PARP-1 regulates expression of TGF-β receptors in T cells

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Abstract

TGF-β receptors (TβRs) are essential components for TGF-β signal transduction in T cells, yet the mechanisms by which the receptors are regulated remain poorly understood. We show here that Poly(ADP-ribose) polymerase-1 (PARP-1) regulates TGF-β receptor I (TβRI) and II (TβRII) expression in CD4+ T cells and subsequently affect Smad2/3-mediated TGF-β signal transduction. Inhibition of PARP-1 led to the up-regulation of both TβRI and TβRII, yet the underlying molecular mechanisms were distinct; PARP-1 selectively bound to the promoter of TβRII, whereas the enzymatic activity of PARP-1 was responsible for the inhibition of TβRI expression. Importantly, inhibition of PARP-1 also enhanced expression of TGF-β receptors in human CD4+ T cells. Thus, PARP-1 regulates TβRs expression and TGF-β signaling in T cells.
INTRODUCTION

TβRI and TβRII are essential components of TGF-β signaling and play an indispensable role in generation of regulatory T cells (Tregs). In mice, selective deletion of TβRI or TβRII in T cells results in severe defect in Treg generation. However, the underlying mechanisms are poorly understood. The expression of TβRs in T cells determines TGF-β signal strength, which has profound effects on T cell responses and differentiation. Thus, insights into the mechanisms that regulate TβR expression is not only essential for understanding Treg generation, but also important for treatment of autoimmune diseases, transplant rejection, cancer and infection.

PARP-1 is a nuclear enzyme that is conventionally linked to DNA repair. However, PARP-1 has also been shown to function as a transcription factor involved in the transcription of many genes. Inhibition of PARP-1 activity by inhibitors or gene mutation has been shown to lead to both suppression and exacerbation of chronic inflammation and autoimmune disease models. Recently, it was shown that deletion of PARP-1 inhibited NF-κB activation, decreased TNF-α and inducible NO synthesis in macrophages. However, the role of PARP-1 in T cell mediated immune responses remains elusive.

Here we show that PARP-1 regulates the expression of TβRs and thereby controls Treg generation in T cells. Deletion of PARP-1 in mice (PARP-1-/-) results in a T cell-intrinsic preference to generate more thymic Tregs (tTregs) and convert more naive T cells into induced Tregs (iTregs) in vitro and in vivo. Tregs increase was attributed to enhanced sensitivity of CD4+ T cells to TGF-β1 signals by upregulation of both TβRI and II, and
subsequent Smad2/3 activation in PARP-1–/–T cells. We show that PARP-1 inhibits TβRI expression through its enzymatic function, and modulates TβRII by directly binding to TβRII gene. In addition, PARP-1 deficiency enriched the binding of Smad3 at the enhancer of Foxp3 gene. Importantly, inhibition of PARP-1 enzyme activity resulted in increased Foxp3 and TβRs expression in human CD4+ T cells. Together, these data reveal an unrecognized role for PARP-1 in the regulation of TβRs expression.

Materials and Methods

Mice. Generation of PARP-1–/– (sv/129 x C57BL6 background) mice was previously described. PARP-1–/– mice on C57BL/6 background were obtained by backcrossing with C57BL/6 mice for at least six generations and used in the experiments unless otherwise stated. Rag-1–/– and C57BL/6 (CD45.2+ or CD45.1+) mice were from Jackson Laboratory. Mice were performed per NIH guidelines for use and care of live animals and approved by Animal Care and Use Committee of NIDCR.

Antibodies and reagents. Mouse anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51), anti-CD16/CD32 (clone 93), PE or APC-conjugated anti-CD25 (clone PC61.5), FITC or Perp-conjugated anti-CD4 (clone GK1.5), FITC or Perp-conjugated anti-CD8 (clone 53-6.7) mAb were from BD Biosciences. APC-conjugated anti-TGF-β receptor I and PE or APC-conjugated anti-TGF-β receptor II and anti-TGF-β1,2,3 mAb were from R&D Systems. Anti-PARP-1 (B-10) mAb was from Santa Cruz biotechnology. Anti-Smad3 (ab28379) and rabbit control IgG ChIP grade antibodies were from Abcam Company. Phospho-Smad2 (S465/467), Smad2 (L16D3) antibodies were from Cell Signaling Technology (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
antibody was from IMGENEX. Mouse and human CD4^+CD25^+ T isolation Kit were from MiltenyiBiotec. APC or PE-conjugated anti-Foxp3 (clone FJK-16s) and Rat IgG2a isotype control, IL-6 ELISA kits were from eBiosciences. TGF-β receptor I kinase inhibitor II was from Calbiochem (Darmstadt, Germany).

