Role of IFN-γ in induction of Foxp3 and conversion of CD4+CD25− T cells to CD4+ Tregs

Zhaojun Wang,[1] Jian Hong,[1,2] Wei Sun,[2] Guangwu Xu,[1] Ningli Li,[1] Xi Chen,[1] Ailian Liu,[1] Lingyun Xu,[1] Bing Sun,[3] and Jingwu Z. Zhang[1,2,4]

[1]Joint Immunology Laboratory of Institute of Health Sciences and Shanghai Institute of Immunology, Shanghai JiaoTong University School of Medicine, and Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, People's Republic of China. [2]Department of Neurology, Baylor College of Medicine, Houston, Texas, USA. [3]Institute of Biochemistry and Cell Biology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, People's Republic of China. [4]E-Institutes of Shanghai Universities, Shanghai, People's Republic of China.

IFN-γ is an important Th1 proinflammatory cytokine and has a paradoxical effect on EAE in which disease susceptibility is unexpectedly heightened in IFN-γ–deficient mice. In this study, we provide what we believe is new evidence indicating that IFN-γ is critically required for the conversion of CD4+CD25− T cells to CD4+ Tregs during EAE. In our study, the added severity of EAE in IFN-γ knockout mice was directly associated with altered encephalitogenic T cell responses, which correlated with reduced frequency and function of CD4+CD25+Foxp3+ Tregs when compared with those of WT mice. It was demonstrated in both human and mouse systems that in vitro IFN-γ treatment of CD4+CD25− T cells led to conversion of CD4+ Tregs as characterized by increased expression of Foxp3 and enhanced regulatory function. Mouse CD4+CD25− T cells, when treated in vitro with IFN-γ, acquired marked regulatory properties as evidenced by suppression of EAE by adoptive transfer. These findings have important implications for the understanding of the complex role of IFN-γ in both induction and self regulation of inflammatory processes.

Introduction

EAE is an established animal model for MS and is mediated by activated T cells specific for various myelin autoantigens, such as myelin oligodendrocyte glycoprotein (MOG) (1). Predominant Th1 immunity of encephalitogenic T cells represents one of the main immunologic features of EAE (2). As an important member of the family of Th1 cytokines typically seen in EAE, IFN-γ is thought to play an important role in the activation of encephalitogenic T cells and CNS inflammation. For example, IFN-γ has been demonstrated to upregulate MHC class II expression that facilitates migration of T cells into CNS (3, 4). However, it is entirely unexpected that blocking of IFN-γ does not ameliorate the disease but exacerbates the clinical severity and the pathology of EAE, which seems paradoxical when one considers the Th1 paradigm of EAE (5–7). This phenomenon is not limited to EAE and is found in other Th1 autoimmune conditions, including experimental autoimmune uveitis (8, 9), which implies a generalized role of IFN-γ in the regulation of autoimmune T cell responses. Studies over the past 10 years have not yet offered a convincing explanation of this puzzle, even though some observations have been made to partially account for the effect of IFN-γ in relationship to heightened susceptibility to EAE (10–14). For example, Willenborg and colleagues previously described IFN-γ as downregulating EAE by enhancing inducible NO synthase and subsequently NO production in macrophages in the periphery and microglia and astrocytes in the target tissue (14). Other investigators suggested that the role of IFN-γ in EAE is related to T cell suppression and the production of chemokines (12, 13). These studies, however, have not satisfactorily addressed the critical role of IFN-γ and the exact mechanism of its deficiency in relation to heightened susceptibility to EAE.

In a recent study, to analyze the induction of transcription factor Foxp3 expression in CD4+ T cells, a marker associated with CD4+CD25+ Tregs, we discovered that only IFN-γ and TGF-β but not other Th1 and Th2 cytokines could characteristically induce the expression of Foxp3 in CD4+CD25− T cells (unpublished observations). This initial finding prompted us to address the potential role of IFN-γ in the induction of CD4+CD25+ Tregs and its potential association with the unexplained paradox in EAE. The CD4+CD25+ Treg network has been recognized recently as an important regulatory mechanism that keeps autoreactive T cells in check (15–19). There is evidence indicating that CD4+CD25+ Tregs are characteristically associated with activation and expression of transcription factor Foxp3 (20–22). Gene transfer of Foxp3 directly results in conversion of naive CD4+ T cells to CD4+CD25+ Tregs (15, 20). Thus, the expression of Foxp3 distinguishes CD4+CD25+ Tregs from T cells without regulatory function that are also present in the CD4+CD25+ T cell pool, and Foxp3 expression has therefore been used as a marker for CD4+CD25+ Tregs (21, 23–26).

In this study, we first examined whether altered encephalitogenic T cell responses and added severity of EAE in IFN-γ KO mice was associated with impaired CD4+CD25+ Treg function compared with that of WT mice. Detailed investigation was carried out to further address whether IFN-γ is required for the induction of Foxp3 expression in CD4+ T cells and subsequent conversion of CD4+CD25− T cells into CD4+ Tregs in both mouse experiments, including adoptive transfer experiments, and human experimental systems. Our initial observation indicates that lack of IFN-γ is associated with reduced frequency and function of CD4+ Tregs, leading to heightened susceptibility to and added severity of EAE

Nonstandard abbreviations used: GKO, IFN-γ gene KO; MOG, myelin oligodendrocyte glycoprotein.

Conflict of interest: The authors have declared that no conflict of interest exists.

