The Influence of Excessive IL-6 Production In Vivo on the Development and Function of Foxp3+ Regulatory T Cells

Minoru Fujimoto, Mayumi Nakano, Fumitaka Terabe, Hirohisa Kawahata, Tomoharu Ohkawara, Yongmei Han, Barry Ripley, Satoshi Serada, Teppei Nishikawa, Akihiro Hirohisa Kawahata, Tomoharu Ohkawara, Yongmei Han, Barry Ripley, Satoshi Serada, Teppei Nishikawa, Akihiro Kimura, Shintaro Nomura, Tadamitsu Kishimoto and Tetsuji Naka

J Immunol 2011; 186:32-40; Prepublished online 24 November 2010;
doi: 10.4049/jimmunol.0903314
http://www.jimmunol.org/content/186/1/32

References
This article cites 25 articles, 7 of which you can access for free at:
http://www.jimmunol.org/content/186/1/32.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Influence of Excessive IL-6 Production In Vivo on the Development and Function of Foxp3+ Regulatory T Cells

Minoru Fujimoto,* Mayumi Nakano,† Fumitaka Terabe,‡ Hirohisa Kawahata,§ Tomoharu Ohkawara,§ Yongmei Han,§ Barry Ripley,§ Satoshi Serada,* Teppei Nishikawa,‖ Akihiro Kimura,¶ Shintaro Nomura,¶ Tadamitsu Kishimoto,¶ and Tetsuji Naka*

IL-6 is a proinflammatory cytokine and its overproduction is implicated in a variety of inflammatory disorders. Recent in vitro analyses suggest that IL-6 is a key cytokine that determines the balance between Foxp3+ regulatory T cells (Tregs) and Th17 cells. However, it remains unclear whether excessive IL-6 production in vivo alters the development and function of Foxp3+ Tregs. In this study, we analyzed IL-6 transgenic (Tg) mice in which serum IL-6 levels are constitutively elevated. Interestingly, in IL-6 Tg mice, whereas peripheral lymphoid organs were enlarged, and T cells exhibited activated phenotype, Tregs were not reduced but rather increased compared with wild-type mice. In addition, Tregs from Tg mice normally suppressed proliferation of naive T cells in vitro. Furthermore, Tregs cotransferred with naive CD4 T cells into SCID–IL-6 Tg mice inhibited colitis as successfully rather increased compared with wild-type mice. Collectively, these results suggest that IL-6 overproduced in vivo inhibits inducible Treg generation from naive T cells, but does not affect the development and function of natural Tregs. The Journal of Immunology, 2011, 186: 32–40.

Copyright 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00

Received for publication October 13, 2009. Accepted for publication October 16, 2010.

This work was supported by a grant-in-aid for the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation and in part by the Ministry of Education, Science, Sports and Culture with a grant-in-aid for Scientific Research (to M.F.) and research funding from Chugai Pharmaceutical (to A.K. and T.K.).

Address correspondence and reprint requests to Dr. Tetsuji Naka, National Institute of Biomedical Innovation, 7-6-8 Saitoasagi, Ibaraki City, Osaka 567-0085, Japan. E-mail address: tnaka@nibio.go.jp

Abbreviations used in this paper: GITR, glucocorticoid-induced TNFR-related protein; IgLN, inguinal lymph node; iTreg, inducible regulatory T cell; MesLN, mesenteric lymph node; N.D., not detected; nTreg, natural regulatory T cell; Sp, spleen; Tg, transgenic; Thy, thymus; Treg, regulatory T cell; Tresp, responder T cell.
maintained. However, although TGF-β and retinoic acid has been implicated in Foxp3+ iTreg generation, the regulation of Foxp3 expression in vivo remains largely unknown.

Because excessive IL-6 production is a common feature of chronic inflammatory diseases (1), it is of importance to determine if IL-6 plays a role in vivo in the regulation of Foxp3 expression and Treg development. In this study, using IL-6 transgenic (Tg) mice in which IL-6 is constitutively expressed (11), we investigated the impact of IL-6 overproduction in vivo on the development and function of Foxp3+ Tregs.