**Cell isolation, Cell culture experiments, Mixed bone marrow chimeras, Flow cytometry analysis, Chromatin immunoprecipitation (ChIP) assay, Luciferase assay, and HDM-induced Asthma, Real-time PCR, Oral tolerance, Immunoblot analysis and Isolation of subsets of human CD4^+ T cells and cell culture** are described in supplementary methods.

**Statistical analysis.** Statistical significance of differences was determined by unpaired 2-tailed Student’s t-test unless otherwise stated.

**Online supplementary Figures:** Supplementary Figures include Supplemental Figures (1-6) and legends.

**RESULTS**

**Deletion of PARP-1 results in enhanced sensitivity to TGF-β1 in CD4^+ T cells**

To study the role of PARP-1 in T cells in response to TGF-β1 signaling, we first investigated Treg generation in PARP-1^-/- mice, as TGF-β1 signaling is crucial in Foxp3^+ Tregs generation 18, 19. We observed that PARP-1^-/- mice had significantly higher frequencies of CD4^+Foxp3^+ Tregs in the spleen, thymus and peripheral lymph nodes, compared to wild type (PARP-1^+/+) littermates (data not shown). PARP-1^-/- Tregs exhibited similar levels of apoptosis (**Supplementary Fig. 1a-b**), cell proliferation (**Supplementary Fig. 1c**), and activation markers CD44, CD45RB, CD62L and CD69.
compared to PARP-1\(^{+/+}\) Tregs (data not shown). These results suggest a role for PARP-1 in controlling Treg generation, which is consistent with a recent report\(^{20}\). However, whether the increase in Tregs in PARP-1\(^{-/-}\) mice was due to a T-cell intrinsic mechanism was undetermined in the recent report. Therefore we generated bone marrow chimeras to address this question. We injected a mixture of C57BL/6 (CD45.1\(^{+}\)) and PARP-1\(^{-/-}\) (CD45.2\(^{+}\)) bone marrow or a mixture of C57BL/6 (CD45.1\(^{+}\)) and PARP-1\(^{+/+}\) (CD45.2\(^{+}\)) bone marrow at 1:1 ratio, into sub-lethally irradiated recipient Rag1\(^{-/-}\) mice. Both spleen and thymus reconstituted with PARP-1\(^{-/-}\) (CD45.2\(^{+}\)) bone marrow contained a higher percentage of CD4\(^{+}\)Foxp3\(^{+}\)Tregs than did those transplanted with PARP-1\(^{+/+}\) (CD45.2\(^{+}\)) bone marrow (Fig. 1a-c). There was no significant increase of total Treg numbers in PARP-1\(^{-/-}\) bone marrow chimers since the number of total splenocytes in PARP-1\(^{-/-}\) bone marrow chimers was lower (data not shown). These data confirm that PARP-1 deficiency confers T cells with an increased capacity to differentiate toward Treg fate in vivo.

Furthermore, we determined whether PARP-1 deficiency affected Treg generation in culture. We induced Foxp3 expression in naïve CD4\(^{+}\)CD25\(^{-}\) T cells by culturing cells with anti-CD3 antibody, antigen presenting cells (APCs), and TGF-\(\beta\)1\(^{18}\). There were higher amounts of Foxp3 mRNA (Fig. 1d, Supplementary Fig. 2a) and protein (Fig. 1e, Supplementary Fig. 2b-c) in PARP-1\(^{-/-}\) CD4\(^{+}\) T cells compared to PARP-1\(^{+/+}\) CD4\(^{+}\) T cells. PARP-1\(^{-/-}\) CD4\(^{+}\) T cells also expressed higher levels of Foxp3 mRNA and protein in response to lower concentrations of TGF-\(\beta\)1 suggesting PARP-1\(^{-/-}\) T cells are more sensitive to TGF-\(\beta\)1 stimulation (Fig. 1d, Supplementary Fig. 2b). In addition, PARP-1\(^{-/-}\) T cells converted to Foxp3 expressing cells much faster than PARP-1\(^{+/+}\) T cells in response to 2ng/ml TGF-\(\beta\)1 (Supplementary Fig. 2c). A similar increase in Tregs was
seen in PARP-1−/− T cells when they were cultured with anti-CD3 and CD28 antibodies and TGF-β1 (data not shown).

Enhanced Treg generation in PARP-1−/− T cells in vitro prompted us to explore whether this phenomenon would also occur in vivo. We addressed this in an oral tolerance model 21-23. We transferred PARP-1−/− or PARP-1+/+ CD4+CD25− T cells from OTII TCR transgenic mice (CD45.1+) into C57BL/6 (CD45.2+) mice and fed the mice with chicken OVA protein (1.5%) in drinking water for five constitutive days. The mice were sacrificed to assess Treg induction in the lamina propria (LP). We analyzed the Foxp3 expression in the CD4+CD45.1+ OTII TCR transgenic T cells and observed that PARP-1−/− CD4+ OTII T cells showed significantly higher frequencies of Foxp3+ Tregs than PARP-1+/+ OTII T cells (Fig. 1f). As expected, there was no significant difference in Tregs in the spleens and peripheral lymph nodes in CD45.2+ host CD4+ T cells. Collectively these data indicate that PARP-1 expression in CD4+ T cells affects Treg generation.

