Synergy between IL-6 and TGF-β signaling promotes FOXP3 degradation

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Abstract: The forkhead family transcription factor FOXP3 is critical for the differentiation and function of CD4+CD25+ regulatory T cells (Treg). How FOXP3 protein level is negatively regulated under the inflammatory microenvironment is largely unknown. Here we report that the combination of transforming growth factor-beta (TGF-β) and IL-6 treatment (IL-6/TGF-β) can synergistically downregulate FOXP3 at the posttranslational level by promoting FOXP3 protein degradation. In our FOXP3 overexpression model, we found that IL-6/TGF-β treatment upregulated IL-6R expression but did not affect the stability of FOXP3 mRNA. Moreover, we found that the proteasome inhibitor MG132 could inhibit IL-6/TGF-β-mediated downregulation of FOXP3 protein, which reveals a potential pathway for modulating Treg activity by preventing FOXP3 degradation during inflammation.

Keywords: FOXP3, Treg, instability, IL-6, TGF-β, proteasome

Introduction

Naïve CD4+ T cells can be differentiated into various effector cell types, including T helper 1 (Th1), Th2, Th9, Th17 and induced regulatory T (iTreg) cells, all of which polarize according to the local cytokine environment in which they are stimulated [1]. Th17 cells are proinflammatory, characterized by the production of inflammatory cytokines such as interleukin (IL)-17, IL-6 and tumor necrosis factor (TNF), which are not only involved in mediating host defense mechanisms but can also promote the development of autoimmune disease [2]. In contrast, iTreg cells, marked by expression of the forkhead/winged helix transcription factor forkhead box P3 (FOXP3) are capable of suppressing autoimmunity and can limit excessive tissue damage due to inflammation [3]. Transforming growth factor-β (TGF-β), along with T cell receptor stimulation, is required for the induction of FOXP3 expression in conventional T cells to become fully differentiated into iTreg cells [4-7].

TGF-β, in conjunction with IL-6, stimulates the differentiation of Th17 cells where IL-6 signaling inhibits the generation of FOXP3+ Treg cells induced by TGF-β [8, 9]. The vitamin A metabolite retinoic acid, as a key regulator of TGF-β-dependent immune responses, can inhibit IL-6-driven induction of Th17 cells, promote iTreg cell differentiation and prevent nTreg to Th17 cell conversion [10-12]. Previous studies have reported that IL-1β and IL-6, but not TGF-β, are responsible for Th17 differentiation in humans [13], but others have suggested that TGF-β dosage plays an essential role in the differentiation of naive human CD4+ T cells toward the Th17 lineage [14]. Serum-free medium seems important for cell culture during in vitro T cell differentiation [15] as serum may contain traceable amounts of TGF-β. Furthermore, TGF-β can upregulate the expression of the IL-6 receptor [10], inhibit the production of IL-4, IFN-γ and suppressor of cytokine signaling 3 (SOCS3) [16, 17]-a major negative feedback regulator of the STAT3 signaling pathway.

IL-6 trans-signaling can augment the expression of the TGF-β signaling inhibitor SMAD7 which renders naïve CD4+ T cells resistant to
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the induction of FOXP3 [18]. At present, the exact molecular mechanisms underlying IL-6 and TGF-β-mediated regulation of Th17 differentiation remain unclear. Here, we used a FOXP3 overexpression system to demonstrate that the combination of TGF-β and IL-6 (IL-6/TGF-β) signaling can directly mediate the degradation of FOXP3 protein. We also show how the ubiquitin-proteasome pathway [19, 20] is involved in this process, since the proteasome inhibitor MG132 circumvented IL-6/TGF-β-mediated FOXP3 degradation. Our finding may have important implications for understanding the molecular mechanisms underlying the differential plasticity between Treg cell subsets with Th17 and other T effector cell types.

