Cbl-b mediates TGFβ sensitivity by downregulating inhibitory SMAD7 in primary T cells

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Abstract

T cell-intrinsic transforming growth factor β (TGFβ) receptor signaling plays an essential role in controlling immune responses. The RING-type E3 ligase Cbl-b has been shown to mediate the sensitivity of T cells to TGFβ; however, the mechanism underlying this process is unknown. This study shows that SMAD7, an established negative regulator of TGFβ receptor (TGFbR) signaling, is a key downstream effector target of Cbl-b. SMAD7 protein levels, but not SMAD7 mRNA levels, are upregulated in cblb−/− T cells. Cbl-b directly interacts with and ubiquitinates SMAD7, suggesting that Cbl-b posttranscriptionally regulates SMAD7. In support of this notion, concomitant genetic loss of SMAD7 in cblb−/− mice restored TGFβ sensitivity on T cell cytokine responses and abrogated the tumor rejection phenotype of cblb−/− mice. These results demonstrate an essential and non-redundant role for Cbl-b in controlling TGFβR signaling by directly targeting SMAD7 for degradation during T cell responses in vitro and in vivo.

Keywords: TGFβ signaling, Cbl-b, SMAD

Introduction

Cytokines of the transforming growth factor β (TGFβ) family play important roles in many cellular functions, including cell-cycle control, embryogenesis, differentiation, extracellular matrix formation, hematopoiesis, angiogenesis, chemotaxis, apoptosis, and immunity (Schuster and Kriegstein, 2002). Three TGFβ isoforms have been described in mammals (Govinden and Bhoola, 2003). Of these isoforms, TGFβ1 (herein referred to as TGFβ) is predominantly expressed in the immune system and is one of the key regulators of immune homeostasis and peripheral T cell tolerance (Lucas et al., 2000; Bommireddy et al., 2003; Li et al., 2006; Lan et al., 2012). TGFβ also suppresses T cell activity, thereby limiting autoimmunity (Shull et al., 1992; Gorelik and Flavell, 2000). Furthermore, TGFβ secreted by tumor cells or tumor-infiltrating regulatory T cells (T reg) generates an immunosuppressive milieu that contributes to immune escape of tumor cells (Thomas and Massague, 2005).

In addition to the immunosuppressive properties of TGFβ, this cytokine is pivotal to T cell differentiation (Tran, 2012). TGFβ alone induces Foxp3 expression and I T reg differentiation (Chen and Konkel, 2010), whereas the inclusion of IL-6 induces RORγt and drives naïve CD4+ T cells toward the T H17 lineage (Bettelli et al., 2006; Ivanov et al., 2006). Conversely, TGFβ inhibits T H1 and T H2 differentiation by downregulating the transcription factors T-bet and GATA-3, respectively (Heath et al., 2000; Gorelik et al., 2002). This suggests that the T H1 lineage represents a default effector pathway for CD4+ T cells in the absence of TGFβ signaling (Li and Flavell, 2008).

At molecular level, binding of TGFβ to the receptor serine/threonine kinases TGFβRI and TGFβRII leads to the phosphorylation of TGFβRI by the constitutively active TGFβRII (Wrana et al., 1994). SMAD2 and SMAD3 are subsequently recruited to the activated receptor and phosphorylated by TGFβRI. These receptor-activated SMADs (R-SMADs) form a complex with common (Co-)SMAD4, thereby targeting multiple gene promoters for either activation or repression (Massague et al., 2005). This signaling pathway is subject to negative regulation by the inhibitory (I-)SMADs, i.e. SMAD6 and SMAD7. SMAD7 is the predominant I-SMAD in the TGFβ pathway, and SMAD7 expression is induced by TGFβ itself, thereby constituting a negative feedback loop (Hayashi et al., 1997; Nakaoka et al., 1997; Stopa et al., 2000). SMAD7 is posttranslationally regulated by acetylation and ubiquitination. It has been shown to be ubiquitinated by Smurf2 and Arkadia, which leads to SMAD7 proteasomal degradation (Kavsak et al., 2000; Koinuma et al., 2003).

SMAD7 antagonizes TGFβ signaling at several levels. SMAD7 has been shown to compete with SMAD2 and SMAD3 for TGFβRI binding, hence inhibiting the recruitment and phosphorylation of R-SMADs (Hayashi et al., 1997; Nakaoka et al., 1997). Furthermore, SMAD7 acts as an adaptor that allows the HECT-type E3 ligase

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Smurf1/2 to associate with TGFβRI, targeting the receptor for ubiquitination and degradation (Kavasak et al., 2000; Yamaguchi et al., 2006). Another adaptor function has been described where SMAD7 recruits the phosphatase complex GADD34-PP1c to activated TGFβRI, followed by dephosphorylation of the receptor (Shi et al., 2004). In addition to this membrane-proximal inhibition of TGFβ signaling, SMAD7 has been shown to function in the nucleus by binding to DNA via its MH2 domain and interfering with functional SMAD-DNA complex formation (Zhang et al., 2007).