Materials and Methods

**Mice**

IL-6 Tg mice on a C57BL/6 background and on a C.B-17 SCID background (11) were kindly provided by Y. Ohsugi (Chugai Pharmaceutical, Sizouka, Japan). Wild-type BALB/c mice were purchased from Clea Japan (Tokyo, Japan). To generate donor IL-6 Tg mice for transfer experiment, Tg mice on a C.B-17 SCID background were crossed with wild-type BALB/c mice. Mice were maintained in specific pathogen-free facilities, and, unless specified, 8–11-wk-old mice were used for analysis. All animal experiments were performed with the approval of the institutional Animal Care and Use Committee.

**Cell preparations**

Single-cell suspensions were obtained from murine lymphoid organs as reported previously (12). To obtain naive CD4 T cells, splenocytes and lymph node cells were labeled with CD4 MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and positively selected with MS-5 columns according to the manufacturer’s instruction. Enriched CD4+ cells were further labeled with Abs against CD4 (BD Biosciences, Tokyo, Japan), CD62L, and CD25 (Biolegend). Two populations and CD4+CD25+ populations were obtained with the FACSARia cell sorter (BD Biosciences) and used as naive CD4 T cells and Tregs, respectively. For T cell transfer experiments, CD4+CD62L+CD45RBhi and CD4+CD25+ populations were obtained as naive CD4 T cells and Tregs, respectively. For T cell transfer experiments, CD4+CD62L+CD45RBhi and CD4+CD25+ populations were obtained as naive CD4 T cells and Tregs, respectively. For T cell transfer experiments, CD4+CD62L+CD45RBhi and CD4+CD25+ populations were obtained as naive CD4 T cells and Tregs, respectively. In some cases, highly purified CD4+CD62L+CD25+CD45RBhi population was used as donor naive CD4 T cells. The CD45RBhi population was defined as the most brightly stained cells consisting of 40–50% of non-Tg CD4+ cells. The purity of these naive CD4 T cells and Tregs was routinely >95%.

**ELISA**

Serum levels of human IL-6 were determined by human IL-6 US ELISA kit from Invitrogen (Carlsbad, CA).

**Flow cytometry**

Surface Ags were stained as reported previously (12) with the following Abs: anti-CD4, CD8, CD25 (BD Biosciences), and glucocorticoid-induced TNFR-related protein (GITR) (Biolegend). For intracellular staining of Foxp3 and cytokines, a Foxp3 staining kit (eBioscience, San Diego, CA) was used according to the manufacturer’s instructions. Cells were stained with Abs against Foxp3, IFN-γ (Biolegend), IL-17, Helios, or CD127 (Biolegend). Cells were analyzed with FACS Canto (BD Biosciences), and FACS data were analyzed with FlowJo software (Tree Star, Ashland, OR). For intracellular detection of p-STAT3, harvested cells were immediately fixed with 1.6% paraformaldehyde and permeabilized with ice-cold methanol as reported previously (13) and stained with Abs against p-STAT3 and TCRαβ (BD Biosciences).

**In vitro suppression assay**

Sorted naive CD4 T cells were labeled with 3 μM CFSE (Molecular Probes, Eugene, OR) and cultured in RPMI 1640 (Wako, Osaka, Japan) supplemented with 10% FCS (Hyclone, Irvine, CA), 2-ME (Nacalai Tesque, Eugene, OR) and cultured in RPMI 1640 (Wako, Osaka, Japan) supplemented with 10% FCS (Hyclone, Irvine, CA), 2-ME (Nacalai Tesque, Kyoto, Japan), penicillin G, and streptomycin. CD4 T cells were stimulated with anti-CD3e (0.5 μg/ml; BD Biosciences) in the presence of irradiated CD4-depleted splenocytes with or without sorted CD4+CD25+ Tregs from wild-type mice or IL-6 Tg mice. After 3 d, cells were harvested, stained with Abs against CD4 and Foxp3, and analyzed with FACS Canto (BD Biosciences).