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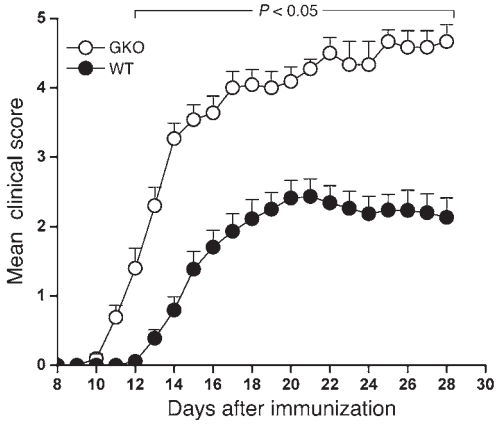

Figure 1
Severity of EAE in IFN-γ GKO and WT mice. WT (n = 27) C57BL/6 mice (IFN-$\gamma^{+/+}$, open circles) and IFN-γ GKO (n = 21) mice (IFN-$\gamma^{-/-}$, closed circles) were immunized under the same experimental conditions on day 0 with the encephalitogenic peptide of MOG (residues 35–55). Pertussis toxin (200 ng/mouse) was administered i.v. on the day of immunization and 2 days later. The mice were monitored daily and scored clinically as described in Methods. Data are representative of 3 independent experiments.

in IFN-γ–deficient mice. The study provides what we believe is new evidence demonstrating that IFN-γ is critical for conversion of $CD4^+CD25^-$ T cells to $CD4^+$ Tregs during EAE. Our findings suggest that IFN-γ is important for homeostasis of the immune system and acts by inducing a self-regulating mechanism in response to overt inflammation (EAE) in which high production of IFN-γ is seen by the immune system as a danger signal. The results provide what we believe are new insights into the complex role of IFN-γ in autoimmune disease and inflammatory processes as well as in other pathological conditions, such as tumor and infection.

Results

Characteristics of encephalitogenic T cell responses in IFN-γ gene KO mice and WT EAE mice. EAE was induced in both IFN-γ gene KO (GKO) mice and WT mice of the same C57BL/6 background under the same experimental conditions. GKO mice exhibited more severe EAE with respect to clinical score (Figure 1) and spinal cord pathology (Figure 2) compared with WT mice. The results were in agreement with those reported previously (5, 12). Furthermore, T cells derived from GKO EAE mice exhibited a significantly augmented proliferative response to the eliciting MOG peptide than those of WT EAE mice (Figure 3A). The increased encephalitogenic T cell response seen in GKO EAE mice was accompanied by a seemingly altered cytokine profile of encephalitogenic T cells characterized by Th1 deviation, as evidenced by markedly increased production of TNF-α and a decrease in IL-10 and IL-4 production (Figure 3B).

Impaired expression of Foxp3 and regulatory function of $CD4^+CD25^+$ T cells in IFN-γ KO mice during EAE. We then examined the expression of transcription factor Foxp3 and inhibitory function of purified $CD4^+$ and $CD4^+CD25^+$ T cells obtained from GKO and WT mice experiencing acute EAE. The percentage of $CD4^+CD25^+$ T cells rose slightly in $CD4^+$ T cells of splenocytes derived from GKO mice compared with those from WT mice (mean 8.83% versus mean 6.95%). Purified $CD4^+CD25^+$ T cells derived from GKO mice exhibited markedly decreased inhibitory activities on the encephalitogenic T cell response to the MOG peptide when compared with their counterparts obtained from WT EAE mice (Figure 4, A and B) and failed to express comparable levels of Foxp3 in relation to that of WT mice (Figure 4C). Similarly, there was decreased expression of Foxp3 in T cells isolated from CNS tissue of GKO mice when compared with that of WT mice during acute EAE (Figure 4D). Furthermore, intracellular Foxp3 expression in $CD4^+CD25^+$ T cell populations obtained from WT or GKO EAE mice was analyzed in the context of other cellular markers (CD62L, GITR, and CTLA-4). The results confirmed impaired expression of Foxp3 in GKO mice during acute EAE (Figure 4E).

Role of IFN-γ in the conversion of $CD4^+CD25^-$ T cells to $CD4^+$ Tregs during EAE. We then determined whether the reduced frequency and function of $CD4^+CD25^+$ Tregs seen in IFN-γ GKO mice potentially correlated with impaired conversion of $CD4^+CD25^-$ T cells to $CD4^+$ Tregs in the absence of IFN-γ during EAE. To directly address this question, purified $CD4^+CD25^-$ T cells from naive GKO or WT mice were cultured in the presence of recombinant mouse IFN-γ in an attempt to convert $CD4^+CD25^-$ T cells to $CD4^+$ Tregs. The analyses revealed that the resulting T cell populations markedly inhibited the proliferation of syngeneic T cells induced by antibodies to CD3 and CD28 (Figure 5A). The effect correlated with a high level of Foxp3 expression by real-time PCR (Figure 5B). As shown in Figure 5C, treatment of $CD4^+CD25^-$ $Foxp3^-$ T cells of GKO mice with IFN-γ alone led to the conversion of approximately 10% ± 2% of these cells into $Foxp3^+CD4^+$ T cells as measured by intracellular staining; this percentage was enhanced to 42% ± 4% $Foxp3^+CD4^+$ T cells within this population by additional TCR costimulation. The observed effect of IFN-γ was more pronounced for T cells derived from GKO, which might be related to differential expression of IFN-γ receptors on $CD4^+CD25^-$ T cells obtained from WT or GKO mice. Surface

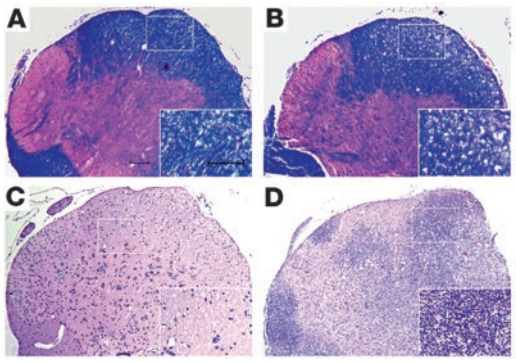

Figure 2
Histology of spinal cord obtained from acute EAE of IFN-γ GKO and WT mice. Histology of spinal cords was analyzed on day 16 after immunization for WT mice (IFN-$\gamma^{+/+}$, left panels) and GKO mice (IFN-$\gamma^{-/-}$, right panels). Pictures shown are representative of spinal cord histology of 3 to 4 mice in each group. (**A** and **B**) Luxol fast blue staining: degree of demyelination in WT mice (**A**) and GKO mice (**B**). (**C** and **D**) H&E staining: degree of inflammation in WT mice (**C**) and GKO mice (**D**). Magnification, ×50; magnification of insert, ×100. Scale bar: 100 μm. The degree of demyelination and inflammatory infiltrates was quantified on an average of 3 spinal cord transverse sections per mouse for a total of 5 mice per group. Demyelination score was 0.83 ± 1.74 for WT mice and 2.7 ± 1.77 for GKO mice. Inflammatory infiltrates are 0.86 ± 1.21 for WT mice and 2.9 ± 0.74 for GKO mice (mean ± SD). Differences are statistically significant (P < 0.05).