Cbl-b is an E3 ubiquitin ligase that regulates T cell activation thresholds by regulating the requirement for CD28 costimulation (Bachmaier et al., 2000; Chiang et al., 2000). Cbl-b plays an essential role in immunotolerance and consequently limits autoimmunity (Jeon et al., 2004). In this context, Cbl-b has also been shown to mediate the immunosuppressive effects of TGFβ. Cblb-deficient T cells are less sensitive to TGFβ and to inhibition by Treg (Wohlfert et al., 2004, 2006; Chiang et al., 2007; Loenser et al., 2007; Adams et al., 2010; Lutz-Nicoladoni et al., 2012). Moreover, cblb-/- mice display enhanced responses to a TGFβ-secreting tumor compared with WT mice (Wohlfert et al., 2006).

Although these studies have revealed a non-redundant role of Cbl-b in TGFβ signaling, very little is known regarding the underlying molecular mechanisms. Therefore, we examined cblb-deficient primary T cells in greater detail in this study. We report here that Cbl-b is necessary for efficient TGFβ-mediated DNA binding of SMAD2/3 to the SMAD-binding element (SBE), which is the consesus core element of many SMAD-responsive promoter regions (Zawel et al., 1998). Consistently, binding of SMAD2 and SMAD3 to the upstream sites of the Il2 gene is strongly diminished in cblb-deficient T cells. By analyzing components of the TGFβ/SMAD pathway, we found that SMAD7 protein levels are significantly elevated in cblb-/- primary T cells. We show that Cbl-b physically interacts with SMAD7 and SMAD7 is directly ubiquitinated in a Cbl-b E3 ligase-dependent fashion and subsequently degraded, thereby increasing canonical TGFβ receptor/SMAD signaling in T cells. In accord with these results, resistance of cblb-/- T cells to TGFβ is mostly abrogated by the concomitant genetic loss of smad7 in vitro and in vivo, providing strong genetic evidence for a functional antagonism between Cbl-b and SMAD7. We, therefore, propose a model in which Cbl-b sets TGFβR signaling thresholds in T cells by acting as a critical signaling intermediate in the canonical TGFβ signaling pathway by regulating I-SMAD7 protein stability at posttranslational level.

Results

Cblb-/- CD4+ T cells differentiated under Tn17 conditions exhibit increased levels of the Tn17 signature cytokine IFNγ and the transcription factor T-bet

Cbl-b has been shown to be highly expressed in CD4+ and CD8+ T lymphocytes (GNF SymAtlas, http://biogps.org/#goto=gene report&id=868) and to exert an essential function in these T cell types (Jeon et al., 2004; Loenser et al., 2007).

While examining the expression of cblb in CD4+ T helper cell subsets in vitro, we observed upregulation of cblb mRNA in CD4+ T cells grown under conditions that promote Tn2, Tn17 and Tn_reg cell development (Figure 1A); this upregulation was not detected in T cells cultured under Tn1-polarizing conditions, leading us to hypothesize that Cbl-b might be important in balancing T helper cell differentiation.

To determine the functional consequences of the absence of Cbl-b in Tn17 effector cells, we differentiated naïve cblb-/- and WT CD4+ T cells toward Tn17 in vitro. We then analyzed the levels of secretion of the key Tn17 cytokine IL-17A and found that the IL17A secretion levels were not significantly different between WT and cblb-deficient Tn17 cells (Figure 1B). Surprisingly, when we measured the levels of secretion of the Tn1 key cytokine IFNγ, we observed significantly enhanced IFNγ levels in cblb-deficient Tn17 cells compared with WT (Figure 1C). Because anti-IFNγ was added to the differentiation medium to enable proper Tn17 differentiation, we restimulated Tn17-polarized cells with anti-CD3 and anti-CD28 for 24 h in the absence of cytokines and blocking antibodies, and measured the IFNγ secretion levels. Consistent with Figure 1C, the IFNγ levels were also strongly enhanced in restimulated cblb-deficient Tn17 cells compared with WT (Figure 1D and E). Interestingly, significantly more Foxp3+ regulatory T cells were generated under Tn17 conditions when Cbl-b was absent (Supplementary Figure S1). This could be a consequence of the observed hyperproduction of IL-2 in cblb-deficient T cells (not shown). Because IFNγ expression is regulated by the Tn1-specific transcription factor T-bet, we analyzed the expression of T-bet in differentiated cblb-deficient Tn17 cells. Consistent with IFNγ production, T-bet expression was significantly enhanced in cblb-deficient Tn17 cells (Figure 1F and G). Notably, this was not a result of secondary effects because normal survival rates were observed in WT and cblb-deficient genotypes (Figure 1K). In contrast to these observations, the expression of T-bet and secretion of IFNγ were unaltered in cblb-deficient T cells during Tn1 differentiation (Figure 1H and I). To further determine the specificity of Tn17-differentiated cells, we investigated the key Tn17 transcription factor RORγt. The expression of RORγt was similar between WT and cblb-deficient genotypes, but was strongly induced in Tn17-polarized cells compared with Tn1 cells (Figure 1J). Taken together, these results emphasize the importance of Cbl-b in the downregulation of Tn1 markers during Tn17 cell differentiation. The fact that T-bet and IFNγ expression have been shown to be suppressed by TGFβ (Gorelik et al., 2002) supports our hypothesis that Cbl-b plays a central role in TGFβR signaling. Cblb-/- T cells display defective TGFβR signaling.