**Western blot analysis**

Thymocytes obtained from wild-type mice or IL-6 Tg mice were lysed in ice-cold RIPA buffer immediately after harvest or 30 min poststimulation with human IL-6 (100 ng/ml; PeproTech, London, U.K.) plus soluble IL-6R (14) (100 ng/ml). Cell lysates were subjected to an SDS-PAGE as described previously (12). Samples transferred onto a filter were stained with anti-p-STAT3 (Cell Signaling Technology, Beverly, MA) or anti-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA) Abs and visualized as described previously (12).

**Quantitative PCR**

Complimentary DNA was synthesized from total RNA using a Quantitect Reverse Transcription kit (Qiagen, Valencia, CA). PCR reactions were performed in triplicate using FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). Levels of IL-10, TGF-β, and the control β-actin were determined by the 7900HT Real-time PCR system (Applied Biosystems, Foster City, CA) using specific primers: IL-10 forward 5'-CCCTTTGCTATGGTGTCCTT-3' and reverse 5'-TGGTTTCTCTCC-CAAGACC-3'; TGF-β forward 5'-ACCATGCAAATTCTGCTG-3' and reverse 5'-CGGGTTTGTGTTGTTGATA-3'; and β-actin forward 5'-GACACCACATCTTCTAAGT-3' and reverse 5'-GGTTTGGAAGTGTC- TCAAAC-3'.

**Induction of T cell transfer colitis**

T cell transfer colitis was induced as described previously (15) with minor modifications. Briefly, FACs-sorted CD4+CD62L+CD45RBhi cells from wild-type BALB/c mice or CD4+CD62L+CD25+CD45RBhi cells from IL-6 Tg mice or their non-Tg littermates were injected i.p. into SCID or SCID–IL-6 Tg mice (4 × 10⁵ cells/mice). In some cases, sorted CD4+CD25+ Tregs (1 × 10⁵ cells/mice) from BALB/c mice were cotransferred. All donors and recipients were housed in the same specific pathogen-free facility with comparable microbiota. Mice were euthanized for histological and cytological examinations 7 to 8 wk after T cell transfer.

**Histological analysis**

Tissues were fixed with 10% formaldehyde and sections were stained with H&E.

**CD4 T cell culture**

Sorted naive CD4+CD62L+CD25+ T cells were stimulated in vitro with plate-bound anti-CD3 (1 μg/ml; Biolegend) and anti-CD28 (1 μg/ml; BD Biosciences) in the presence or absence of cytokines as indicated in the figure legend. All cytokines were obtained from PeproTech. Anti–IL-6R Ab was described previously (7).

**FIGURE 1.** Excessive IL-6 signaling in T lineage cells of IL-6 Tg mice. A, Serum levels of human IL-6 in wild-type mice (n = 5) and IL-6 Tg mice (n = 5) are shown (mean ± SD). B, Freshly isolated thymocytes from wild-type mice, and IL-6 Tg mice were lysed pre- and poststimulation with IL-6 plus soluble IL-6R for 30 min, and lysates were subjected to Western blot analysis. C, Intracellular pSTAT3 levels in thymic and splenic TCRαβ T cells of wild-type mice and IL-6 Tg mice were determined by flow cytometry. N.D., not detected.

Downloaded from http://www.jimmunol.org/ by guest on July 28, 2017
**Statistics**
Experimental groups were compared with the Student unpaired t test, and p values <0.05 were considered significant.