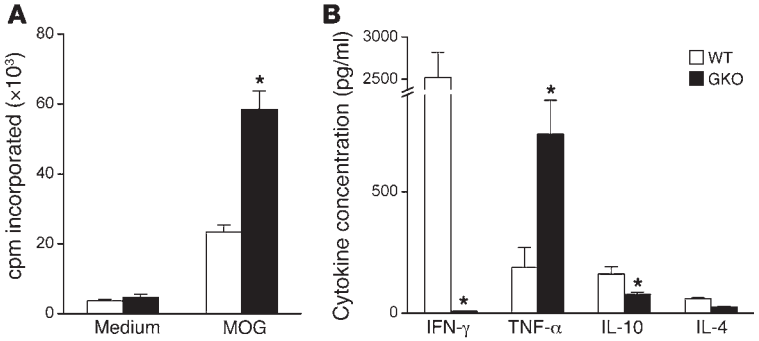

Figure 3
Encephalitogenic T cell response and cytokine profile in EAE mice. (**A**) Splenocytes isolated from GKO (IFN-$\gamma^{-/-}$) and WT (IFN-$\gamma^{+/+}$) mice at day 16 after immunization were examined for proliferation in the presence and absence (medium control) of the MOG peptide (5 μg/ml). Data are presented as mean cpm ± SD of triplicates. (**B**) Supernatants were collected from the above-mentioned culture after 48 hours and measured for the concentration of the indicated cytokines using ELISA. Values represent mean concentrations (pg/ml ± SD) of triplicates. Results were reproduced in 3 independent experiments. Asterisks represent statistical differences between groups; *$P < 0.05$.

expression of IFN-γ receptors was 41% ± 4% in $CD4^+CD25^-$ T cells derived from WT mice and 51% ± 3% in those from GKO mice as measured in 3 independent flow cytometric analyses using an anti-CD119 antibody. Purified mouse $CD4^+CD25^-$ T cells did not express significant levels of Foxp3 upon stimulation with antibodies to CD3/CD28 (data not shown). As illustrated in Figure 6, adoptive transfer of $CD4^+CD25^{high}$ T cells derived from $CD4^+CD25^-$ T cell cultures treated with IFN-γ and TCR costimulation exhibited a significant inhibitory property in the suppression of EAE induced in GKO mice while PBS control or untreated $CD4^+CD25^+$ T cells had no inhibitory effect.

The induction of Foxp3 expression and subsequent conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Tregs were further characterized in human $CD4^+CD25^-$ T cells using an in vitro experimental system. As illustrated in Figure 7, A–C, recombinant human IFN-γ, but not IL-10 and TNF-α, selectively induced the expression of Foxp3 in $CD4^+CD25^-$ T cells derived from healthy volunteers as shown by real-time PCR, immunoblot analysis, and intracellular staining. It is shown in Figure 7C that intracellular staining of Foxp3 in IFN-γ–induced $CD4^+CD25^+$ T cells displayed a shifted population as confirmed in our repeated experiments. The pattern was somewhat different from that of naturally occurring Tregs exhibiting 2 distinguishable or segregated populations. The observed discrepancy in staining patterns may be explained by staining of homogenously converted T cells by IFN-γ induction versus that seen in naturally occurring Tregs. Figure 7B shows the dose-response pattern of Foxp3 expression in purified $CD4^+CD25^-$ T cells induced by IFN-γ. The observed effect of IFN-γ in Foxp3 expression could be inhibited significantly by a blocking antibody to IFN-γ but not antibodies to other cytokines, excluding the possibility that the observed effect might be attributable to other cytokines (e.g., TGF-β) in the experimental system (Figure 7D). Furthermore, the resulting $CD4^+CD25^+$ T cells converted from $CD4^+CD25^-$ T cells after in vitro treatment with IFN-γ exhibited significantly increased inhibition of autologous T cell proliferation induced