These observations prompted us to study the mechanistic basis underlying the de-regulation of IFNγ in cblb-/- Tn17 cells. Given the central role of TGFβ in Tn17 differentiation, we investigated potential effects of cblb-deficiency on the TGFβR signaling pathway. We studied the effects of TGFβ stimulation on SMAD2/3 DNA-binding activity to the SBE in CD3+ T cells using electrophoretic mobility shift assay (EMSA). Cblb-/- CD3+ T cells showed a strong defect in SMAD2/3 DNA-binding activity compared with WT cells (Figure 2A). This defect was apparent not only in TCR/CD28/TGFβR-stimulated cells but also in cells treated with TGFβ alone (not shown), indicating that the observed difference in SBE binding is not due to aberrant TCR signaling. In light of this result and considering the role of TGFβ in repressing IL-2 expression in a SMAD3-dependent manner (McKarns et al., 2004), we analyzed the Il2 promoter region for potential SMAD2/3 binding sites using a transcription factor prediction program (TRANSFAC® database). Two potential binding regions were
whether the diminished sensitivity of cblb-sensiveness in leprosy patients (Kumar et al., 2011). To evaluate the effects of cblb on T cell activation, we used Bio-Plex technology for the presence of IL-17 or IFNγ on Day 3 of differentiation or after restimulation with anti-CD3/anti-CD28 for 24 h (n = 3–9). (E) Tg17-polarized cells were analyzed for IFNγ expression by intracellular staining on Day 3 after restimulation with PdBu/ionomycin for 4 h (n = 2). (F, I, and J) Tg17- and Tg1-polarized cells were examined for the transcription factors T-bet and ROR-γt, and were analyzed by flow cytometry or qRT–PCR, respectively (n = 3). (G) Nuclear extracts of Tg17-polarized cells were analyzed using western blotting for T-bet expression. The blot shown is representative of two independent experiments with similar outcomes. (K) The survival rate of Tg17-differentiated cells is shown (n = 3). The data are presented as mean ± SEM (A–F and H–K); *** P = 0.0002 (C); * P = 0.023 (D); P = 0.11 (E); * P = 0.03 (F).

**Figure 1** The key Tg1 cytokine IFNγ and transcription factor T-bet are significantly upregulated in cblb-deficient Tg17-differentiated cells. (A) Naive CD4⁺ T cells from WT or cblb⁻/⁻ mice were differentiated under Tg10-, Tg11-, Tg12-, Tg17-, and Itreg-polarizing conditions for 3 days and were analyzed for cblb expression by qRT–PCR upon restimulation with anti-CD3 for 4 h. Tg0 values were set as 1 (n = 2). (B–D) Supernatants from Tg17 and Tg1-polarized cells were analyzed by Bio-Plex technology for the presence of IL-17 or IFNγ on Day 3 of differentiation or after restimulation with anti-CD3/anti-CD28 for 24 h (n = 3–9). (E) Tg17-polarized cells were analyzed for IFNγ expression by intracellular staining on Day 3 after restimulation with PdBu/ionomycin for 4 h (n = 2). (F, I, and J) Tg17- and Tg1-polarized cells were examined for the transcription factors T-bet and ROR-γt, and were analyzed by flow cytometry or qRT–PCR, respectively (n = 3). (G) Nuclear extracts of Tg17-polarized cells were analyzed using western blotting for T-bet expression. The blot shown is representative of two independent experiments with similar outcomes. (K) The survival rate of Tg17-differentiated cells is shown (n = 3). The data are presented as mean ± SEM (A–F and H–K); *** P = 0.0002 (C); * P = 0.023 (D); P = 0.11 (E); * P = 0.03 (F).

SMAD7 protein levels are strongly increased in Cblb⁻/⁻ T cells

Because none of the critical components of the TGFβR signaling pathway was found to be deregulated in cblb⁻/⁻ T cells, we next investigated the expression of inhibitory SMAD7. Surprisingly, analysis of whole cell extracts from CD3⁺ T cells indicated that SMAD7 protein levels were significantly increased in cblb⁻/⁻ T cells compared with WT (Figure 3A). Because SMAD7 is not expressed in Cblb⁻/⁻ T cells, we next investigated whether SMAD7 protein expression was upregulated in cblb⁻/⁻ T cells upon TGFβ stimulation, and considerable levels of Cbl-b were detected in the nuclear cell fractions of WT cells (Figure 3B and C). Next, we analyzed smad7 mRNA expression in cblb⁻/⁻ T cells. No substantial difference in smad7 mRNA expression was observed between the genotypes, indicating that SMAD7 is regulated by Cbl-b at protein level rather than at transcript level (Figure 3D). We then investigated whether SMAD7 protein levels were also upregulated in cblb⁻/⁻ Tg17-polarized cells by FACs analysis and western blotting. Indeed, SMAD7 protein levels were also significantly enhanced in cblb⁻/⁻ Tg17 cells (Figure 3E–G). Consistent with naive CD3⁺ T cells, smad7 mRNA expression in Tg17-polarized cells was similar between the two genotypes (Figure 3H).
Importantly, cblb\(^{-/-}\) T\(_{17}\)-polarized cells showed a strong defect in SMAD\(_2/3\) DNA-binding activity compared with WT cells (Figure 3I), whereas this defect was not observed in iT\(_{reg}\) cells (Figure 3J). Overall, these data indicate a Cbl-b-dependent posttranscriptional regulation of SMAD\(_7\) protein levels in both naïve CD\(_3^+\) and T\(_{17}\)-polarized T cells.