**Results**

**Overproduction of IL-6 perturbs homeostasis of T cells in IL-6 Tg mice**

In IL-6 Tg mice, human IL-6 is produced under the control of MHC class I promoter. As reported previously (16), serum concentrations of IL-6 were constitutively elevated in IL-6 Tg mice (Fig. 1A). To examine the effect of IL-6 overproduction in IL-6 Tg mice, phosphorylation of STAT3 in freshly isolated thymocytes was evaluated by Western blot analysis (Fig. 1B). As expected, STAT3 in thymocytes of IL-6 Tg mice was phosphorylated without stimulation. In addition, flow cytometric analyses revealed that p-STAT3 levels in T cells of thymus and spleen were also elevated in IL-6 Tg mice (Fig. 1C). These results indicate that IL-6 overproduction affects T lineage cells in IL-6 Tg mice.

One of the representative characteristics in IL-6 Tg mice is marked enlargement of peripheral lymphoid organs (Fig. 2A). As shown in Fig. 2B, total cell counts of spleen cells, but not of thymocytes were significantly increased in IL-6 Tg mice compared with those of wild-type mice. In addition, increased percentages of T cells exhibited CD62L low phenotype (Fig. 2C), suggesting that IL-6 overproduction leads to aberrant activation and expansion of T cells in the periphery.

Given the established role of IL-6 in Th17 differentiation (5), we examined IL-17 production in CD4 T cells from IL-6 Tg mice using flow cytometry. Indeed, IL-17–producing CD4 T cells were increased significantly in IL-6 Tg mice compared with wild-type mice (Fig. 2D,2E). However, we also noted that IFN-γ–producing Th1 cells were also significantly increased in IL-6 Tg mice (Fig. 2D, 2E). These results suggest that IL-6 overproduction in vivo...
has an ability to enhance Th17 differentiation but does not skew Th cell differentiation toward Th17 pathway.

**Foxp3**+ Treg development is not impaired under conditions of excessive IL-6 production in vivo

To examine the effect of IL-6 on the development of Foxp3**+** Tregs, the expression of Foxp3 in CD4 T cells in IL-6 Tg mice was determined by flow cytometry. As shown in Fig. 3A and 3C, the percentage of Foxp3**+** Tregs among CD4 single-positive thymocytes in IL-6 Tg mice was not reduced but rather increased compared with that in wild-type mice. Similarly, the percentage of Foxp3**+** Tregs in peripheral lymphoid organs of IL-6 Tg mice was also increased compared with those of wild-type mice (Fig. 3B, 3C). Moreover, because the absolute number of peripheral lymphoid cells was markedly increased in IL-6 Tg mice (Fig. 2B), the absolute number of Foxp3**+** Tregs was much higher in IL-6 Tg mice than in wild-type mice (Fig. 3D). Thus, in contrast to the established inhibitory effect of IL-6 in vitro on Foxp3**+** iTreg generation, IL-6 overproduction in vivo did not inhibit natural Foxp3**+** Treg development in the thymus but rather led to a slight increase of this population. In addition, increased frequency and number of Foxp3**+** Tregs are maintained in the periphery even under conditions of IL-6 overproduction.

**Foxp3**+ Tregs from IL-6 Tg mice retain normal suppressor function in vitro

Flow cytometric analysis of CD25 and GITR two surface markers highly expressed on Foxp3**+** Tregs revealed that Foxp3**+** Tregs in IL-6 Tg mice, in comparison with those in wild type mice, express comparable levels of CD25 and increased levels of GITR (Fig. 4A). In addition, Foxp3**+** Tregs in IL-6 Tg mice express CTLA-4, a molecule crucial for Treg suppressor function (8), similar to or even higher than Tregs in wild-type mice (Fig. 4A). Foxp3**+** Tregs in Tg mice also express immunosuppressive cytokines IL-10 and TGF-β at the levels comparable to wild-type mice (Fig. 4B). To examine the function of Foxp3**+** Tregs, in vitro suppression activity of these cells was examined by CFSE dilution assay using wild-type naive CD4 T cells as responder cells. As expected, coculture of wild-type Tregs inhibited expansion of responder cells dose dependently (Fig. 4C, upper panel). Similarly, division of responder T cells was inhibited when Tregs from IL-6 Tg mice were cocultured (Fig. 4C, lower panel). These results suggest that Foxp3**+** Tregs in IL-6 Tg mice retain in vitro suppressor function. Interestingly, as shown in Fig. 4D, the addition of exogenous IL-6 during coculture of responder T cells with Tregs did not restore cell division of responder cells, suggesting that suppressor function of Tregs in vitro is maintained even in the presence of IL-6. However, because increased number of responder cells was retrieved from IL-6–supplemented culture (data not shown), IL-6 is likely to act not on Tregs but on responder cells and enhance their survival.