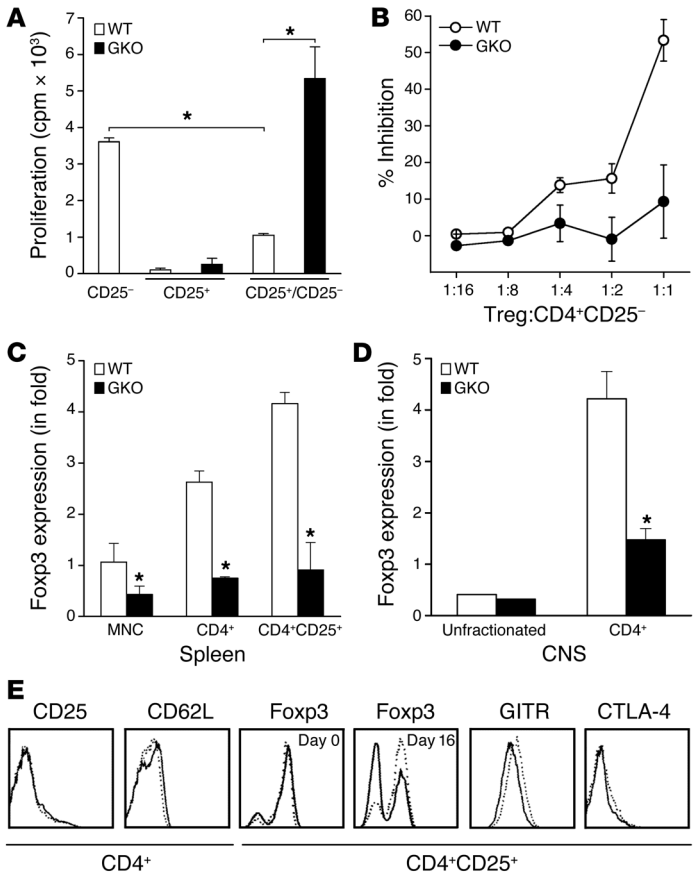

Figure 4
Foxp3 mRNA expression and the regulatory properties of $CD4^+CD25^+$ T cells obtained from EAE mice and $CD4^+CD25^-$ T cells. $CD4^+CD25^+$ T cells were purified from splenocytes of GKO and WT mice at day 16 after immunization and used as inhibitor. The resulting $CD4^+CD25^+$ T cells were assayed for inhibitory activity on the proliferation of $CD4^+CD25^-$ T cells (responder) purified from WT EAE mice in the presence of the MOG peptide (5 μg/ml) and APCs. Data are presented as mean cpm ± SD at a fixed ratio of inhibitor ($CD25^+$) to responder ($CD25^-$) of 1 (**A**) or percentage of inhibition at the indicated ratio of inhibitor to responder (**B**). (**C**) Splenocytes and purified $CD4^+$ and $CD4^+CD25^+$ T cells were obtained from GKO (IFN-$\gamma^{-/-}$) and WT (IFN-$\gamma^{+/+}$) mice at day 16 after immunization and subject to real-time PCR analysis for the expression of Foxp3. (**D**) mRNA expression of Foxp3 in $CD4^+$ T cells purified from CNS tissue of GKO and WT EAE mice. Data are presented as relative expression of Foxp3 in reference to β-actin. (**E**) Splenocytes were derived from WT (dotted lines) or GKO (solid lines) EAE mice on day 0 and day 16 after immunization. Cells were analyzed for intracellular Foxp3 staining and surface expression of the indicated cellular markers in the gated T cell populations by flow cytometry. Plots shown are representative of 6 independent experiments. Asterisks represent statistical differences between groups; *$P < 0.05$.

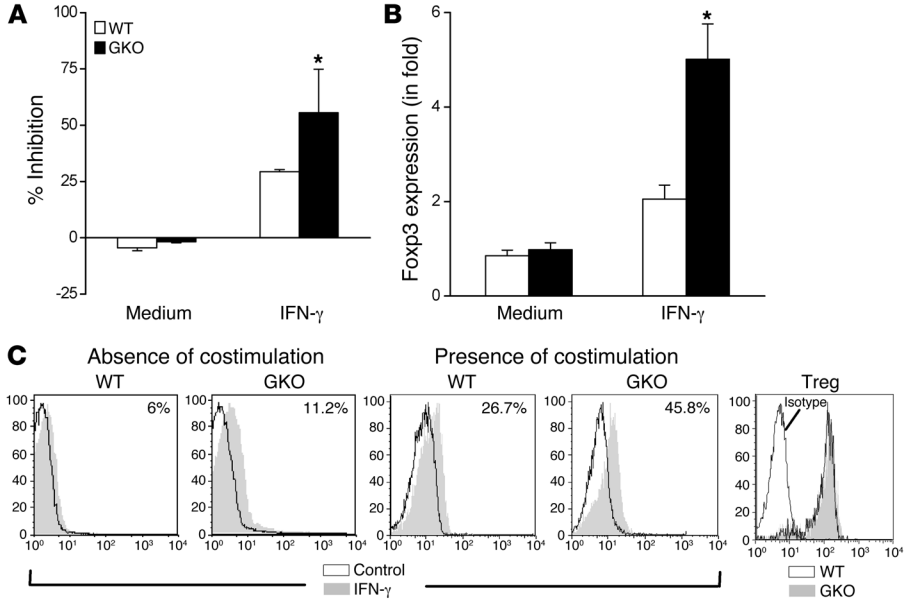

Figure 5

Conversion of CD4⁺CD25⁻ T cells to CD4⁺CD25⁺ Tregs in response to IFN-γ in vitro. (**A**) CD4⁺CD25⁻ T cells were purified from splenocytes of unprimed GKO (IFN-$\gamma^{-/-}$) and WT (IFN-$\gamma^{+/+}$) mice, respectively, and cultured in the presence and absence (medium control) of recombinant mouse IFN-γ for 24 hours. The resulting T cells (inhibitor) were examined for their ability to inhibit the proliferation of syngeneic CD4⁺CD25⁻ T cells (responder) induced by antibodies to CD3/CD28. Results are given as percentage of inhibition rate. (**B**) Aliquots of the T cell preparations described above were analyzed in parallel experiments for the expression of Foxp3 by real-time PCR. (**C**) CD4⁺CD25⁻ T cells described above were cultured with (gray contours) or without IFN-γ (open contours) in the presence or absence of anti-CD3 antibody. Intracellular expression of Foxp3 was measured in gated CD4⁺ T cells or CD4⁺CD25⁺ T cells. Indicated percentage refers to percentage of change between experimental groups with or without IFN-γ treatment. Purified CD4⁺CD25⁺ T cells (Tregs) from WT or GKO mice were stained for intracellular Foxp3 expression. Asterisks indicate that differences between groups are statistically significant; $^*P < 0.05$.

by antibodies to CD3 and CD28 (Figure 7E). Taken together, the results indicate that IFN-γ is critically required for conversion of CD4⁺CD25⁻ T cells to CD4⁺ Tregs.