The Cbl-b E3 ligase physically interacts with and ubiquinates SMAD\(_7\)

To more thoroughly investigate the function of Cbl-b in the TGF\(_{B}\)R signaling pathway, we examined the physical interaction between Cbl-b and SMAD\(_7\) under endogenous conditions in murine CD\(_3^+\) T cells. Immunoprecipitation analysis confirmed a constitutive interaction between Cbl-b and SMAD\(_7\) (Figure 4A). Additionally, analysis of endogenously stained Cbl-b and SMAD\(_7\) by confocal microscopy (Figure 4B) revealed that these proteins are not solely located in the cytosol, but also detectable in the nucleus of primary human CD\(_4^+\) T cells (calculation of the mean gray values showed no significant difference in the cytosolic and nuclear distribution, data not shown). Moreover, Cbl-b and SMAD\(_7\) showed a substantial constitutive colocalization, which slightly but significantly increased upon TGF\(_{B}\) stimulation in the cytosol as well as in the nucleus (Figure 4C). Cbl-b possesses an ubiquitin-associated (UBA) domain at its C-terminal end, which serves as a putative binding site for ubiquitinated proteins (Davies et al., 2004). As the Cbl-b/SMAD\(_7\) interaction might be due to a secondary effect of ubiquitinated SMAD\(_7\) binding to the UBA domain of Cbl-b, we analyzed whether Cbl-b/SMAD\(_7\) complex formation can be achieved in an ubiquitin-independent fashion using a Cbl-b DUBA mutant. HA-tagged Cbl-b DUBA and WT HA-tagged Cbl-b overexpressed in HEK293T cells coimmunoprecipitated with FLAG-tagged SMAD\(_7\) to similar extents, indicating that Cbl-b/SMAD\(_7\) binding is UBA-independent (Figure 4D).
Based on this newly identified Cbl-b/SMAD7 complex formation, we hypothesized that SMAD7 could be a potential ubiquitination target for Cbl-b. We performed in vitro ubiquitination assays using purified recombinant full-length Cbl-b and recombinant GST- SMAD7. SMAD7 ubiquitination was clearly detected in a Cbl-b E3 ligase-dependent manner because the capacity of the E2 (UbcH10) to ubiquitinate SMAD7 was virtually abolished when Cbl-b was absent (Figure 4E). Because the ubiquitin moieties are randomly biotinylated at lysine residues in this assay, mainly monoubiquitination could be detected. We also checked polyubiquitination of SMAD7 in primary WT and cblb-deficient T cells and demonstrated that SMAD7 is polyubiquitinated in a Cbl-b-dependent manner in primary T cells (Figure 4F, compare lanes 2 and 3).

Resistance of cblb-deficient T cells to TGFβ is abrogated by the conditional deletion of smad7 in vitro and in vivo

To evaluate the non-redundant role of Cbl-b in SMAD7 regulation, we cross-bred established cblb and CD4Cre-smad7fl/fl knockout mice (Gruber et al., 2009; Kleiter et al., 2010) to generate animals with a T cell-specific deficiency in both smad7 and cblb and characterized their immune phenotype. QRT–PCR confirmed smad7 and cblb deletion in purified T lymphocytes (Supplementary Figure S3). Cblb−/−/CD4cre-smad7fl/fl double-knockout (DKO) mice were viable and fertile. Flow cytometric analysis of thymocytes, splenocytes, and lymph nodes revealed comparable distributions of CD4, CD8, and T cell receptor (TCR) expressing cells among the different genotypes (Table 1). Analysis of T cell activation under increasing...
TGFβ concentrations, indicated by IL-2 and IFNγ secretion in primary T cells, showed that concomitant loss of smad7 substantially abrogated the resistance of cblb-deficient T cells to TGFβ in vitro (Figure 5A and B). Moreover, the hyperproduction of IFNγ under TH17 conditions was also completely reversed in dKO cells (Figure 5C). In contrast, Foxp3+ iTreg generation, which is positively regulated by Cbl-b (Harada et al., 2010), was only partially rescued to WT level by the concomitant loss of smad7 (Supplementary Figure S4). CblbKO mice have been shown to reject tumors in a T cell-dependent fashion (Chiang et al., 2007; Loeser et al., 2007; Paolino et al., 2011; Lutz-Nicoladoni et al., 2012). Interestingly, overexpression of smad7 seems to exert effects reminiscent of the anti-tumor immunity observed in cblbKO mice (Rizzo et al., 2011). T cell-specific smad7 single-knockout mice displayed slightly decreased survival rates compared with WT mice, whereas cblb-deficient mice were able to efficiently and spontaneously reject TC-1 tumors and exhibited strongly increased survival rates. However, TC-1 tumors grew substantially in T cell-specific dKO mice have been shown to reject tumors in a T cell-dependent fashion.
mice that displayed comparable survival rates to WT mice (Figure 5D and E). These results indicate that T cell-specific loss of smad7 abrogates the survival advantage observed in cblb-deficient animals.