Higher expression of TβRs in PARP-1−/− CD4+CD25− T cells

As PARP-1−/− T cells showed an increased sensitivity to TGF-β1, we hypothesized that PARP-1 could affect TGF-β1 signaling by altering TβR expression. To test this hypothesis, we first examined the mRNA expressions of TβRI and TβRII in CD4+ T cells. Freshly isolated PARP-1−/− CD4+CD25− T cells expressed higher levels of TβRI and TβRII mRNA compared to PARP-1+/+ CD4+CD25− T cells (Fig. 2a, Supplementary Fig. 3). TCR stimulation of PARP-1−/− CD4+CD25− T cells with anti-CD3 and CD28 antibodies for 15-90 mins also resulted in higher TβRI and TβRII mRNA expressions compared PARP-1+/+ CD4+CD25− T cells (Fig. 2b).
CD4^+CD25^- T cells with TGF-β1 (2ng/ml) plus anti-CD3 (0.5μg/ml) and CD28 (0.2μg/ml) antibodies also led to higher levels of TβRI and TβRII mRNA compared to PARP-1^+/+ CD4^+CD25^- T cells (Fig. 2c). Intriguingly, freshly isolated PARP-1^+/+ CD4^+CD8^-CD25^- thymocytes showed much higher expression (2-3 fold) of TβRI and TβRII mRNA than splenic CD4^+CD25^- T cells from the same mice (Fig. 2d). Again, deletion of PARP-1 led to slightly, but reproducibly higher expression levels of TβRI and TβRII mRNA on PARP-1^-/- CD4^+CD8^-CD25^- thymocytes compared to PARP-1^+/+ cells (Fig. 2d). In contrast to CD4^+CD25^- T cells, freshly isolated PARP-1^-/-Tregs from the spleen (Fig. 2e) and thymus showed similar levels of TβRI and TβRII mRNA to Tregs from PARP-1^+/+ mice. We also found that PARP-1 deficiency up-regulated the surface protein expression of TβR I and II on splenic CD4^+CD25^+ T cells, as well as CD4^+CD25^- T cells (Fig. 2f-h). Together these data demonstrate that a deficiency in PARP-1 led to increased expression of TβRI and TβRII in CD4^+ T cells, suggesting that PARP-1 may have an inhibitory role in the control of TβR expression.

PARP-1 binds to TβRII but not to TβRI gene

We next determined how PARP-1 regulated TβR expression. We first determined whether PARP-1 could directly bind to TβRI and TβRII genes and thereby regulate their transcription in CD4^+ T cells. Using freshly isolated CD4^+CD25^- T cells from the spleen of wild type mice, we assessed the binding of PARP-1 to TβRI and TβRII genes by chromatin immunoprecipitation (ChIP)-coupled quantitative PCR. We designed a series
of primers which covered -2 kb to +1 kb from the TSS of TβRI and TβRII genes (Supplementary Fig. 4a). We observed binding of PARP-1 to the regions close to the TSS and +1 kb from the TSS at the TβRII gene (Fig. 2i, Supplementary Fig. 4b). However we did not detect enrichment of PARP-1 binding to the same regions of the TβRI gene. The data suggest that PARP-1 differentially regulates TβRI and TβRII expression; directly interacting with the promoter of the former, but not the latter.

To further confirm the hypothesis that PARP-1 proteins control TβR gene expression, we overexpressed PARP-1 protein and co-transfected it with pGL4-based constructs which contained a fragment of murine TβR promoter to EL4/LAF cell lines. We found that overexpression of PARP-1 protein reduced the activity of both TβRI and II in the presence of TGF-β1 (Supplementary Fig. 5). These data together further support our hypothesis that PARP-1 negatively regulates TβR signaling.

Inhibition of PARP-1 enzymatic activity increases TβRI but not TβRII expression

We next investigated the mechanisms by which PARP-1 regulated TβRI expression. Since PARP-1 possesses enzymatic activity in addition to its transcriptional function, we determined whether PARP-1 could influence TβRI expression through its catalytic function. We pre-treated wild-type (C57BL/6) CD4+CD25− T cells with PARP-1-specific inhibitor 5-aminoisoquinoline (5-AIQ) 24, followed by stimulation of these T cells with anti-CD3 and CD28 antibodies for 30 minutes. 5-AIQ treatment can significantly up-regulate TβRI expression in wild-type CD4+CD25− T cells (Fig. 2j). Surprisingly, 5-AIQ treated CD4+CD25− T cells showed no significant changes in TβRII expression compared
to untreated CD4+CD25− T cells (Fig. 2j). These data reveal that PARP-1 controls TβRI expression through its catalytic activity.