Materials and methods

Antibodies and reagents

The following antibodies were used: anti-CD4-FITC (Biolegend, 300506), anti-CD25-PE (Biolegend, 317706), anti-CD127-PE/Cy7 (ebioscience, 25-1278-41), anti-HA (Santa Cruz, USA, F-7), anti-IL-6RA (Santa Cruz, USA, BN-12), anti-STAT3 (Cell Signaling, 79D7), Anti-pSTAT3 (Cell Signaling, 79D7), anti-β-actin (Sigma AC-15), anti-α-Tubulin (Sungene). Recombinant human IL-6 and TGF-β were purchased from R&D Systems. The proteasome inhibitor MG132 (474790) was purchased from Merck Biosciences and reconstituted in dimethylsulfoxide (DMSO).

Cell preparation and culture

To isolate Treg cells, human PBMC were stained in FACS buffer (PBS 1% FBS) with anti-CD4-FITC, anti-CD25-PE and anti-CD127-PE/Cy7 for 30 min on ice, washed, then resuspended in 3ml FACS buffer. Treg were purified using a FACS ARIA II cell sorter (BD). The purity of the sorted cells was 95–99%.

The Jurkat-HA-FOXP3 stable cell line was generated in our laboratory via lentiviral transduction. HA-FOXP3 is expressed under the control of the ubiquitin promoter, and puromycin resistance was used to select for FOXP3+ cells. Human Treg cells were cultured in X-VIVO medium (Lonza) supplemented with 10% AB serum, 1% Glutamax, 1% non-essential amino acids (NEAA), 1% sodium pyruvate and 1% penicillin/streptomycin.

HA-FOXP3-Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% NEAA, 1% sodium pyruvate and 10mM HEPES. Cell culture reagents were purchased from Invitrogen (Gibco) unless otherwise indicated.

Western blotting

Stimulation of HA-FOXP3-Jurkat T cells: cells (5x10⁵) were seeded into 6-well plates then stimulated with IL-6, TGF-β or the combination of TGF-β and IL-6 treatment (IL-6/TGF-β) for 0 h, 0.5 h, 2 h, 4 h, 12 h or 24 h. The cells were then harvested, washed and lysed with RIPA buffer (20mM Tris-HCl, 150mM NaCl, 1mM NaEDTA, 1% NP-40, 0.5% NaDoc, 10% Glycerol), supplemented with protease inhibitor cocktail (0.1M PMSF, 1M NaF, 1mM Na3VO4, Roche). Cell lysates were subsequently treated with 2X SDS loading buffer and then separated on SDS-PAGE before being transferred onto nitrocellulose membranes. After blocking (PBS-Tween 5% milk), the membranes were probed with HRP-conjugated anti-mouse HA mAb (F-7) then treated with ECL Solution (Millipore). To confirm sample loading and transfer efficiency,
membranes were reprobed with anti-β-actin/α-Tubulin antibody. To detect STAT3 or phosphorylated STAT3 (pSTAT3), HA-FOXP3-Jurkat T cells (1×10⁶) were cultured in 6-well plates and treated with IL-6 or IL-6/TGF-β. For pSTAT3 detection, we used 5% BSA to block the membranes overnight. HA-FOXP3 Jurkat T cells (5x10⁶) were also cultured in 6-well plates and treated with IL-6/TGF-β/MG132 or IL-6/TGF-β/DMSO for 0 h, 12 h and 24 h. FOXP3 mRNA expression was examined by quantitative Real-Time PCR.

Flow cytometry

HA-FOXP3-Jurkat T cells (5x10⁶) were cultured in 12-well plates. To confirm IL-6R expression, cells were stimulated with IL-6, TGF-β or IL-6/TGF-β for 0 h and 12 h. Cells were then harvested and incubated with anti-IL-6R mAb for 1h, washed with PBS, then labeled with a PE-conjugated secondary mAb (A21422, Invitrogen). All samples were acquired and analyzed on an LSR II flow cytometer (Becton Dickenson) and FlowJo software (Tree Star), respectively.