### Discussion

The RING-type E3 ligase Cbl-b has been established to be a negative regulator of T cell activation. Without adequate costimulation, Cbl-b targets critical signaling molecules for ubiquitination, thereby increasing the threshold for productive T cell activation and ultimately leading to a state of unresponsiveness, termed anergy (Macian et al., 2002; Heissmeyer et al., 2004; Jeon et al., 2004). Costimulation via the antigen receptor and CD28 leads to the degradation of Cbl-b as a prerequisite for potent T cell responses (Zhang et al., 2002; Gruber et al., 2009). Consequently, loss of Cbl-b uncouples TCR stimulation from the necessity of CD28 costimulation for effective T cell activation (Bachmaier et al., 2000; Chiang et al., 2000; Krawczyk et al., 2000; Naramura et al., 2002; Jeon et al., 2004). In addition to the well-documented role in immunotolerance, Cbl-b also contributes to the maintenance of self-tolerance by mediating the immunosuppressive effects of TGFβ (Wohlfert et al., 2004, 2006).

Given the numerous observations that suggest an important role for Cbl-b in TGFβ signaling, we considered whether T<sub>17</sub> differentiation was also affected by the absence of Cbl-b. In concert with IL-6, TGFβ induces RORγt and drives T<sub>17</sub> differentiation of naive CD<sub>4</sub><sup>+</sup> cells (Bettelli et al., 2006; Ivanov et al., 2006). Interestingly, we found that the induction of RORγt and production of IL-17 are normal in cblb<sup>−/−</sup> T<sub>17</sub> T cells, but the secretion of the T<sub>1</sub> signature cytokine IFNγ is strongly enhanced under T<sub>17</sub>-skewing conditions. Consistently, the expression of the T<sub>1</sub>-related transcription factor T-bet was also significantly augmented. These data suggest that Cbl-b plays a pivotal role in the suppression of the T<sub>1</sub> lineage under T<sub>17</sub> conditions. TGFβ has been shown to strongly inhibit T<sub>1</sub> differentiation of naive CD<sub>4</sub><sup>+</sup> T cells (Gorelik et al., 2002), and this suppression has been shown to be dependent on SMAD2/3 signaling (Takimoto et al., 2010). Accordingly, SMAD7, an established inhibitor of TGFβ signaling, is induced during T<sub>1</sub> differentiation and inhibited during T<sub>17</sub> differentiation (Kleiter et al., 2010).

Our data indicate that cblb-deficiency in T cells has disturbed TGFβ/SMAD signaling during T<sub>17</sub> differentiation. The relatively normal T<sub>17</sub> phenotype of these cells argues for alternative pathways for T<sub>17</sub> differentiation that are not influenced by the loss of Cbl-b. Indeed, it has been suggested that SMAD2 and SMAD3 are not involved in the induction of RORγt by TGFβ (Takimoto et al., 2010). The resistance of cblb<sup>−/−</sup> T cells to TGFβ has been shown to be context-dependent; these cells were resistant to TGFβ-mediated suppression during anti-tumor immune responses (Wohlfert et al., 2006) but were responsive to TGFβ-mediated T<sub>17</sub> differentiation (Adams et al., 2010). Moreover, it has been proposed that TGFβ is dispensable for the differentiation of human T<sub>17</sub> cells but it indirectly favors their expansion by inhibiting T<sub>1</sub> cells (Santarlasci et al., 2009). This notion was confirmed by the observation that TGFβ did not promote the differentiation of murine T<sub>17</sub> cells directly, but did so indirectly by preventing T<sub>1</sub> and T<sub>12</sub> differentiation (Das et al., 2009). In this context, our data indicate that Cbl-b plays a crucial role in TGFβ-mediated suppression of the T<sub>1</sub> lineage during T<sub>17</sub> differentiation.

Nevertheless, virtually nothing is known regarding the molecular mechanisms by which Cbl-b conveys TGFβ sensitivity to T cells. One study has reported impaired SMAD2 phosphorylation in cblb-deficient CD4<sup>+</sup> cells (Wohlfert et al., 2006), but these results were contradicted in another study (Harada et al., 2010).