**PARP-1 deletion leads to increased Smad2/3 phosphorylation**

As PARP-1−/− CD4+ T cells showed increased TβR expression, and therefore an increased sensitivity to TGF-β signaling, we next determined the effect of PARP-1-deficiency on the phosphorylation and function of Smad2/3 (Smad2 and/or Smad3). Smad2/3 is immediate downstream mediators of TβRs and key factors in mediating TGF-β-induced Foxp3 gene transcription. We used an anti-phosphorylated Smad2 (P-Smad2) as an indicator for Smad2/3 phosphorylation, and observed that even freshly isolated PARP-1−/− CD4+ CD25− T cells showed substantially higher amounts of P-Smad2 than PARP-1+/+ CD4+CD25− T cells (Fig. 3a-b). This corresponded to the higher levels of TβRs in PARP-1−/− T cells (Fig. 2a). Stimulation of PARP-1−/− CD4+CD25− T cells with anti-CD3 and CD28 antibodies for 20-30 minutes substantially enhanced P-Smad2 compared to that of PARP-1+/−CD4+CD25− T cells (Fig. 3a-b). As expected, addition of exogenous TGF-β1 increased P-Smad2 in both PARP-1−/− and PARP-1+/− CD4+ T cells compared to stimulation with TCR alone, but the level of P-Smad2 in PARP-1−/− T cells was still higher than that in PARP-1+/− T cells (Fig. 3a-b). Total Smad2 levels were similar between the PARP-1−/− and PARP-1+/− CD4+ T cells irrespective of treatment (Fig. 3a-b). These data suggest PARP-1 regulates Smad2/3 phosphorylation, which is likely due to increased expression of TβR.
PARP-1 regulates Smad3 binding to the Foxp3 transcriptional enhancer

Smad3 has been shown to preferentially binds to the enhancer region of Foxp3 gene\(^27\), and plays a critical role in the initiation and mediation of the cascade of transcription factors which drive Foxp3 gene activation\(^{25, 27}\). We next investigated whether PARP-1 affected Smad3 binding to the enhancer region of the Foxp3 gene\(^27\). Recently, it has been reported that PARP-1 is a Smad-interacting partner and can attenuate Smad-mediated transcription by blocking the binding of Smad3 to its target gene and/or dissolving the bound Smad3 from its target gene in tumor cells\(^29\). We hypothesized that PARP-1 might also interfere with Smad3 binding to the Foxp3 gene enhancer in naïve CD4\(^+\) T cells and that a deficiency in PARP-1 protein would increase Smad3 binding at the enhancer of the Foxp3 gene and induce more Foxp3 transcription. We cultured PARP-1\(^{-/-}\) and PARP-1\(^{+/+}\) CD4\(^+\)CD25\(^-\) T cells with anti-CD3 and CD28- antibodies plus TGF-\(\beta\)1 for 30 mins (an optimal time for Smad3 binding at the Foxp3 enhancer)\(^25\) and examined the binding of Smad3 to the enhancer region (\(+2085\) to \(+2231\)) of Foxp3 gene by chromatin immunoprecipitation (ChIP)-coupled quantitative PCR. PARP-1 deficiency significantly enhanced the Smad3 binding to the enhancer of Foxp3 gene in naïve CD4\(^+\)CD25\(^-\) T cells in response to TCR and TGF-\(\beta\)1 treatment (Fig. 3c).

More Th17 cells in PARP-1\(^{-/-}\) T cells in response to TGF-\(\beta\)1 and IL-6 in vitro

Since TGF-\(\beta\)1 is also required for Th17 cell differentiation in the presence of pro-inflammatory cytokine IL-6 \(^6, 30-32\), we next examined whether deletion of PARP-1 affected Th17 cell differentiation. We found that treatment with TGF-\(\beta\)1 and IL-6 in the
presence of anti-CD3 antibody plus WT APCs resulted in significantly more IL-17 production from PARP-1−/− than PARP-1+/+ CD4+CD25− T cells (Fig. 4a-b, Supplementary Fig. 6b). Stimulation with TGF-β1 plus IL-6 in the presence of immobilized anti-CD3 and CD28 antibodies increased IL-17 producing as well, although the overall frequency was lower than that in the cultures with APCs (data not shown). Consistent with the enhanced sensitivity to TGF-β1 treatment in Foxp3 Tregs generation, PARP-1−/− CD4+CD25− T cells also showed significantly higher Th17 cell polarization over a wide range of TGF-β1 concentrations (Supplementary Fig. 6a). In line with IL-17 production, treatment with TGF-β1 plus IL-6 also induced higher levels of Rorc expression and RORγt protein in PARP-1−/− T cells (Fig. 4c-d). Together our data indicate that in the absence of PARP-1, CD4+ T cells have an increased ability to differentiate into the Th17 fate in vitro, although this is unlikely to occur in naive PARP-1−/− mice in the steady state, because naïve PARP-1−/− mice are unlikely to show increased IL-6 production in the absence of exogenous inflammatory stimuli. Indeed, naïve PARP-1−/− splenocytes and purified CD4+CD25− T cells secreted substantially lower amounts of IL-6 in response to TCR stimulation in vitro compared with PARP-1+/+ control mice (Supplementary Fig. 6c).