Quantitative real-time PCR

Human Treg (1x10⁶) were cultured in 12-well plates and treated with IL-6/TGF-β for 0 h, 12 h or 24 h. Jurkat-HA-FOXP3 T cells (1x10⁶) were cultured in 12-well plates and treated with IL-6, TGF-β or IL-6/TGF-β for 0 h, 12 h or 24 h. Cells were then harvested at 24 h and total RNA was extracted using Trizol Reagent (Invitrogen). Complementary DNA (cDNA) was prepared from 1μg of total RNA using the PrimeScript RT reagent kit (TaKaRa).

FOXP3 forward primer: 5'-TCCCAGAGT-TCTCCACAAC-3'; FOXP3 reverse primer: 5'-ATTGAGTGTCGGCTGTTCT-3'; β-actin-forward primer: 5'-GGACTTCGAGCAA-GAGATGG-3'; β-actin-reverse primer: 5'-AGCACTGTTGGCGTACAG-3'; mRNA levels of FOXP3 were assessed relative to β-actin mRNA levels by quantitative RT-PCR (ABI, 7900HT) using the SYBR Premix Ex Taq Reagent (TaKaRa).

Results

Synergy between IL-6 and TGF-β signaling downregulates FOXP3 expression

Given that IL-6 and TGF-β induces the differentiation of Th17 cells from naïve CD4 T cells, we sought to address the direct effect of IL-6 and TGF-β signaling on FOXP3 expression and protein stability. We constructed a HA-FOXP3 Jurkat stable cell line and treated these cells with IL-6, TGF-β or their combination (IL-6/TGF-β) for different time-points. FOXP3 expression level was then detected by Western blotting. FOXP3 expression level had no change by IL-6 or TGF-β treatment alone, but IL-6/TGF-β co-treatment downregulated FOXP3 protein level after 12 h stimulation (Figure 1). As FOXP3 protein expression was under the control of the ubiquitin promoter, rather than its natural promoter, this suggests that the change in FOXP3 expression in our system was directed at the protein level.

IL-6/TGF-β treatment significantly downregulates FOXP3 mRNA in human nTreg, but not in HA-FOXP3-Jurkat T cells

To further determine whether the changes in FOXP3 protein expression occurred at the pro-
tein level, we evaluated FOXP3 mRNA expression in cells treated with IL-6/TGF-β. The HA-FOXP3-Jurkat T cells were treated with IL-6/TGF-β, IL-6 only or TGF-β only. FOXP3 mRNA level was detected by RT-PCR (Figure 2A). We found that TGF-β alone, or in combination with IL-6, had no effect on FOXP3 mRNA levels. However, IL-6 modestly induced FOXP3 mRNA level at 24 h. Primary nTregs from human PBMC, treated with IL-6/TGF-β, downregulated FOXP3 mRNA level as detected by RT-PCR (Figure 2B). These data suggest that in our model, IL-6/TGF-β-mediated downregulation of FOXP3 protein was not due to the regulation of FOXP3 expression at the mRNA level.

The combination of TGF-β and IL-6 is a prerequisite for the upregulation of the IL-6 receptor in the Jurkat T cell line

As TGF-β enhances IL-6-induced STAT3 activation by upregulating the expression of the murine IL-6 receptor (IL-6R) [6], we decided to treat HA-FOXP3 Jurkat T cells with IL-6 in the presence or absence of TGF-β, or TGF-β alone, to analyze the expression of human IL-6R in these cells. By flow cytometry, we found that IL-6 or TGF-β treatment alone could not increase the surface expression of IL-6R (alpha chain); however, the surface expression of IL-6R was enhanced at 12 h after IL-6/TGF-β treatment (Figure 3). This result coincides with the above observations which showed how FOXP3 levels started to reduce after 12 h of IL-6/TGF-β treatment.