We report here that Cbl-b acts as a positive regulator of the TGFβ signaling pathway by downregulating inhibitory SMAD7. Nuclear SMAD7 protein levels are significantly elevated in cblb<sup>−/−</sup> T cells. SMAD2/3 complex formation on the SBE is impaired in naive and T<sub>17</sub>-differentiated cblb-deficient T cells but is intact in iT<sub>reg</sub>. Consistently, Harada et al. (2010) have reported that Cbl-b regulates Foxp3 expression through a Foxo3a-dependent, yet SMAD-independent, mechanism. Our data confirm the defective iT<sub>reg</sub> differentiation of cblb-deficient T cells (Harada et al., 2010) and a negative regulatory role of SMAD7 on iT<sub>reg</sub> generation (Dominitzki et al., 2007; Kleiter et al., 2010). Interestingly, cblb/smad7<sup>−/−</sup> KO cells are considerably rescued, suggesting that loss of smad7 and the resulting hyperactivation of the Smad signaling pathway can partially compensate for the defective FOXO activity.

Of note, SMAD2 and SMAD3 phosphorylation is unaffected by the loss of Cbl-b, which suggests that proximal TGFβ signaling is Cbl-b-independent. Consistently, TGFβR1 levels are not altered in T cells from cblb<sup>−/−</sup> mice. In the current model, SMAD7 acts upstream of SMAD2/3 by competing with R-SMADs for receptor binding and additionally promoting TGFβRII degradation (Hayashi et al., 1997; Nakao et al., 1997; Kavask et al., 2000; Yamaguchi et al., 2006). We and others have observed enhanced SMAD2 phosphorylation in smad7-deficient T cells (Kleiter et al., 2010), which is in accordance with a proximal inhibitory role of SMAD7. Altogether, our data suggest that Cbl-b specifically regulates the membrane-distal effects of SMAD7. We propose that SMAD7 accumulates in the nuclei of cblb-deficient T cells and competes with SMAD2/3 for DNA binding. This can explain why SMAD2/3 DNA binding to the SBE is diminished in cblb<sup>−/−</sup> T cells while proximal events such as SMAD2/3 phosphorylation are unaffected. In accordance with a nuclear role of Cbl-b and SMAD7, we demonstrate that these proteins are both located in the nucleus of T cells.

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**Table 1** Thymocytes, splenocytes, and lymph nodes show comparable distributions of CD4, CD8, and TCR expressing cells among the different genotypes.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>WT (%)</th>
<th>cblb&lt;sup&gt;−/−&lt;/sup&gt; (%)</th>
<th>Smad7&lt;sup&gt;−/−&lt;/sup&gt; (%)</th>
<th>DKO (%)</th>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>5.0 ± 0.0</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;TCR&lt;sup&gt;β&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>11.5 ± 2.0</td>
<td>13.0 ± 2.0</td>
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<td>CD8&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>CD8&lt;sup&gt;+&lt;/sup&gt;TCR&lt;sup&gt;β&lt;/sup&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
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Thymocytes, splenocytes, and lymph nodes stained for CD4, CD8, and TCRβ were analyzed by flow cytometry. Data are shown as mean ± SEM from two independent experiments.
The enhanced SMAD7 protein levels observed in cblb<sup>−/−</sup> T cells are not due to transcriptional deregulation because smad7 mRNA levels are normal compared with WT controls, suggesting that SMAD7 protein is regulated posttranscriptionally, likely through modulation of its stability. Indeed, we showed that Cbl-b co-precipitates with SMAD7 in mouse T cell extracts, indicating endogenous Cbl-b/SMAD7 complex formation. This association is not mediated simply by interaction of an ubiquitinated form of SMAD7 with the UBA domain of Cbl-b because a UBA-deleted Cbl-b/UBA mutant was still able to bind SMAD7. This result was confirmed by confocal analysis of human naïve CD4<sup>+</sup> T cells, which showed substantial constitutive colocalization of Cbl-b and SMAD7 that significantly increased upon TGFβ stimulation.

Moreover, we further confirmed that SMAD7 is a direct ubiquitination target of Cbl-b by demonstrating the Cbl-b-dependent endogenous polyubiquitination of SMAD7.

The cytokine responses of cblb/smad7 double-deficient T cells under the influence of TGFβ showed that concomitant loss of smad7 largely abolished the resistance to TGFβ observed in naïve cblb<sup>−/−</sup> T cells. Furthermore, the suppression of IFNγ expression...
under T₅₁₀ conditions, which is abolished in ccblb⁻/- T cells, was completely restored in ccblb/smad7 double-deficient T cells. These data indicate that SMAD7 is a major target of Cbl-b in the TGFβ signaling pathway and highlight the presented biochemical data.