Increased expression of Foxp3 and TβRI in human CD4+ T cells following PARP-1 inhibition

Finally, to determine whether the pathways we identified in mouse are present in humans, we assessed whether reduction of PARP-1 activity in human CD4+ T cells affected Foxp3
expression, as TGF-β1 signaling is also required for Foxp3 induction in human CD4⁺CD25⁻ T cells. We pretreated CD4⁺CD25⁻ human T cells with the PARP-1 inhibitor 5-AIQ and then cultured with anti-CD3 and CD28 antibodies overnight, which was documented to induce Foxp3 transcription by an endogenous TGF-β1-dependent mechanism. Inhibition of PARP-1 activity with 5-AIQ significantly enhanced Foxp3 expression in human CD4⁺CD25⁻ T cells (Fig. 5a), suggesting PARP-1 controls the sensitivity of human T cells to TGF-β signaling.

We next investigated whether inhibition of PARP-1 activity affected the expression of TβRs. We cultured human CD4⁺CD25⁻ T cells with anti-CD3 and CD28 antibodies in the absence and presence of 5-AIQ for 90 minutes. Analysis of TβR expression revealed that PARP-1 inhibitor significantly increased TβRI expression in 5-AIQ-treated resting CD4⁺CD25⁻ T cells compared to untreated controls (Fig. 5b), which confirmed our results from mice (Fig. 2j). In contrast to TβRII expression in mouse T cells, inhibition of PARP-1 with 5-AIQ in TCR-stimulated human CD4⁺CD25⁻ T cells resulted in higher levels of TβRII expression compared to control T cells receiving TCR stimulation alone (Fig. 5b). Nevertheless, the data indicate that PARP-1 also controls the expression of TβRs in human T cells, and therefore could also potentially influence Foxp3 expression in human T cells.
DISCUSSION

TβRs are essential components in transducing TGF-β1 signal that is required for Tregs differentiation² ³ ³⁵, yet the molecular mechanisms is poorly understood. In this paper we have shown that PARP-1 can control expression of TβRs and therefore affect Smad2/3 mediated signaling in CD4⁺ T cells. We show that PARP-1 regulates TβRI expression by its catalytic activity, yet regulates TβRII expression by its transcriptional function. We also demonstrate that there is increased binding of Smad3 to the Foxp3 enhancer in PARP-1⁻/⁻ CD4⁺ T cells; a critical step in initiating Foxp3 gene transcription in response to TGF-β1⁵. Our data also show that PARP-1 regulates the expression of TβRs in human CD4⁺ T cells.

Several conclusions can be drawn from the present study. Firstly, PARP-1 controls TGF-β1 signaling by restraining TβR expression in CD4⁺ T cells on mRNA level. We also observed an increased TβR staining on cells surface in the absence of PARP-1 by flow cytometry. However, this data need to be confirmed by more experiments since the reliability of antibodies to detect cell surface TGF-β receptors is still controversial. The up-regulation of TβRI and TβRII was evident in fresh and stimulated PARP1⁻/⁻ CD4⁺CD25⁻ T cells, which is in line with the enhanced sensitivity of these knockout T cells to TGF-β1. Likewise, PARP-1⁻/⁻ CD4⁺CD8⁻CD25⁻ thymocytes also showed increased expression of TβRs compared with those from PARP-1⁺/+ mice. Intriguingly, CD4⁺CD25⁺Foxp3⁺ Tregs from PARP-1⁻/⁻ mice exhibited no increase in TβRI or TβRII expression compared to PARP-1⁺/+ Tregs. The reason why PARP-1 is able to control TβR expression in naïve CD4⁺CD25⁻ T cells but not in CD4⁺CD25⁺Foxp3⁺ Tregs remains
unknown. However, it could be because CD4+CD25+Foxp3+ Tregs already express optimal levels of TβRs. This is in line with data showing that TGF-β1 is unable to further up-regulate Foxp3 expression in isolated CD4+CD25+Foxp3+ Tregs. Consistent with the increase in TβR expression, PARP-1−/− T cells show increased levels of activated Smad2/3. Short TCR stimulation alone (15-90 minutes) without TGF-β1 substantially increased phosphorylated Smad2/3 (P-Smad2/3) in PARP-1−/− T cells.