**CD4**+CD25**+** Tregs exert their suppressor function under conditions of excessive IL-6 production in vivo

We next evaluated the effect of IL-6 on the suppressor function of Tregs in vivo using a T cell transfer model of colitis. In this analysis, we used IL-6 Tg mice on an SCID background that were established previously (11). First, colitis was induced in SCID mice or SCID–IL-6 Tg mice by the transfer of wild-type naive CD4 T cells. In accordance with the pathogenic role of IL-6 in this model (15), body weight loss of SCID–IL-6 Tg mice progressed slightly but significantly faster than that of SCID mice after CD4 T cell transfer (Fig. 5A). Wild-type CD4+CD25+ Tregs were then cotransferred with naive CD4 T cells into SCID mice or SCID–IL-6 Tg mice. As reported previously, cotransfer of Tregs inhibited wasting disease and colitis in SCID mice (Fig. 5B, 5C). Interestingly, Treg cotransfer also successfully inhibited disease in SCID–IL-6 Tg mice in a similar manner (Fig. 5B, 5C). We also

---

**FIGURE 3.** Unimpaired development of Foxp3**+** Tregs in IL-6 Tg mice. CD4/CD8 profile and Foxp3 expression in gated CD4 single-positive T cells in thymus (A) and spleen (B) from wild-type mice, and IL-6 Tg mice were analyzed by flow cytometry. Representative results are shown. C, The percentages of Foxp3**+** Tregs in thymus and spleen from wild-type mice (n = 7 to 8) and IL-6 Tg mice (n = 9 to 10) are shown (mean + SD). D, The cell numbers of Foxp3**+** Tregs in thymus and spleen from wild-type mice and IL-6 Tg mice were estimated (mean + SEM). *p < 0.05 compared with the wild-type mice.
examined the frequency of Foxp3+ Tregs in these mice. As expected, sorted naive CD4 T cells before the transfer contained negligible frequency of Foxp3+ Tregs (data not shown). Eight weeks posttransfer, CD4 T cells retrieved from SCID mice contained ∼7% of Foxp3+ Tregs (Fig. 5D). However, CD4 T cells from SCID–IL-6 Tg mice contained slightly but significantly reduced frequency of Foxp3+ Tregs (∼5%; Fig. 5D), suggesting that Foxp3+ Treg induction from naive T cells were partially impaired in SCID–IL-6 Tg mice. When Tregs were cotransferred, increased frequencies of Foxp3+ Tregs were retrieved from both types of mice compared with naive CD4 T cells alone (Fig. 5E). Importantly, the frequencies of Foxp3+ Tregs were not different between SCID mice and SCID–IL-6 Tg mice after Treg cotransfer (Fig. 5E). These results suggest that transferred Foxp3+ Tregs can expand and function normally even under conditions of excessive IL-6 production in vivo.