To assess the in vivo relevance of this Cbl-b/SMAD7 relationship, we monitored the outgrowth of subcutaneously injected TC-1 tumor cells in different mice. Intriguingly, ccblb⁻/- mice could completely reject the tumors, which is in accordance with previous reports (Wohlfert et al., 2006; Chiang et al., 2007; Loeser et al., 2007; Paolino et al., 2011). In contrast, we observed that TC-1 tumors were able to grow substantially in ccblb⁻/-/CD4cre-smad7fl/fl mice. Of note, only cells that expressed CD4 during their lifetime were ccblb/smad7 double-deficient in these mice (CD4⁺ and CD8⁺ T cells in particular), whereas all other cells only lacked ccblb. T cell-specific loss of smad7 almost completely abrogated the survival advantage observed in ccblb-deficient animals. There has been clear evidence that the tumor rejection seen in ccblb KO mice can be at least partially ascribed to the hyposensitivity of T cells to TGFβ. Wohlfert et al. (2006) reported that ccblb⁻/- mice show a significantly enhanced response to a tumor that is strictly regulated by TGFβ. They used EL-4 tumor cells, which were also rejected by transgenic mice that have TGFβ-resistant T cells due to a dominant negative TGFβRI (Gorelik and Flavell, 2001). We chose a tumor model (TC-1) that secretes significant amounts of TGFβ and uses TGFβ as an immune escape mechanism (Terabe et al., 2009). Loeser et al. (2007) demonstrated that TC-1 tumors are efficiently rejected by ccblb⁻/-/- mice, which is mechanistically ascribed in part to the resistance of ccblb⁻/-/- CD8⁺ T cells to the suppression by regulatory T cells, a major source of TGFβ. Our in vivo tumor experiments were performed to complement the in vitro results that clearly show that the resistance of ccblb⁻/-/- T cells to TGFβ was completely reversed by the concomitant loss of smad7. Although these results are not sufficient to determine whether in vivo SMAD7 is a target of Cbl-b downstream of the TGFβR or Cbl-b and SMAD7 are acting through two entirely different pathways, the complete reversal of the immune response toward a TGFβ-secreting tumor in dKO mice argues for a physiological relevance of the Cbl-b/SMAD7 interaction in the TGFβR signal transduction pathway. However, further studies are required to elucidate the potential crosstalk between TCR/CD28 and TGFβR signaling in Cbl-b-mediated tumor rejection.

Collectively, our data suggest that in T cells, Cbl-b associates with SMAD7 and promotes its ubiquitination. SMAD7 is subsequently degraded, allowing efficient canonical TGFβR/SMAD signaling. In non-hematopoietic cell lines, the RING-type E3 ligase Arkadia has been shown to mediate the polyubiquitination and degradation of SMAD7 (Koinuma et al., 2003), but this capacity has not been validated specifically in primary T cells. Therefore, our study is the first to provide evidence for Cbl-b-dependent posttranslational regulation of SMAD7 in primary T lymphocytes and proposes a molecular explanation for the resistance to TGFβ observed in ccblb-deficient T cells. As the level of SMAD7 in T cells determines T₅₁₀ polarization (Kleiter et al., 2010), our model also provides an explanation for the strong bias of ccblb⁻/-/- CD4⁺ T cells toward T₅₁₀ differentiation despite T₅₁₀-skewing conditions.

Material and methods

**Mice and reagents**

Cbl-b and CD4Cre-Smad7fl/fl knockout mice were described previously (Bachmaier et al., 2000; Kleiter et al., 2010). The dKO animals were generated by mating the respective single-knockout mice. Mice were maintained under specific pathogen-free (SPF) conditions. Experiments were performed using 6- to 12-week-old mice and complied with the current laws of Austria.

RPMI medium, DMEM medium, and fetal calf serum were from Biochrom, IMDM medium and MG132 were from Sigma, and X-vivo 20 was from Lonza. Anti-CD3 was in-house made and anti-CD28 was from BD Bioscience. [γ-³²P] adenosine 5’-triphosphate (ATP) was purchased from Amersham. TGFβ, IL-6, IL-23, αIL-4, and αIFNγ were from eBioscience. Recombinant human Cbl-b was from Abnova. Metafectene was from Biontex.

**T cell stimulation and skewing**

Cells were cultured in RPMI or IMDM (for T₅₁₀ polarizations), each supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. T cells were stimulated at a density of 5 × 10⁶ cells/ml with TGFβ alone, or TGFβ with plate-bound anti-CD3 (2–5 μg/ml; ZC11) and soluble anti-CD28 (1 μg/ml), or TGFβ with soluble anti-CD3 (2 μg/ml) and soluble anti-CD28 (1 μg/ml) cross-linked with anti-Hamster IgG1 (2 μg/ml) as indicated. Polarization of T cells into T₅₁₀ cells was performed in the presence of polarizing cytokines TGFβ (5 ng/ml), IL-6 (40 ng/ml), IL-23 (10 ng/ml), αIL-4 (2 μg/ml), and αIFNγ (2 μg/ml). For T₅₁₀ polarization, the following conditions were used: IL-12 (10 ng/ml) and αIL-4 (5 μg/ml). For T₅₁₀ polarization: IL-4 (10 ng/ml), αIL-12 (5 μg/ml), and αIFNγ (5 μg/ml). For iTreg polarization: TGFβ (10 ng/ml), IL-2 (10 ng/ml), αIL-4 (5 ng/ml), αIFNγ (5 μg/ml), αIL-10 (5 μg/ml), and αIL-12 (5 μg/ml). Supernatants were collected on Day 3 of differentiation and 24 h after restimulation with anti-CD3 plus anti-CD28, and analyzed via Bio-Plex multianalyte technology (BioRad).