**IL-6/TGF-β co-treatment significantly downregulates the protein level of FOXP3 and activates STAT3**

To test the potential downstream signals that affected FOXP3 protein levels, we tested the expression of STAT3 upon IL-6/TGF-β treatment. Although FOXP3 level was downregulated, STAT3 levels showed little change (Figure 4A). We also observed a higher degree of STAT3 phosphorylation (pSTAT3) when cells were treated with IL-6/TGF-β compared to IL-6 alone (Figure 4B). This could be due to the upregulation and sustaining of IL-6R expression and signaling, respectively.

**MG132 inhibits IL-6/TGF-β-mediated downregulation of FOXP3**

As the ubiquitin-proteasome and autophagylsosome pathways are the two main routes of protein and organelle degradation in eukaryotic cells [21], we hypothesized that the decrease in FOXP3 expression was due to FOXP3 protein degradation via the proteasome. We treated HA-FOXP3 Jurkat T cells with IL-6/TGF-β/ MG132 for 12 h, with DMSO as a control. We found that the proteasome inhibitor MG132, but not the DMSO control, could inhibit IL-6/TGF-β-mediated downregulation of FOXP3 expression (Figure 4C). Our data suggests that FOXP3 protein is degraded by the ubiquitin-proteasome-dependent pathway upon IL-6/TGF-β treatment.

**Discussion**

FOXP3 plays an important role in the differentiation and function of regulatory T cells [22]. In humans, mutations in the FOXP3 gene lead to...
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The development of the fatal autoimmune disease Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX). Previous studies have found that TGF-β, retinoic acid and IL-2 serve to maintain or upregulate FOXP3 transcription and Treg function [4, 10, 23]. In contrast, IL-6, IL-4, IL-27 and IL-21 are negative regulators of TGF-β-induced FOXP3 expression [23-28]. However, few studies have investigated the direct role of these signaling pathways on the post-translational control of FOXP3 expression.

Although it has been previously shown how IL-6 promotes nTreg to Th17 conversion, and how this process depends on TGF-β receptor signaling [29], we show a complementary system of how TGF-β and IL-6, acting in concert, promotes the degradation of FOXP3 protein. It still remains unclear as to which signaling pathways are directly involved in FOXP3 degradation. CD4+ T cells stimulated with TGF-β significantly increase the activation of ERK and JNK but not p38; but unlike Foxp3+ iTreg cell differentiation, Th17 cell development induced by TGF-β and IL-6 requires the JNK and p38 MAPK pathways [30]. Whether FOXP3 protein degradation is associated with the JNK and p38 MAPK pathways requires further investigation.

There is a reciprocal relationship between iTreg and Th17 cell differentiation and function, where IL-6 has a pivotal role in dictating the balance between these two cell populations [31, 32]. STAT3 is a transcription factor critical for Th17 differentiation and regulates the expression of retinoic acid receptor-related orphan receptor yt (RORyt). RORyt is a key transcription factor for the differentiation of Th17 cells that can induce the transcription of the genes encoding for IL-17a and IL-17F in naïve CD4+ T helper cells [33, 34]. The inhibitory effect of IL-6 on FOXP3 expression is dependent on STAT3 [24]. However, FOXP3 can interact with phosphorylated STAT3, and is necessary for Treg cell suppressive function towards Th17 responses [35]. Here we show that IL-6/TGF-β signals have no effect on STAT3 protein level, but they can promote the activation of STAT3 more rapidly than IL-6 alone. This process could be explained by the upregulation of IL-6R by TGF-β.