Discussion

In this study, we provide compelling evidence indicating that IFN-γ plays an essential role in conversion of CD4⁺CD25⁻ T cells to CD4⁺ Tregs. First, in an EAE model induced in both WT and IFN-γ GKO mice, we demonstrated that heightened susceptibility of IFN-γ GKO mice to EAE is attributed to uncontrolled and altered encephalitogenic T cell responses, which directly result from impaired conversion of CD4⁺CD25⁻ T cells to CD4⁺ Tregs in the absence of IFN-γ in the disease state. This conclusion is based on the following observations: (a) there is reduced frequency and function of CD4⁺CD25⁺ Tregs accompanied by decreased Foxp3 expression as a result of impaired conversion of CD4⁺CD25⁻ T cells to CD4⁺ Tregs during acute EAE in IFN-γ GKO mice; (b) there is direct evidence that in vitro treatment of CD4⁺CD25⁻ T cells with IFN-γ in the presence or absence of TCR costimulation leads to conversion of CD4⁺CD25⁻ T cells to CD4⁺ Tregs, as shown by increased regulatory function and Foxp3 expression in both mouse and human experimental systems; (c) CD4⁺CD25⁻ T cells converted through in vitro treatment with IFN-γ acquire the ability to suppress EAE by adoptive transfer.

It seems that a proportion of CD4⁺CD25⁻ T cells undergo conversion to CD4⁺ Tregs during acute EAE, a process that requires the function of IFN-γ. In GKO mice, failure to produce IFN-γ and the resulting deficit in CD4⁺CD25⁺ Treg function during acute inflammation have significant immunological and pathological consequences. The findings provide convincing evidence indicating that IFN-γ plays an essential role in the self-regulatory mechanisms of the immune system in response to acute inflammation through the induction of transcription factor Foxp3. Our observations offer, for what we believe is the first time, a satisfactory explanation for the paradoxical role of IFN-γ in the development of EAE and provide insight into the mecha-

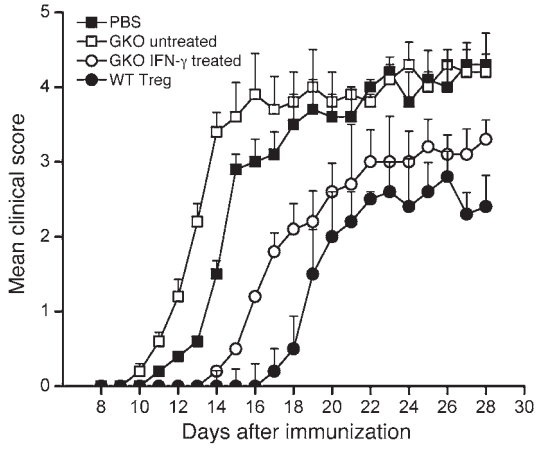

Figure 6

Suppressing effect of IFN-γ–treated CD4⁺CD25⁻ T cells in EAE. CD4⁺CD25⁻ T cells were purified from GKO mice and cultured in vitro with or without recombinant IFN-γ for 72 hours in the presence of anti-CD3 antibody. The resulting CD4⁺CD25^{high} T cells from both groups were purified and subsequently transferred to GKO mice (2×10^6 cells per mouse) at day 9 and day 11 after immunization. CD4⁺CD25⁺ T cells purified from naive WT mice (Tregs) were transferred at the same cell number (2×10^6 cells per mouse) and under the same experimental conditions without IFN-γ. GKO mice given PBS instead of cells were included as a reference (PBS). Each group consisted of at least 4 mice. Mice were monitored clinically every day for EAE development and progression. The data are presented as EAE clinical scores.

