

## Overproduction of IL-2 by Cbl-b deficient CD4<sup>+</sup> T cells provides resistance against regulatory T cells

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### ABSTRACT

Regulatory T cells are integral to the regulation of autoimmune and anti-tumor immune responses. However, several studies have suggested that changes in T cell signaling networks can result in T cells that are resistant to the suppressive effects of regulatory T cells. Here, we investigated the role of Cbl-b, an E3 ubiquitin ligase, in establishing resistance to Treg-mediated suppression. We found that the absence of Cbl-b, a negative regulator of multiple TCR signaling pathways, rendered T cells impartial to Treg suppression by regulating cytokine networks leading to improved anti-tumor immunity despite the presence of Treg cells in the tumor. Specifically, Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells hyper-produced IL-2 and together with IL-2 R $\alpha$  upregulation served as an essential mechanism to escape suppression by Treg cells. Furthermore, we report that IL-2 serves as the central molecule required for cytokine-induced Treg resistance. Collectively our data emphasize the role of IL-2 as a key mechanism that renders CD4<sup>+</sup> T cells resistant to the inhibitory effects of Treg cells.

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### Introduction

Regulatory T cells (Tregs) play a pivotal role in the pathogenesis of human diseases.<sup>1,2</sup> Loss of Treg function promotes a breakdown in immune tolerance, and facilitates early onset multi-organ autoimmunity in humans with IPEX syndrome and in scurfy mice.<sup>3-7</sup> In contrast, Treg cells also constrain the beneficial anti-tumor immune responses thereby serving as a negative prognostic indicator.<sup>8-13</sup> Tregs exploit diverse contact-dependent and/or cytokine-mediated mechanisms, including TGF- $\beta$  secretion, IL-2 consumption, and downregulation of co-stimulatory molecules on antigen presenting cells, to restrict T cell function in a context-dependent manner.<sup>1,2,14,15</sup> Thus, numerous strategies have been proposed to reverse the suppressive effects of Treg cells in the context of tumor immunity.<sup>15</sup> One particular approach involves rendering T cells refractory to the suppressive effects of Treg cells.

Studies have demonstrated that there are a variety of molecular pathways and cellular mechanisms which render T cells resistant to Treg suppression.<sup>16-25</sup> First, TLR4 and TLR9-mediated stimulation of dendritic cells and the subsequent increase in IL-6 production by DCs have been shown to render T cells resistant to the suppressive effects of CD4<sup>+</sup>CD25<sup>+</sup> Tregs *in vitro*.<sup>16</sup> This is consistent with other studies demonstrating that IL-6 inhibited the suppressive ability of Treg cells in lupus-prone and graft-versus-host disease (GVHD) murine models, through mechanisms that have not been elucidated.<sup>23,25</sup> Furthermore, cognate ligand-induced stimulation of glucocorticoid-induced TNFR-related protein (GITR) on activated CD4<sup>+</sup> T cells and subsequent production

of IL-2 by the CD4<sup>+</sup> T cells have been shown to potentially mediate Treg resistance.<sup>17,18</sup> Similarly, several other T cell stimulating cytokines such as IL-1 $\beta$ , IL-4, IL-7, IL-15, and IL-21, have also been shown to render T cells resistant to Treg-mediated suppression.<sup>19-22,24</sup> Although the specific mechanism utilized by these cytokines to render T cells resistant to Treg cells has not been defined, evidence suggests that the phosphatidylinositol 3-kinase (PI3 K)/Akt signaling pathway may play a role.<sup>26,27</sup>

Beyond the role of cytokines in T cell resistance to Treg cells, intracellular molecules such as Casitas B- Lineage Lymphoma-b (Cbl-b), p105 (NF $\kappa$ B precursor protein), miR-155, Akt1, MyD88, TRAF6 and SHP-1 have also been reported to render effector T cells resistant or sensitive to Treg-mediated suppression.<sup>28-35</sup> In particular, studies involving Cbl-b deficient T cells have been one of the first to demonstrate the role of intracellular molecules in rendering T cells resistant to the suppressive effects of Treg cells *in vitro* and *in vivo*.<sup>28,30,33</sup>