Our surprising findings are that PARP-1 regulates TβRI and TβRII through different molecular mechanisms and reveals an unrecognized regulation pathway for TβRs. We show that PARP-1 functions as both a transcription factor and an enzyme in controlling TβR expression. These data are in line with the reported functions of PARP-1. Intriguingly, PARP-1 binds directly to TβRII gene, but not TβRI gene. This indicates that PARP-1 could directly limit TβRII gene activation through transcriptional regulation which is consistent with a previous report of PARP-1 regulation of TβRII in cancer cells.

The lack of binding of PARP-1 at TβRI gene prompted us to investigate a possible role of the enzymatic activity of PARP-1 in regulating TβRI expression. Indeed, blockade of PARP-1 enzymatic activity with inhibitor 5-AIQ up-regulated TβRI expression in CD4+CD25− T cells, but had less impact on TβRII expression. Although it remains to be determined how PARP-1 executes this dual function, it is not without precedence that enzyme can also be transcription factor; it was recently reported that indoleamine 2,3-dioxygenase (IDO) also functions as signal protein in pDCs.

We have shown that Smad2/3 proteins are constitutively phosphorylated. In addition, the deficiency of PARP-1 leads to the enhanced binding of Smad3 at the enhancer of the
The enhanced binding of Smad3 at the enhancer may be partially responsible for the increased Foxp3 expression in PARP-1–/– T cells. Enhanced Treg generation in PARP-1–/– T cells was also confirmed in a model of oral tolerance in mice.

The increased differentiation of Th17 cells in PARP-1–/– T cells in vitro further validate the role for PARP-1 in controlling sensitivity to TGF-β1 signals. However, this up-regulation of Th17 cells is unlikely to occur in the steady state in PARP-1–/– mice as PARP-1–/– mice showed decreased levels of IL-6 compared to PARP-1+/+ mice. However, increased Th17 differentiation could occur where mice encounter infections and/or inflammatory stimuli that trigger IL-6 production from APCs. Indeed, PARP-1 deficiency in mice showed no protective effect upon immunization to induce EAE despite PARP-1–/– mice having elevated Tregs, as PARP-1–/– t mice showed elevated levels of Th17 cells (data not show). We would like to explore the function of PARP-1 in Th17 differentiation in the future and this might help to resolve a controversial issue of whether responsiveness to TGF-β makes more or less pathogenic Th17 cells.

We also show that PARP-1 affects TβRs expression in human T cells and thereby regulates Foxp3 gene expression. Our data shows that inhibition of PARP-1 activity in human T cells substantially enhanced Foxp3 induction and this was likely attributable to enhanced TGF-β1. Indeed, treatment of human CD4+CD25– resting T cells with 5-AIQ significantly enhanced TβRI expression, consistent with mouse CD4+ T cells. Intriguingly, in contrast to mouse, inhibition of PARP-1 with 5-AIQ also caused up-regulation of TβRII expression. It should be noted that the effect results from the prevention of TCR-
mediated down-regulation of TβRII mRNA, as TCR stimulation for 90-min was shown to cause downregulation of TβRII in human CD4+ T cells.

Our data shows that PARP-1 restrains TGF-β signaling in T cells by decreasing TβR expression. Except for responding to DNA-damage, PARP-1 has been shown to regulate both chromatin structure and gene transcription. Here we add to these studies and show that in the absence of PARP-1 TGF-β signaling is enhanced. Our data therefore suggests that PARP-1 functions to dampen regulatory signals in T cells. Although considered to be activated following DNA-damage, PARP-1 has been shown to be active in resting cells and by both ERK1/2 and Notch signaling. Interestingly, PARP-1 has been shown to be activated during T cell stimulation. As such it is possible that PARP-1 plays a role in directly promoting immune responses. This would be achieved at two discreet points, first at sites of inflammation where reactive oxidant species are present, and secondly in T cells activated in lymph nodes. Thus PARP-1 could function to dampen immunoregulatory pathways and promote T cell activation in the lymph node and further protect T cell from any regulatory mechanisms at the site of inflammation. This fits with previous data showing that PARP-1 inhibition reduces cytokine production and allergic asthma. Further suggesting a role for PARP-1 in promoting immune responses is data that shows increased tumor formation in PARP-1−/− mice. PARP-1 has also been known to act as a transcriptional co-regulator of NK-kB and is important for activation of this vital mediator of inflammatory response.

Our data have uncovered an unrecognized role for PARP-1 in regulating Treg differentiation and development by regulation of TβRs expression. This provides a starting point to further understand the network of factors regulating TβRs and their
expression. Our data also have implications for exploring the possibility of blocking PARP-1 as a potential therapy in human autoimmune diseases. Collectively we show a vital role for PARP-1 in restraining TGF-β1 signaling in T cells.