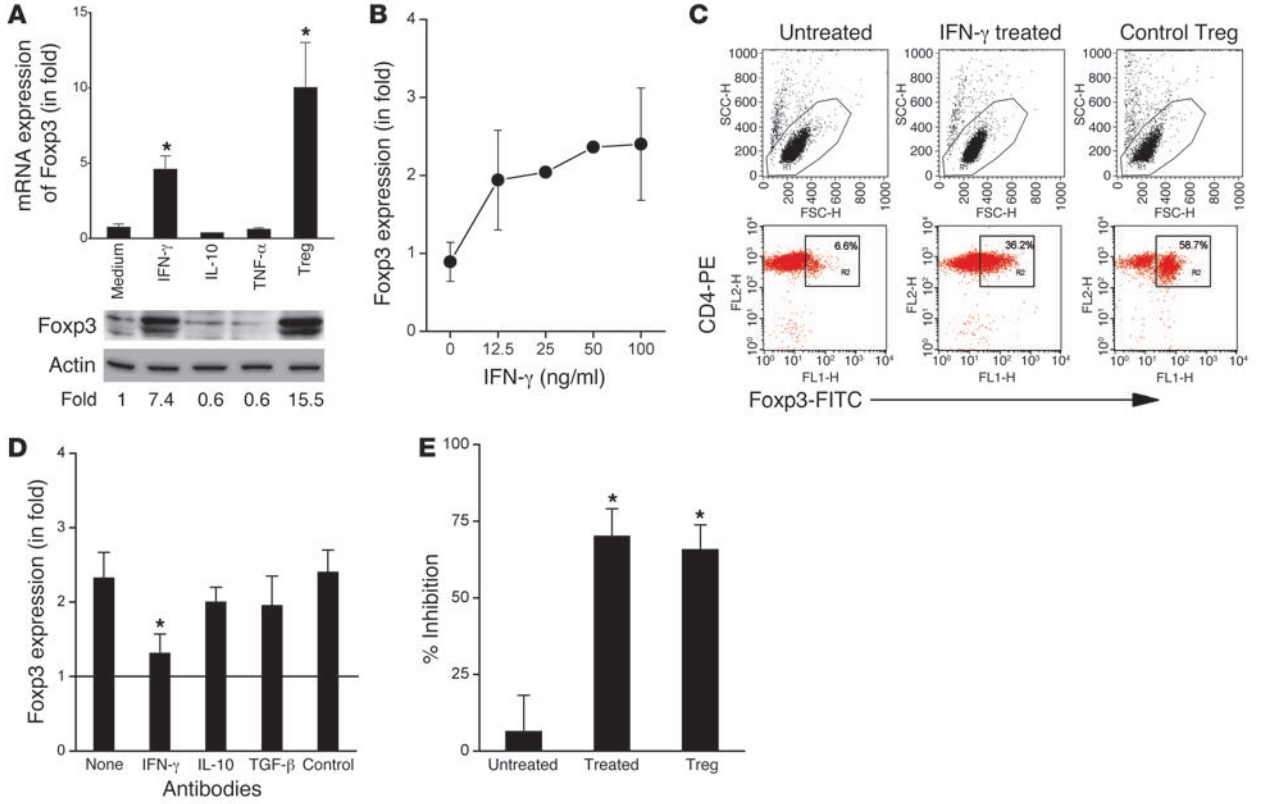

Figure 7

Induction of Foxp3 expression and regulatory properties by IFN-γ in human cells. (**A**) PBMC preparations were obtained from 10 healthy individuals and cultured in the presence or absence of the indicated cytokines (25 ng/ml) for 24 hours. $CD4^+CD25^+$ Tregs were used as a reference. The resulting cells were analyzed for the expression of Foxp3 by real-time PCR and immunoblot. The real-time PCR histogram represents analysis of 10 individual specimens. Relative change in Foxp3 expression in immunoblot is presented in folds (intensity of experimental band/intensity of control band). (**B**) Purified $CD4^+CD25^-$ T cell preparations (n = 10) were cultured in the presence of IFN-γ at the indicated concentrations for 24 hours and measured for Foxp3 expression. (**C**) $CD4^+CD25^-$ T cells treated in the presence or absence of IFN-γ (25 ng/ml) were analyzed for intracellular Foxp3 expression by flow cytometry. $CD4^+CD25^+$ Tregs were used as a reference. (**D**) $CD4^+CD25^-$ T cells were cultured in the presence of IFN-γ and the indicated antibodies (10 μg/ml). The resulting T cells were analyzed for mRNA expression of Foxp3 by real-time PCR. Horizontal line represents the level of Foxp3 expression in untreated $CD4^+CD25^-$ T cells. (**E**) Purified $CD4^+CD25^-$ T cells were treated with IFN-γ under the experimental conditions described above. The resulting T cells were FACS sorted and assayed for inhibitory activity on the proliferation of autologous $CD4^+CD25^-$ T cells. Results are expressed as mean percentage inhibition ± SEM from 5 independent experiments. Asterisks indicate that differences between groups are statistically significant; $^*P < 0.05$.

nism of the added severity in the encephalitogenic responses, altered cytokine profile, and clinical course of EAE in IFN-γ GKO mice. Our findings were in accordance with previous attempts by other investigators to define the underlying mechanism for heightened susceptibility of IFN-γ GKO mice to EAE and experimental autoimmune uveitis (10–12, 27). For example, Chu and colleagues demonstrated that there was a 10- to 16-fold increase of activated T cells ($CD4^+$, $CD44^{high}$) accumulated in the central nervous system at the onset of EAE in IFN-γ–deficient mice compared with those in WT mice and that these T cells proliferated extensively in response to antigen stimulation, which could be inhibited by IFN-γ (12). The authors hinted that IFN-γ might limit the extent of EAE by suppressing expansion of activated $CD4^+$ T cells. In this study, $CD4^+CD25^-$ T cells from IFN-γ–deficient mice appear more susceptible to IFN-γ in the induction of Foxp3 expression when compared with their WT counterparts. One of the possibilities to explain this discrepancy may relate to differential expression of IFN-γ receptors in $CD4^+CD25^-$ T cells derived from WT or GKO mice, which renders them more or less susceptible to IFN-γ treatment.

Our findings indicate that the induction of Foxp3 in $CD4^+CD25^-$ T cells and subsequent conversion to $CD4^+$ T cells of regulatory potential do not necessarily require costimulation with the T cell receptors or other cytokines. A similar property of TGF-β in the conversion of $CD4^+CD25^-$ T cells was described recently. However, in that case, costimulation of $CD4^+CD25^-$ T cells with T cell receptors is required to achieve conversion to $CD4^+CD25^+$ Tregs through the induction of Foxp3 expression (28). Furthermore, the role of IFN-γ in the conversion of $CD4^+CD25^-$ T cells to $CD4^+$ Tregs is consistent with a recent report indicating that STAT1, a signaling molecule involved in the IFN-γ signaling pathway, is critical to the induction of $CD4^+CD25^+$ Tregs. The authors demonstrated that STAT1-deficient mice expressing a transgenic T cell receptor against myelin basic protein spontaneously developed EAE, which was attributable to a functional impairment of $CD4^+CD25^+$ Tregs in STAT1-deficient mice (29). More recently, Kelchtermans and

colleagues demonstrated that heightened susceptibility of IFN-γ receptor KO mice to collagen-induced arthritis is associated with impaired function of $CD4^+CD25^+$ Tregs (30). These studies provide further support to the role of IFN-γ in the induction and conversion of $CD4^+CD25^+$ Tregs as described here.

The study described here has important implications in the understanding of the complex role of IFN-γ in the immune response and immune regulation, which has direct relevance to various pathological conditions, including autoimmune disease, tumor, and infectious pathology. In this regard, IFN-γ may act as a double-edged sword in immune responses and inflammatory processes. On one hand, it promotes a Th1 response and T cell migration to the site of inflammation and amplifies an inflammatory cascade through its ability to mediate a variety of signaling events, leading to the production of inflammatory molecules (3, 4, 31). On the other hand, the intensity of Th1-induced inflammation as quantifiable by the production of IFN-γ potentially triggers the immune system to call for a controlling mechanism. In such a scenario, IFN-γ produced at a high concentration either locally or systemically may be seen as a danger signal to the immune system that is programmed to activate various cellular and molecular events leading to peripheral conversion of $CD4^+CD25^-$ T cells to $CD4^+$ Tregs for the purpose of regulating overt inflammation. The observed role of IFN-γ in immune regulation may represent, in part, the tremendous ability of the immune system to regulate itself to prevent overheated immune responses in various pathological conditions.

