxp3-transduced T cells. A and B, Naive mice received transfer of 1×10^5 Foxp3^{high}-transduced (n = 5) or Foxp3^{low}-transduced (n = 5) T cells primed with CII-pulsed dendritic cells (DCs) (A) or 1×10^5 Foxp3^{high}-transduced (n = 5) or Foxp3^{low}-transduced (n = 5) T cells primed with ovalbumin (OVA)–pulsed DCs (B) intravenously, 1 day prior to the primary immunization. Boosters were administered to the experimental mice as well as control mice, which did not receive Foxp3-transduced T cells (n = 10 in A and B). Joint swelling in the hind limbs was measured at the indicated days after booster immunization, and the mean values for the right and left sides of all mice in the experimental groups were plotted. Differences in CIA scores, ankle width, and paw thickness between the control group and the Foxp3^{high} and Foxp3^{low} groups were significant ($P < 0.01$ by repeated-measures analysis of variance [ANOVA]). C–E, Mice received transfer of 1×10^5 Foxp3^{high} (n = 5) or Foxp3^{low} (n = 5) T cells (C), 5×10^4 (n = 3) or 1×10^4 (n = 5) Foxp3^{high} T cells (D), or 1×10^5 Foxp3^{high} T cells (n = 3) (E) primed with CII-pulsed DCs, intravenously 20 days after the primary immunization (C and D) or 4 days after the booster immunization (E). A group of control mice did not receive T cells (n = 10 in C, n = 8 in D, and n = 10 in E). C, $P < 0.01$, control versus Foxp3^{high}, and $P < 0.05$, control versus Foxp3^{low}, by repeated-measures ANOVA. D, $P < 0.01$, control versus Foxp3^{high} (5×10^4) and Foxp3^{high} (1×10^4), by repeated-measures ANOVA. E, $P < 0.01$, control versus Foxp3^{high}, by repeated-measures ANOVA. Data below the figures represent the mean ± SD day of onset (a), the frequency (%) of arthritis induced (b), the mean ± SD maximum score (c), and the mean ± SD day on which the maximum score was reached for each mouse (d) among the mice in each group and are representative of 2 individual experiments. ND = not determined.

the fact that the antigen-specific Treg used in this study suppressed CIA more efficiently, because type II collagen–specific antibodies are known as the direct effectors giving rise to arthritis in the CIA model (36,37).

Treg can affect antibody production (38). Generating Foxp3-transduced T cells also circumvented the difficulty in preparing sufficient numbers of CD4+,CD25+ T cells, because this population occurs only as a small portion of peripheral T cells. Non–antigen-specific CD4+,CD25+ T cells (1×10^6) derived from 20 donors were required to treat 1 nonlymphopenic mouse with CIA (25). The Foxp3-transduced T cells needed to treat 1 CIA mouse (1×10^5 cells) in this study were generated from ~5×10^6 lymph node cells, which can be derived from 1 donor. At the moment, in vitro generation of Treg appears to be more practical for cell transfer therapy.

BDC2.5 T cell receptor–transgenic CD4+,CD25+ T cells, which presumably recognize an antigen derived from β cells in pancreatic islets, were shown to be capable of suppressing diabetes induced by diabetic NOD mouse splenocytes (21,22). Thus, Treg specific to a single antigen suppress immune responses induced by

polyclonal effectors. We assume that Foxp3-transduced T cells reactive to a single antigen specifically expressed in joint tissue could suppress T cell reactions against multiple autoantigens in the joints; the original target antigens of the arthritis may not need to be determined.

The Foxp3 expression level in Foxp3-transduced T cells directly correlated with their antiarthritic activity. A previous study showed that CD4 T cells from Foxp3-transgenic mice expressed 10–15-fold higher Foxp3 messenger RNA compared with those from wild-type mice. However, their in vitro suppressive activity was slightly reduced relative to that of wild-type CD4+,CD25+ T cells (6). Those investigators speculated that excessive Foxp3 expression interfered with the functional activation of Treg. In our experiments, the Foxp3 gene was retrovirally introduced after activating the T cells. This technique, combined with use of 2 different retrovirus vectors, allowed us to observe that the Foxp3 expression level dictated the suppressive activity of Foxp3-transduced Treg, both in vitro and in vivo.

It has been shown that stimulated CD4+,CD25− responder T cells were more resistant to in vitro suppression by Treg than were fresh CD4+,CD25− T cells (39,40). We observed that a larger number of Foxp3-transduced T cells was required to suppress CIA when transfer occurred after the primary immunization. Moreover, $Foxp3^{high}$ cells suppressed arthritis when the same dose of $Foxp3^{low}$ cells failed to have an effect. These findings demonstrate that in vivo resistance to Treg was induced after effector activation. In the CIA model, various other effectors besides T cells are involved in pathogenesis, including synovial fibroblasts. Insensitivity of these effectors to suppression by Treg may also contribute to the observed resistance. It may be possible that transfer of even larger numbers of $Foxp3^{high}$ cells can ameliorate full-blown arthritis. However, based on our results, current techniques would require multiple donors to treat a single host.

Our results suggest a possible problem in terms of the clinical application of Treg transfer therapy for patients with RA. Because advanced-stage disease would be refractory to such therapy, Treg transfer should be most effective for the induction of remission in the early stage of RA and for remission maintenance later. In contrast, $Foxp3^{high}$ T cells overcame, to some extent, the insensitivity of the immunized mice. If Treg are generated using Foxp3 gene transfer, transfer of T cells expressing high levels of Foxp3 would be desirable in Treg therapy for autoimmune diseases.

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AUTHOR CONTRIBUTIONS

Dr. Ohata had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Ohata, Ziegler, Kohsaka, Hori.
Acquisition of data. Ohata, Miura.
Analysis and interpretation of data. Ohata, Ziegler, Kohsaka.
Manuscript preparation. Ohata, Johnson, Kohsaka.
Statistical analysis. Johnson, Kohsaka.

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