TGF-β can induce both FOXP3 and RORyt expression in CD4+ T cells but FOXP3 can inhibit RORyt function. IL-6 treatment alone has been shown to induce low levels of RORyt but the addition of TGF-β greatly enhances RORyt expression [36, 37]. The degradation of FOXP3

Figure 4. IL-6/TGF-β downregulates FOXP3 protein level, activates STAT3, and FOXP3 expression is rescued by the proteasome inhibitor MG132. A. HA-FOX3-Jurkat T cells were treated with IL-6 (20ng/ml) or IL-6 (20ng/ml)/TGF-β (10ng/ml) for the indicated incubation times. Cells were then harvested and lysed with RIPA buffer. FOXP3 and STAT3 expression was detected by Western Blotting. B. HA-FOX3-Jurkat T cells were treated with IL-6 (20ng/ml) or IL-6 (20ng/ml)/TGF-β (10ng/ml) for the indicated incubation times. Cells were harvested and then lysed with RIPA buffer and the corresponding markers detected by Western Blotting. C. HA-FOX3-Jurkat T cells were treated with IL-6 (20ng/ml)/TGF-β (10ng/ml)/DMSO or IL-6 (20ng/ml)/TGF-β (10ng/ml)/MG132 (5µmol/ml) for the indicated incubation times. Cells were harvested, and then lysed with 2x SDS loading buffer. Samples were then separated via PAGE and FOXP3 protein level detected by Western Blotting.
induced by TGF-β/IL-6 signaling may release
the negative regulation of RORγt activation by
FOXP3 and thus lead to Th17 cell differentia-
tion. It has been reported that upon activation,
Treg cells produce high amounts of TGF-β, and
with the addition of IL-6, CD4+CD25 FOXP3+ T
cells are able to differentiate into Th17 cells;
Treg cells themselves may also differentiate
into Th17 cells in the presence IL-6 due to the
loss of FOXP3 expression [38]. Although it is
still unclear at present, this phenomenon may
be partly due to the direct control of FOXP3 pro-	ein degradation. We constructed a ubiquitin
promoter driven FOXP3-expressing Jurkat sta-
ble cell line, as this allowed us to analyze the
modulation of FOXP3 expression at the post-
transcription level. Using this stable cell line, it
excluded the possibility that any modulation in
FOXP3 protein expression was due to changes
in gene regulation. Although IL-6/TGF-β down-
regulated FOXP3 mRNA in primary nTregs, we
consistently found little or no effect on the
expression level of FOXP3 mRNA in our FOXP3
Jurkat cell line. However, a slight upregulation
of FOXP3 mRNA expression was observed upon
IL-6 treatment.

Post-translational regulation is crucial for mod-
ulating FOXP3 stability and function. FOXP3
acetylation can regulate its protein level by
impairing proteasome-mediated Foxp3 degra-
dation, but little is known regarding the differ-
et modifications to which FOXP3 can be sub-
jected, as well as the mediators that regulate
these modifications [39]. Our previous studies
indicated that short time treatment (4 h) by
IL-6/TGF-β could downregulate the association
of FOXP3 to chromatin, which could be reversed
by HDAC inhibitors [40]. Here we found that a
longer treatment with IL-6/TGF-β for 12 h could
upregulate IL-6R expression, promote the
downregulation of FOXP3, and could be pre-
vented by the proteasome inhibitor MG132.
Protein ubiquitination can be mediated by ubiq-
uitin-activating enzymes (E1), ubiquitin conju-
gating enzymes (E2), and ubiquitin ligases (E3).
In naïve CD4+ T cells, IL-6 stimulation induces
the expression of the E3 ubiquitin ligase
MARCH-7 to downregulate the IL-6 superfamily
member LIF receptor gp190 [41]. We propose
that FOXP3 can be modified with ubiquitin,
which in turn leads to its degradation as MG132
could inhibit IL-6/TGF-β-mediated FOXP3 deg-
radation. It should be of great interest to eluci-
date the E3 ligase responsible for this process
as it may serve as a target to regulate FOXP3
expression and Treg function.

Abbreviations

Treg: Regulatory T cells; IL-6: Interleukin 6; TGF-
β: Transforming growth factor beta.

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