**Generation of Foxp3+ iTregs is inhibited under conditions of excessive IL-6 production in vivo**

A recent report has shown that a transcription factor Helios is a useful marker to discriminate thymus-derived nTregs from iTregs (17). We therefore examined the expression of Helios among Foxp3+ Tregs by flow cytometry. In accordance with a previous observation (17), ∼30% of Foxp3+ Tregs in spleen did not express Helios in wild-type mice (Fig. 6A, B). The frequency of this population in thymus was much lower (Fig. 6A, B), likely reflecting a few iTregs reentered in thymus from the periphery (17). In contrast, we found that the percentage of Helios−Foxp3+ population was higher in wild-type mesenteric lymph nodes, conceivably due to increased iTreg generation driven by gut-derived Ags (Fig. 6A, 6B). Strikingly, in IL-6 Tg mice, the frequencies of Helios−Foxp3+ cells in all these organs were significantly lower compared with wild-type mice (Fig. 6A, 6B). This result supports the notion that iTreg development is impaired under excessive IL-6 production in vivo.

We next investigated whether naive CD4 T cells in IL-6 Tg mice retain the ability to differentiate into Foxp3+ Tregs. For this purpose, FACS-sorted naive CD4 T cells from IL-6 Tg mice and wild-type mice were cultured in vitro in the presence of various concentration of TGF-β. As shown in Fig. 6C, dose-dependent induction of Foxp3+ Tregs were observed in both cells obtained from Tg mice and wild-type mice. In addition, in both cell types, Foxp3+ Treg induction was not enhanced by anti–IL-6R Ab treatment, and Th17 cell differentiation was induced only in the presence of exogenous IL-6 (Fig. 6C). We then transferred highly purified naive CD4 T cells of IL-6 Tg mice or non-Tg littermate controls into SCID mice and investigated their differentiation in vivo. As shown in Fig. 6D, the induction of Foxp3+ Tregs was comparable between mice received Tg donors and control donors. We also found that naive CD4 T cells from IL-6 Tg mice preferentially differentiated into Th1 cells as did control cells and did not show marked skewing toward Th17 cells (Fig. 6D, 6E). These results suggest that naive CD4 T cells persistently exposed to IL-6 in vivo retain their ability to differentiate into various Th subsets including Foxp3+ Tregs.
Discussion

IL-6 is robustly produced during inflammatory processes and regulates immune responses both locally and systemically. Recently, in vitro studies have highlighted a role of IL-6 in Foxp3+ Treg development and/or function, but it remained unknown if excessive IL-6 production has an effect on Foxp3+ Tregs in vivo. In this study, we used IL-6 Tg mice as a model mimicking excessive IL-6 production during chronic inflammation and investigated the effect of IL-6 overproduction in vivo on the development and function of Foxp3+ Tregs.

In IL-6 Tg mice, serum IL-6 levels are constitutively elevated, and the IL-6/STAT3 signaling pathway is activated in thymocytes and splenic T cells. Peripheral lymphoid organs in these mice were enlarged and contained increased number of activated T cell. We showed that IL-17-producing CD4 T cells were significantly increased in IL-6 Tg mice, in accordance with the critical role of IL-6 in Th17 differentiation. However, we also found that IFN-γ-producing CD4 T cells were also significantly increased in Tg mice. These results suggest that excessive IL-6 leads to dysregulated Th responses but does not skew Th differentiation toward a Th17 subset.

In IL-6 Tg mice, the frequency and number of Foxp3+ Tregs in the thymus of IL-6 Tg mice were not reduced but rather increased compared with that of control mice. This result indicates that IL-6 overproduction does not inhibit the generation of thymus-derived nTregs in vivo. In addition, we found that the frequency and absolute number of Foxp3+ Tregs in the periphery were also increased in IL-6 Tg mice compared with control mice. This result suggests that IL-6 overproduction does not inhibit the maintenance of peripheral Foxp3+ Tregs in vivo.