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Figure legends

Figure 1: Mice deficient in PARP-1 gene expression have more Tregs cells. (a-c) Analysis of PARP-1<sup>−/−</sup> (CD45.2<sup>+</sup>) and PARP-1<sup>+/+</sup> (CD45.2<sup>+</sup>) CD4<sup>+</sup> T cells in mixed bone marrow chimeras; representative staining of CD4 versus Foxp3 in CD4<sup>+</sup> cells in the spleen and thymus (a); Frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in spleen (b) and thymus (c) (mean ± s.e.m, PARP-1<sup>+/+</sup> chimeras, n=5; PARP-1<sup>−/−</sup> chimeras, n=5). (d) Quantitative PCR analysis of the expression of Foxp3 (mean ± s.e.m) in freshly isolated (fresh) and CD4<sup>+</sup>CD25<sup>−</sup> T cells stimulated with anti-CD3- (5µg/ml) and CD28- (2µg/ml) specific antibodies in the absence and presence of indicated concentrations of TGF-β1 for 24 hrs. (e) CD4<sup>+</sup>CD25<sup>−</sup> T cells were cultured in the presence of anti-CD3 antibody (0.5 µg/ml) and wild-type APCs with (TGF-β1) or without TGF-β1 (Med) for 2 days. FACS plots show representative staining of CD4 versus Foxp3. (f) Tregs induction in PARP-1<sup>−/−</sup> T cells in vivo. CD4<sup>+</sup>CD25<sup>−</sup> T cells were sorted form the spleens of CD45.1<sup>+</sup> OTII transgenic PARP-1<sup>−/−</sup> or PARP-1<sup>+/+</sup> littermate control mice and 1x10<sup>6</sup> sorted cells were transferred into CD45.2<sup>+</sup> recipient mice. Recipient mice were received OVA protein in the drinking water for 5 days. On day 6 the small intestine lamina propria lymphocytes (LPLs) were isolated and stained with Foxp3 to assess Tregs induction in vivo by flow cytometry analysis. Bar graph shows the frequency of CD45.2<sup>+</sup> (Host) and CD45.1<sup>+</sup> (OT-II) CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in the LPLs of recipient mice. Data is representative of two (a-c, d, f), three (e) independent experiments. *P<0.05, **P<0.01 (unpaired two-tailed Student’s t-test).

Figure 2: Increased expression of TGF-β receptor I and II in CD4<sup>+</sup>CD25<sup>−</sup> T cells of PARP-1<sup>−/−</sup> mice. (a-c) Quantitative PCR analysis of the expression of Tgbr1 and Tgbr2
mRNA in (a) freshly isolated splenic CD4⁺CD25⁻ T cells from PARP-1⁻/⁻ mice and PARP-1⁺/+ littermate controls, in (b) CD4⁺CD25⁻ T cells were stimulated for 15 minutes with CD3- (5μg/ml) and CD28- specific antibodies (2μg/ml) or in (c) CD4⁺CD25⁻ T cells were stimulated for 15 minutes with CD3- (0.5μg/ml), and CD28- (0.2μg/ml) specific antibodies in the presence of TGF-β1 (2ng/ml). (d-e) Quantitative PCR analysis of the expression of Tgbr1 and Tgfbr2 mRNA in CD4⁺CD25⁻ thymocytes and splenocytes (d) and in freshly isolated CD4⁺CD25⁺ Tregs (e). Data is representative of at least three independent experiments (a-e). (mean ± s.e.m of the duplicate measurements). (f-h) Flow cytometry analysis of the expression of TβRI (g) and TβRII (h) in splenocytes which were from both PARP-1⁻/⁻ and PARP-1⁺/+ mice. (f) A representative dot plot showing cells analyzed for CD4 and TβRI or TβRII expressions in CD4⁺CD25⁻ T cells. (g) TbRI expression in CD4⁺CD25⁺ (left two bars) and CD4⁺CD25⁻ (right two bars) cells. (h) TbRII expression in CD4⁺CD25⁺ (left two bars) and CD4⁺CD25⁻ (right two bars) cells. Graphs show mean ± SD. ***p<0.002. (i) ChIP-coupled quantitative PCR analysis of PARP-1 enrichment around 500bp up/downstream (primers No. 3) from Transcriptional Starting Site (TSS) of Tgbr1 and Tgfbr2 genes (mean ± s.e.m of duplicate wells). Data is representative of two independent experiments (j) Quantitative PCR analysis of the expression of Tgbr1 and Tgfbr2 mRNA in 5-AIQ treated CD4⁺CD25⁻ T cells. Data is representative of four independent experiments (mean ± s.e.m) *P<0.05, (unpaired two-tailed Student’s t-test).