Cbl-b is an E3 ubiquitin ligase and directly ubiquitinates PKC $\theta$ , PLC $\gamma$ 1, Vav1, Nedd4 along with several other TCR signaling molecules.<sup>36,37</sup> Thus, Cbl-b serves as a powerful negative regulator of T cell activation.<sup>38</sup> Studies have shown that Cbl-b KO T cells utilize multiple molecular mechanisms which may render T cells resistant to the suppressive effects of Treg cells. Cbl-b directly ubiquitinates SMAD7, a negative regulator of TGF- $\beta$  signaling pathway,<sup>39,40</sup> which has also been implicated in generating CD4<sup>+</sup> T cells that are refractory to Treg-mediated suppression.<sup>41</sup> This is consistent with findings demonstrating that the lack of Cbl-b

results in a robust T cell activation even in the presence of TGF- $\beta$ .<sup>30,40</sup> Furthermore, regulatory T cells utilize several other context-dependent suppressive mechanisms, one of which includes downregulation of CD80/86 expressed on antigen presenting cells.<sup>42,43</sup> Cbl-b KO T cells can be stimulated in a CD28-independent manner and demonstrate impaired induction of T cell anergy,<sup>44,45</sup> therefore Cbl-b KO T cells potentially bypass the requirement for CD80/86-induced co-stimulation. In summary, Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells display context-dependent insensitivity to TGF- $\beta$  signaling, and CD28 independent stimulation, all of which contributes to a robust T cell activation.<sup>30,33,38,44</sup> However, the precise mechanism which renders Cbl-b KO T cells resistant to Treg cells is still under debate.

Despite the emergence of multiple studies characterizing the role of immunoregulatory molecules and cytokines in mediating T cell resistance against Tregs, no consensus was achieved in identifying the specific biological mechanism. This study investigated the role of defined cytokines in rendering Cbl-b deficient T cells resistant to suppression by regulatory T cells.

## Materials and methods

### Mice

C57BL/6 Wild-type (WT), CD25 KO and CD45.1 mice were purchased from The Jackson Laboratory. Cbl-b KO mice in C57BL/6 background were a kind gift from Dr. J.M. Penninger, and DEREK mice in C57BL/6 background were a kind gift from Dr. T. Sparwasser. All mice were maintained and bred under the guidelines and policies set by UHN Animal Resource Center. Mice used for experiments were between 2 to 4 months old.

### In vivo tumor studies

E.G7-Ova tumor cells, previously cultured in complete RPMI 1640 media (10% FCS, 50  $\mu$ M 2-mercaptoethanol, 2 mM glutamine, and 0.1% pen/strep.), were subcutaneously injected into the shaved left flanks of Cbl-b deficient and sufficient mice on day 10 post inoculation. Tumor growth was tracked every 3 days using a caliper. Mice bearing tumors over 225 mm<sup>2</sup> were euthanized and recorded as deceased. For *in vivo* depletion of Treg cells in the tumor study, FoxP3<sup>+</sup> cells were transiently ablated in DEREK mice by administering 10ng per g body weight diphtheria toxin (Merck) intraperitoneally on day 10, 11, 19, and 20 post tumor inoculation.

### Cell isolation

CD4<sup>+</sup> T cells were negatively selected from spleens and lymph nodes of mice using the magnetic purification kit (CAT# 130-104-454 & 130-104-075, Miltenyi Biotec). BD Fluorescence-activated cell sorting (FACS) Aria was used to further separate un-stimulated naive T cells (CD4<sup>+</sup>CD25<sup>-</sup>) and Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>), using FoxP3 expression to confirm the purity of the populations. For purification of APCs, CD5 (Ly-1)

MicroBeads were used to deplete CD5<sup>+</sup> T and B cells (CAT# 130-049-301, Miltenyi Biotec).

### In vitro T cell stimulation

All *in vitro* T cell stimulation was performed using anti-CD3 and irradiated APCs. Prior to co-culture, APCs were irradiated with a dose of 2500cGy using X-RAD 320 (PXI Precision X-Ray). Unless noted otherwise, purified CD4<sup>+</sup>CD25<sup>-</sup> T cells were stained with 10  $\mu$ M cell proliferation dye eFluor<sup>®</sup> 450 (CAT# 65-0842-90, eBioscience) in PBS for 20 min. at 4°C. After three washes, 5  $\times$  10<sup>4</sup> T cells were co-cultured with 2  $\times$  10<sup>5</sup> irradiated APCs and 1  $\mu$ g/ml anti-CD3 Ab (Clone 145-2 C11, CAT# 14-0031-85, eBioscience) in complete RPMI 1640 media (CAT# 11875119, Invitrogen) containing 10% FCS, 1% L-glutamine, 1% Penicillin-Streptomycin, and 0.0004% 2-mercaptoethanol. Cells were incubated in Thermo Scientific<sup>™</sup> Nunc<sup>™</sup> MicroWell<sup>™</sup> 96-well polystyrene microplates (CAT# 12-565-66, Fisher Scientific) in 5% CO<sub>2</sub> and 37°C incubation. Supernatants were harvested on day 1 or 3 post-stimulation for cytokine analysis and flow analysis was also performed either on day 1 or 3 post-stimulation.