Our findings largely contradict a previously proposed role of IL-6 in Foxp3+ Treg development. This discrepancy may in part be due to the fact that recent in vitro analyses have mainly focused on iTregs rather than nTregs. Indeed, signal requirements for iTreg and nTreg generation appear to be different, and iTreg generation is dependent more on TGF-β signaling than thymic nTreg development (18). Because thymus-derived nTregs are the major population of Foxp3+ Tregs in vivo (18), it is reasonable to speculate that our experiments mainly disclosed the effect of IL-6 on nTregs rather than iTregs. In accordance with this, whereas the frequency of Foxp3+ Tregs was not reduced in IL-6 Tg mice, Foxp3+ Treg generation in SCID–IL-6 Tg mice transferred with
sorted naive CD4 T cells alone were reduced compared with those in control SCID mice. In addition, our detailed analysis of Foxp3+ population, using Helios as a marker of nTregs, revealed that the frequency of Helios$^+$ iTregs was significantly reduced in IL-6 Tg mice compared with wild-type mice. These results suggest that IL-6 in vivo interferes not with nTreg development but with peripheral iTreg induction from naive CD4 T cells presumably via counteracting against TGF-β signaling. Given the presence of increased Th17 cells in IL-6 Tg mice, it is conceivable that overexpressed IL-6 enhances Th17 differentiation instead of iTreg generation in vivo. However, as suggested from our findings that Th1 cells are also increased in IL-6 Tg mice, it is likely that IL-6 does not merely regulate iTreg/Th17 balance but also has influence on effector CD4 T cells other than Th17 cells. Detailed analyses are currently underway in our laboratory to elucidate the role for IL-6 in effector Th populations.

It was shown previously that TGF-β also plays a role in the maintenance of peripheral Foxp3$^+$ Tregs, implying that IL-6 may

FIGURE 6. The generation of iTregs in IL-6 Tg mice. A and B, Helios expression in Foxp3$^+$ CD4 T cells in thymus, spleen, and mesenteric lymph nodes of wild-type mice (n = 4) and IL-6 Tg mice (n = 5) were analyzed by flow cytometry. Representative FACS data are shown in A. The mean percentages (± SD) of Helios$^-$ iTregs among Foxp3$^+$ CD4 T cells are shown in B. C, Naive CD4 T cells obtained from wild-type mice and IL-6 Tg mice were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 in the presence of indicated concentrations (0–10 ng/ml) of human TGF-β. Where indicated, IL-6 (50 ng/ml) or anti–IL-6R Ab (10 μg/ml) was also added to the culture. After 5 d of culture, cells were analyzed by flow cytometry. D and E, Highly purified CD4$^+$ CD62L$^+$CD25$^-$CD45RB$^{hi}$ naive CD4 T cells from IL-6 Tg mice or non-Tg control littermates were transferred to SCID mice. Intracellular expression of Foxp3, IL-17, IL-4, and IFN-γ in gated CD4 T cells of mesenteric lymph nodes was determined by flow cytometry 7 to 8 wk posttransfer. Representative FACS profiles of CD4 and Foxp3 or IL-17 and IFN-γ are shown in D. Mean percentages of Th1 (IFN-γ$^+$), Th2 (IL-4$^+$), Th17 (IL-17$^+$), and Foxp3$^+$ Tregs in mesenteric lymph nodes from mice transferred with control donor cells (n = 12) or Tg donor cells (n = 9) are shown in E (mean ± SD). *p < 0.05 compared with the wild-type mice.
have an inhibitory effect on this process. In addition, several groups have recently reported the plasticity of Foxp3+ Tregs including not only iTregs but also a subset of nTregs (18, 20) and suggested that IL-6 is most potent in reprogramming Foxp3+ Tregs and converting them into effector cells (19). However, our finding indicates that chronic overproduction of IL-6 in vivo is insufficient to drive uncontrolled conversion of peripheral Foxp3+ Tregs and perturb the overall balance between Foxp3+ Tregs and effector T cells. In addition, we also found that naïve CD4 T cells chronically exposed to IL-6 in Tg mice still retained their ability to differentiate into Foxp3+ Tregs in vitro and in vivo. Thus, factor(s) other than IL-6, such as cognate Ags and costimulation for Foxp3+ Tregs, may be required to disrupt tightly regulated balance between Foxp3+ Tregs and effector T cells.