The role of IFN-γ as described here has particular relevance to autoimmune conditions, such as MS. It has been demonstrated that there is a functional deficit in $CD4^+CD25^+$ Tregs in patients with MS (32, 33). Our recent study has indicated that there is significant decrease in the expression of Foxp3 in the $CD4^+CD25^+$ T cell pool of MS patients compared with that of healthy individuals (unpublished observations). A large proportion of the $CD4^+CD25^+$ T cell population seen in blood of MS patients is likely to represent activated autoreactive or inflammatory T cells rather than Tregs. High amounts of IFN-γ are likely to accumulate locally in inflammatory lesions, which may trigger the induction of Foxp3 expression in T cells. Hence, it is tempting to speculate that the characteristic self-limiting feature of relapsing/remitting MS, the most inflammatory form of MS, may somehow relate to this mechanism. Furthermore, the possibility of genetic and functional defects potentially involved in the IFN-γ signaling events of transcription factor Foxp3 in MS warrants further investigation. It is conceivable that the present study may have a significant impact on our current thinking regarding the role of IFN-γ in immune response and immune regulation and may help in reexamining the role of IFN-γ in disease mechanism and therapeutic application in various pathological situations.

Methods

Mice. C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. IFN-γ GKO mice of the same C57BL/6 background were provided by B. Sun (Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, People's Republic of China). Mice were maintained under pathogen-free conditions and genotyped prior to use at 6–12 weeks of age.

Induction and evaluation of EAE. The encephalitogenic peptide of MOG used to induce EAE corresponded to residues 35–55 (Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys). The peptide was purchased from BioAsia Biotechnology and had 95% purity. Acute EAE was induced by a subcutaneous immunization with 300 μg of the MOG_{35-55} peptide in CFA containing 5 mg/ml heat-killed H37Ra strain of *Mycobacterium tuberculosis* (BD Diagnostics) in the back region. Pertussis toxin (200 ng/mouse; List Biological Laboratories Inc.) in PBS was administered i.v. on the day of immunization and 48 hours later. Mice were weighed and examined daily for disease symptoms. They were scored for disease severity using the EAE scoring scale: 0, no clinical signs; 1, limp tail; 2, paraparesis (weakness, incomplete paralysis of 1 or 2 hind limbs); 3, paraplegia (complete paralysis of 2 hind limbs); 4, paraplegia with fore limb weakness or paralysis; 5, moribund state or death. The animal protocol was approved by the institutional review board of the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences.

Histology. Tissues for histological analysis were removed from mice 16 days after immunization and immediately fixed in 4% paraformaldehyde. Paraffin-embedded 5- to 10-μm sections of spinal cord were stained with Luxol fast blue or H&E and then examined by light microscopy. The degree of demyelination and inflammatory infiltrates was quantified on an average of 3 spinal cord transverse sections per mouse for a total of 5 mice per group using a previously published procedure (34, 35).

Isolation of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells. For mouse T cells, spleens were removed and prepared for single-cell suspensions. $CD4^+$ T cells were isolated from the resulting splenocytes using magnetic bead separation. Briefly, cells were depleted of $CD8^+$, $B220^+$, $CD16^+$, $Gr\text{-}1^+$, and $Ly76^+$ cells using biotin-labeled specific mAbs (BD Biosciences — Pharmingen), anti-biotin magnetic beads, and an LD magnetic bead column (Miltenyi Biotec). To isolate $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, purified $CD4^+$ T cell populations were incubated with PE-labeled anti-CD25 antibody (BD Biosciences) and anti-PE magnetic beads and were isolated by MACS separation column (Miltenyi Biotec). In some experiments, $CD4^+CD25^+$ T cells were FACS-sorted using a FACSAria instrument (BD). Similar approaches were employed to isolate human $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. $CD4^+$ T cells were first purified from PBMCs using a $CD4^+$ No-touch T cell isolation kit (Miltenyi Biotec), and $CD4^+CD25^-$ T cells were isolated by negative selection using anti-CD25 microbeads (Miltenyi Biotec). The purity of $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^-$ T cell fractions was always greater than 95%. The protocol was approved by the institutional review board of the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences.

Isolation of $CD4^+$ T cells from mouse CNS tissue. To isolate T cells from mouse CNS tissue, mononuclear cells were first prepared from brain and spinal cord using gradient centrifugation as described previously (12). In brief, mice were perfused with 30 ml PBS via the heart to eliminate peripheral blood. The dissociated brain and spinal cord tissue was centrifuged in a Percoll gradient. Mononuclear cells at the interface between the 2 gradients (37% and 70% Percoll) were collected and washed by centrifugation with medium. $CD4^+$ T cells were then isolated using Dynabeads coated with specific antibody to CD4 (Dynal; Invitrogen). The purity of $CD4^+$ T cells obtained from CNS tissue was greater than 93%.

Detection of Foxp3 expression by real-time PCR. Total RNA was isolated from cell pellets using RNeasy Mini Kit (QIAGEN), and first strand cDNA was subsequently synthesized using Sensiscript RT Kit (QIAGEN) according to the manufacture's instructions. mRNA expression of Foxp3 was determined by real-time PCR using SYBR Green Master Mix (Applied Biosystems). Thermocycler conditions comprised an initial holding at 50°C for 2 minutes and a subsequent holding at 95°C for 10 minutes, which was followed by a 2-step PCR program at 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Human GAPDH gene or mouse β-actin gene was used as an endogenous

control for sample normalization. Results were presented as folds relative to the expression of GAPDH or β-actin. Sequences of PCR primer pairs were as follows: human Foxp3, forward 5′-CGGACCATCTTCTGGATGAG-3′ and reverse 5′-TTGTCGGATGATGCCACAG-3′; human GAPDH, forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′; mouse Foxp3, forward 5′-AGGAGCCGCAAGCTAAAAGC-3′ and reverse 5′-TGCCTTCGTGCCCACTGT-3′; mouse β-actin, forward 5′-TGTCCACCTTCCAGCAGATGT-3′ and reverse 5′-AGCTCAGTAACAGTCCGCCTAGA-3′.