**Chromatin immunoprecipitation**

The ChIP assay was performed with a ChIP assay kit according to the recommendations of the manufacturer (Imgenex) in combination with the Cold Spring Harbor protocol (Carey et al., 2009). Briefly, cells were fixed in 1% formaldehyde for 37°C for 10 min and the cross-linking was quenched by the addition of 1.375 M glycine. The cells were then washed twice with ice-cold PBS and lysed in cold cell lysis buffer for ChIP (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP-40) for 10 min. The cell pellets were lysed following centrifugation in 1 ml nuclei lysis buffer for ChIP (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% SDS) supplemented with protease inhibitors and incubated for 10 min on ice. Following sonication with 25 pulses (30 sec each) using a Bioruptor Next Generation (Diagenode), the samples were centrifuged for 10 min at 12000 rpm. The sheared chromatin was used to set up immunoprecipitation reactions with 5 μg of the indicated Abs (IgG Santa Cruz sc-2027; SMAD3 abcam ab28379; SMAD2 Cell Signaling #3003) at 4°C overnight. Magna ChIP protein G magnetic beads were added for 2 h and the samples were sequentially washed once with the buffers provided by the supplier (IMGENEX; high to low salt). The DNA–protein complex was eluted by heating at 65°C overnight,
and the DNA was eluted using the IPure kit (Diagenode). Real-time PCR was performed with the following primers and probes using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). IL2 promoter D (−5184 to −5293 bp): 5′-TGGCATTAGTGGGAAGCCCTTGA-3′ and 5′-ACAGGAGTGAAGCTGTGGA-3′ using the probe 5′-FAM-C TTGAGGATGGGTGCTCCTC-TAMRA-3′; IL2 promoter C (−1624 to −1450 bp): 5′-CAGTTGCATGTAGCTCAA-3′ and 5′-CACCCAC ACCTACCCCATTT-3′ using the probe 5′-FAM-CAGGGATCAGAGAGC AAGGT-TAMRA-3′.

Confocal immunofluorescence

Naive human T cells were incubated with TGFβ (5 ng/ml) for 1 h at 37 °C and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were washed with PBS and permeabilized for SMAD7 and Cbl-b (Santa Cruz Biotechnology) immunocyto-staining. Fluorescently labeled chicken anti-goat and chicken anti-rabbit secondary antibodies ( Molecular Probes) were used. Images were acquired with a FV1000 laser scanning confocal microscope (Olympus). The digital images were processed with NIH Image version 1.37a with the Intensity Correlation Analysis plug-in (Li et al., 2004) to evaluate the colocalization of SMAD7 and Cbl-b. The protein distribution is expressed as ‘mean gray value’ which is calculated as the total fluorescent intensity of the region of interest (ROI) divided by the ROI area. The maximum gray value is 255, at full saturation, and 0 for the black background. 25 to 40 cells were analyzed per time point. Pearson’s coefficients were compared by ANOVA and Bonferroni’s post hoc tests using the GraphPad Prism 4 software.

In vitro ubiquitination assay

For in vitro ubiquitination assays, SMAD7 was fused to GST using pGEX5x-1. Expression of the GST-fusion proteins in Escherichia coli BL21 was induced with 0.3 mM isopropyl-b-D-thiogalactopyranoside at 20 °C overnight. The cells were lysed, and the lysates were incubated overnight with Glutathione Sepharose 4B (GE Healthcare) at 4 °C. The sepharose beads were then washed five times with lysis buffer, and proteins bound to the beads were eluted with elution buffer (50 mM Tris–HCl, pH 8.0, and 20 mM reduced glutathione).

For SMAD7 ubiquitination, the Ubiquitination Kit (Enzo Life Sciences) was used. Specifically, the reaction mixture (50 μl) containing ubiquitination buffer, 1 mM DTT, 20 µl/mL inorganic pyrophosphatase, 5 mM Mg-ATP, 2.5 μM biotinylated ubiquitin, 100 nM E1, 2.5 μM E2 (UbHc10), 100 mM Cbl-b, and 1 μM GST-SMAD7 protein was incubated at 37°C for 1 h. The reaction was quenched by adding 50 μl of 2× non-reducing gel loading buffer. Finally, the samples were immunoblotted with streptavidin-HRP for detecting ubiquitinated SMAD7 and Cbl-b, and SMAD7 served as a loading control.

In vivo TC-1 tumor cell growth

1 × 10⁵ TC-1 cells were injected subcutaneously into the left flank of 8- to 12-week-old female mice. Tumor growth was monitored three times per week by measuring tumor length and width. Tumor volume was calculated according to the following equation: (length × width²) × (π/6). For survival analysis, mice with tumors greater than the length limit of 15 mm were sacrificed and counted as dead.

Statistical analyses

Results are expressed as mean ± standard error of the mean (SEM). Groups were compared using the paired Student’s t-test. Overall survival was expressed using the Kaplan–Meier method, and differences between groups were determined using the log-rank test. Data analysis was performed using SPSS. Significant differences are indicated as *P < 0.05, **P < 0.01, and ***P < 0.001.

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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Conflict of interest: none declared.

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