In this study, we found that the frequency and the number of Foxp3+ Tregs are increased in IL-6 Tg mice. It should be noted that upregulation of Tregs is often observed during inflammatory responses including infection (21, 22) and autoimmunity (6, 7). Because T cells in IL-6 Tg mice were aberrantly activated presumably due to the direct effect of IL-6 on this population, the increase in Tregs of IL-6 Tg mice may be secondary to restrain these activated T cells. A finding supporting this notion is that Tregs cotransferred to SCID–IL-6 Tg mice did not expand more vigorously than those transferred to control SCID mice. In addition, Foxp3+ Tregs obtained from wild-type mice and Tg mice failed to expand in vitro in the presence of IL-6 (data not shown). These experiments suggest that IL-6 does not directly upregulate Foxp3+ Tregs. However, future analysis may be required to determine the effect of IL-6 on intrathymic nTreg development.

Our findings also contradict the previously proposed role of IL-6 in the regulation of Treg function (23). In this study, we found that Foxp3+ Tregs from IL-6 Tg mice have normal suppressor function in vitro and Tregs transferred to IL-6 Tg mice effectively prevented CD4 T cell-mediated colitis in vivo. These results suggest that increased levels of circulating IL-6 do not impair suppressor function of Tregs in vivo. Unexpectedly, we also found that in vitro suppressor function of wild-type Tregs is not abrogated by exogenous IL-6 in culture, because cell division of responder cells in this setting was normally suppressed. Nevertheless, the absolute number of viable responder cells was noticeably increased when exogenous IL-6 was added to the culture (data not shown). These results suggest that IL-6 acts directly on responder CD4 T cells rather than on Tregs and supported their survival. Indeed, lines of evidence indicate that IL-6 has an antiapoptotic property on CD4 T cells (24), although further studies are necessary to exclude the possibility that IL-6 specifically reverses the proapoptotic function of Tregs (25).

Until recently, there is no specific marker distinguishing nTreg and iTregs. Although Helios was newly discovered as an intracellular marker of nTregs, specific roles of these Treg sub-populations in immune regulation remain to be clarified (9). Thus far, lines of evidence indicate that there are many similarities but also substantial differences between these two populations. Interestingly, the TCR repertoire of nTregs only partially overlaps with that of conventional T cells and preferentially exhibits self-reactivity (18). This observation suggests that the TCR repertoire between nTreg and iTregs are different and that nTregs may be important for the maintenance of self-tolerance via recognizing self-Ags, whereas iTregs generated from conventional T cells may be involved in tolerance to non-self Ags. Interestingly, we found that Helios Foxp3+ Tregs were more frequent in mesenteric lymph nodes compared with spleen, suggesting that these iTregs contribute to the tolerance to gut-derived Ags. Because our study suggests that IL-6 acts differently on nTreg and iTregs, IL-6 may promote immune response against foreign Ags by repressing iTreg generation and may retain self-tolerance by maintaining Foxp3+ nTreg development. Future studies using specific markers for each Treg subset will be necessary to address this issue.

In summary, our study indicates that the development and function of Foxp3+ Tregs in vivo is largely unaffected by excessive production of IL-6. However, the effect of IL-6 is likely to be different between subpopulations of Foxp3+ Tregs, and, consistent with previous studies in vitro, IL-6 in vivo exhibited an inhibitory effect on Foxp3+ Treg induction from naïve CD4 T cells. IL-6 may thus enhance foreign Ag-specific immune response by acting differently on nTregs and iTregs. Further studies are required to clarify the intracellular mechanism by which IL-6 signaling regulates Foxp3 expression in thymic nTregs and peripheral iTregs.

Acknowledgments
We thank Y. Ohsugi (Chugai Pharmaceutical) for providing IL-6 transgenic mice, Y. Ito and N. Kawakami for secretarial assistance, and Y. Matsukawa for technical assistance.

Disclosures
T.K. is a holder of anti-human IL-6R Ab (Tocilizumab).

References