**Figure 3:** PARP-1 regulates Smad3 phosphorylation and Smad3 binding at the Foxp3 enhancer. (a) Western Blot of P-Smad2, Smad2 and GAPDH expressions in freshly isolated CD4⁺CD25⁻ T cell and CD4⁺CD25⁻ T cells stimulated for 15 minutes with
CD3- (5µg/ml) plus CD28 (2µg/ml) antibodies in the absence or presence of TGF-β1 (2ng/ml). Data is representative of three independent experiments. (b) Summarization of three times P-Smad2 Western Blot analysis. Western Blot bands were analyzed by Odyssey software, the PARP-1+/+ P-Smad2 expression was set as 1, PARP-1−/− P-Smad2 expression was presented relative to PARP-1+/+. *P<0.05, unpaired one-tailed Student’s t-test. (c) ChIP-coupled quantitative PCR analysis of Smad3 enrichment in the enhancer region of Foxp3 gene assessed using an antibody to Smad3 and presented relative to input and compared with a control IgG. Data is representative of four independent experiments (mean ± s.e.m) ***P<0.001 (unpaired two-tailed Student’s t-test).

Figure 4: Greater differentiation of Th17 in PARP-1−/− T cells in vitro. (a) Scatterplot (left) of staining of IL17 versus IFNγ on sorted CD4+ T cells from PARP-1−/− or PARP-1+/+ littermate controls stimulated with anti-CD3 (0.5µg/ml) antibody and PARP-1+/+ APCs in the absence (Med) or presence of TGF-β1 plus IL6 (TGFβ1+IL6) for 4 days. Data is representative of two independent experiments. The histogram (right) is summarized analysis of two experiments. *P<0.05, (unpaired one-tailed Student’s t-test). (b) IL17 levels in supernatants from cultures described in (a) (mean ± s.e.m of duplicate measurements). (c) Quantitative PCR analysis of RORγt expression in CD4+CD25− T cells from PARP-1−/− or PARP-1+/+ littermate controls stimulated with a anti-CD3 (0.5µg/ml) antibody and PARP-1+/+ APCs in the absence (Med) or presence of TGF-β1 plus IL6 (TGFβ1+IL6) for 2 days. (mean ± s.e.m of duplicate measurements) (d) Staining of RORγt in CD4+T cells from PARP-1−/− or age-matched PARP-1+/+ control littermates which were stimulated with anti-CD3 (0.5µg/ml) antibody and PARP-1+/+ APCs in the absence (Med) or presence of TGF-β1 and IL6 (TGFβ1+IL6) for 4 days. *P<0.05,
**P<0.01 (unpaired two-tailed Student’s t-test).

Figure 5: Inhibition of PARP-1 in human CD4+ CD25- T cells increases the expression of Foxp3 and TβRI. CD4+CD25- T cells were isolated from human peripheral blood and pre-incubated with 5-AIQ for 20 min, then cells were stimulated with human CD3 and CD28 specific antibodies for the indicated time. Quantitative PCR analysis showed the expression of Foxp3, Tgfbr1 and Tgfbr2. (a) Foxp3 expression was determined after 12hrs culture. Data is mean ± s.e.m of three independent experiments. (b) Tgfbr1 and Tgfbr2 mRNA expression was determined after 90 mins of culture. Data is mean ± s.e.m. of three independent experiments. * P<0.05 (unpaired two-tailed Student’s t-test).
Figure 1
Figure 2
Figure 3

(a) Western blot analysis showing the expression of P-Smad2, Smad2, and GAPDH under different conditions: Fresh, αCD3+αCD28, TGF-β1, and Parp1+/+ and Parp1−/−. The blots are normalized to GAPDH.

(b) Bar graph showing the relative P-Smad2 (IPARP-1+/+) for Parp1+/+ and Parp1−/− under Fresh, αCD3+αCD28, TGF-β1. Significant differences are indicated by asterisks (*).

(c) Bar graph showing the relative enrichment (Input) for IgG and Smad3 for Parp1+/+ and Parp1−/−. Significant differences are indicated by asterisks (***).
**Figure 4**

(a) Flow cytometry analysis showing IL17 expression in TGF-β1+IL6 treated Parp1+/+ and Parp1−/− cells.

(b) IL17 levels in Med and TGF-β1+IL6 treated Parp1+/+ and Parp1−/− cells.

(c) RoRγt relative expression in Parp1+/+ and Parp1−/− cells.

(d) Flow cytometry analysis showing RORγt expression in Parp1+/+ and Parp1−/− cells.

**Table**

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL17 (pg/ml)</th>
<th>CD4+IL17+ (%)</th>
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<tbody>
<tr>
<td>Med</td>
<td>0.3</td>
<td>2.9</td>
</tr>
<tr>
<td>TGF-β1+IL6</td>
<td>20.2</td>
<td>30.6</td>
</tr>
</tbody>
</table>

IL17 levels are significantly higher in TGF-β1+IL6 treated Parp1−/− cells compared to Parp1+/+ controls.

**Statistical Significance**

- *p < 0.05
- **p < 0.01

**Notes**

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Figure 5
PARP-1 regulates expression of TGF-β receptors in T cells

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