Immunoblot analysis. T cells were directly lysed in Laemmli sample buffer (Bio-Rad) and separated by 12% SDS-PAGE. Immunoblot analysis was performed by initial transfer of proteins onto nitrocellulose filters using Mini Trans-Blot (Bio-Rad) and followed by a blocking step using tris-buffered saline with 0.1% Tween 20 plus 5% nonfat dried milk for 1 hour at room temperature. The filters were subsequently incubated with a polyclonal rabbit anti-Foxp3 antibody (2 μg/ml) (courtesy of Alexander Rudensky, University of Washington, Seattle, Washington, USA) for 2 hours at room temperature and subsequently washed. After after another incubation with a goat anti-rabbit antibody conjugated with HRP for 1 hour at room temperature and extensive washing, signals were visualized with enhanced chemiluminescence technology (Amersham Bioscience). For immunoblot normalization, the same membrane was stripped off and reprobed with HRP-conjugated anti-actin antibody (Santa Cruz Biotechnology Inc.).

Flow cytometric analysis. T cells were resuspended in PBS containing 1% BSA (Sigma-Aldrich) and 0.1% sodium azide. For surface staining of CD4, CD25, CD62L, and GITR, T cells were incubated with fluorochrome-conjugated antibodies to the indicated cell surface markers (eBioscience) at the recommended dilution of isotype control antibodies for 30 minutes on ice. IFN-γ receptor antibody, anti-CD119 (Clone GR20), was purchased from BD Biosciences — Pharmingen. For intracellular staining of Foxp3, cells were fixed and permeabilized with Foxp3 staining buffer (eBioscience). Permeabilized cells were stained with PE or FITC-conjugated anti-human or anti-mouse Foxp3 mAbs (0.5 μg/10^6 cells; eBioscience). Stained cells were analyzed subsequently using a FACSAria instrument (BD).

Proliferation and inhibition assays. In proliferation assays, mouse splenocytes (5×10^5 per well) were cultured in triplicate in complete DMEM (DMEM with 5% fetal calf serum, HEPES, β-mercaptoethanol, L-glutamine, sodium pyruvate, and penicillin/streptomycin) in 96-well flat-bottomed plates. Cells were cultured in the presence or absence of the MOG peptide (5 μg/ml) at 37°C in 5% CO_2 for 72 hours. Cells were pulsed with 1 μCi [^{3}H]-thymidine during the last 16–18 hours of culture prior to harvest. [^{3}H]-thymidine incorporation was measured as cpm using a β plate counter. To evaluate inhibitory activity, freshly isolated $CD4^+CD25^-$ T cells were used as responder. Accessory cells were obtained by isolating the positive fraction of the $CD4^+$ no-touch magnetic sort and were irradiated with 50 Gy. A total of 2×10^4 $CD4^+CD25^-$ T cells were stimulated with plate-bound anti-CD3 antibody (2 μg/ml; eBioscience) and 1×10^5 APCs in the absence (naive $CD4^+CD25^-$ T cells alone) or presence of $CD4^+CD25^+$ T cells used at the cell density of 2×10^4/well. The ability of $CD4^+CD25^+$ T cells (inhibitor) to suppress the proliferation of $CD4^+CD25^-$ T cells (responder) was determined by [^{3}H]-thymidine incorporation. Percentage of inhibition on the proliferation of responder was calculated as [1 – (experimental cpm/control cpm)] × 100%.

Cytokine measurement. Mouse splenocytes (1.5×10^5 per well) were stimulated with the MOG peptide (5 μg/ml) in complete DMEM in 96-well round-bottom plates for 48–72 hours. Supernatants were collected for measurement of production of IFN-γ, TNF-α, IL-4, and IL-10 using ELISA kits (R&D Systems) according to the manufacturer's instructions. A standard curve was generated using known amounts of the respective purified recombinant murine cytokines.

Conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Tregs. To induce/convert $CD4^+CD25^+$ Tregs from $CD4^+CD25^-$ T cells, purified human or mouse $CD4^+CD25^-$ T cells were cultured at 2×10^6 cells/ml with 25 ng/ml recombinant human or mouse IFN-γ (R&D Systems) in the presence (72 hours) or absence (24 hours) of anti-CD3 antibody (2 μg/ml; eBioscience) as a means of TCR costimulation. The resulting T cells were harvested and washed twice with medium to remove residual IFN-γ. The cells were then subject to analyses as described elsewhere.

Adoptive transfer of GKO $CD4^+CD25^+$ T cells treated with IFN-γ. $CD4^+CD25^-$ T cells were purified from GKO mice and treated in vitro in the presence of anti-CD3 antibody and 25 ng/ml mouse recombinant IFN-γ for 72 hours. The resulting T cells were washed twice with medium to remove residual IFN-γ. $CD4^+CD25^{high}$ T cells were FACS sorted and injected intravenously (2×10^6 cells in 200 μl PBS per mouse) into GKO mice at day 9 and day 11, respectively, after immunization with the encephalitogenic MOG peptide. In parallel groups, the same number of untreated $CD4^+CD25^+$ T cells from GKO mice and $CD4^+CD25^+$ T cells from naive WT mice, respectively, were transferred under the same experimental conditions. Injection of PBS in GKO mice was used as a control. Mice were monitored for clinical score.

Statistics. Differences in the expression of genes between the groups were analyzed by the Mann-Whitney *U* test. Two-tailed Student's *t* test was used to analyze the differences between the groups. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed before using the 2-tailed paired or unpaired Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

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Address correspondence to: Jingwu Zhang, Institute of Health Sciences, 225 South Chongqing Road, Shanghai 200025, People's Republic of China. Phone: 86-21-63848329; Fax: 86-21-63852822; E-mail: jzang@bcm.tmc.edu.

Zhaojun Wang and Jian Hong contributed equally to this work.

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