### Treg suppression assay

Upon FACS sorting of naive CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD25<sup>-</sup>) and Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>), purified CD4<sup>+</sup>CD25<sup>-</sup> T cells were stained with 10  $\mu$ M cell proliferation dye eFluor<sup>®</sup> 450 (CAT# 65-0842-90, eBioscience) in PBS for 20 min, followed by three washes in complete RPMI 1640 media (CAT# 11875119, Invitrogen). 5  $\times$  10<sup>4</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells were subsequently cultured with 2  $\times$  10<sup>5</sup> irradiated APCs, anti-CD3 Ab (1  $\mu$ g/ml), and 5  $\times$  10<sup>4</sup> CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. For Treg suppression assays involving 1:1 to 8:1 Teff: Treg ratios, the quantity of Treg cells was adjusted from 5  $\times$  10<sup>4</sup> to 6.25  $\times$  10<sup>3</sup> cells, respectively. For exogenous supplementation of IL-2 signaling inhibitors/agonist, recombinant IL-2 (CAT# 575404, BioLegend), recombinant IL-15 (CAT# 34-8153-82, eBioscience), anti-IL-2 (S4B6, CAT# 16-7020-85, eBioscience) and anti-CD25 (PC61) were used. For cytokine screening, all cytokines (IFN- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-12, IL-17A, IL-18, IL-23 and TNF- $\alpha$ ) were purchased from BioLegend except for IFN- $\gamma$  purchased from eBioscience. Cells were incubated in Thermo Scientific<sup>™</sup> Nunc<sup>™</sup> MicroWell<sup>™</sup> 96-well polystyrene microplates (CAT# 12-565-66, Fisher Scientific) in 5% CO<sub>2</sub> and 37°C incubation. Unless noted otherwise, supernatants were harvested on day 1 or 3 post stimulation for cytokine analysis and flow analysis was performed either on day 1 or 3 post-stimulation.

### Cytokine analysis

Co-culture supernatants, stored in -80°C, were analyzed using IL-2 and IFN- $\gamma$  ELISA kits (CAT# 88-7024-88, 88-7314-86, eBioscience). LEGENDplex<sup>™</sup> Mouse Th Cytokine Panel cytometric bead array (CAT# 740005, BioLegend) was used for T cell cytokine secretion profiling.

## Surface/Intracellular staining & flow cytometry

Individual cell suspensions were washed twice in FACS buffer (PBS supplemented with 2% FCS and 0.05% sodium azide), followed by FcR blocking (30 min.) using anti-CD16/32 (CAT# 14-0161-85, eBioscience). For surface marker analyses, cells were subsequently stained with Abs for 30 min. on ice followed by two washes. The following antibodies were used for experiments: anti-CD25-PE (PC61), anti-CD122-PE (5H4), anti-CD4-APC (GK1.5), anti-GITR-APC (DTA-1) or anti-MHCII-APC-Cy7/FITC (M5/114.15.2), all purchased from eBioscience; and anti-MHCII-AmCyan (M5/114.15.2) was purchased from BioLegend. Anti-CD132-PE (TUGm2) was purchased from BD Biosciences. For all surface marker staining, cells were fixed using 4% paraformaldehyde after washes.

Intracellular transcription factor staining was performed using FoxP3 Transcription Factor Staining Buffer Set (CAT# 00-5523-00, eBioscience) with the following antibodies: anti-CTLA-4-PE (UC10-489), or anti-FoxP3-PE-Cy7 (FKJ-16 s), from eBioscience. All data were acquired using BD FACSCanto™ II Flow Cytometer (BD Biosciences), and were analyzed on FlowJo software 7.6.1 (FlowJo LLC).

## Statistical analysis

Two-tailed paired Student's t-test or 1-way ANOVA with Tukey's Post-Hoc test was performed for comparisons. All data are presented as mean with standard error (n = 3) using GraphPad Prism 5 (GraphPad Software Inc.). All experiments are representative of 2 or more biological replicates.

## Results

### ***Cbl-b* deficiency enhances anti-tumor immunity despite the presence of regulatory T cells**

Previous studies have demonstrated that *Cbl-b* deficient mice have improved anti-tumor immunity using transplantable tumor models.<sup>46,47</sup> In order to evaluate whether resistance to immune regulation may play a role in the improved response in the *Cbl-b* KO mice, we first compared the ability of *Cbl-b* sufficient and deficient mice in controlling tumor growth using the E.G7 thymoma model. Consistent with a study conducted by Chiang et al. (2007), *Cbl-b* deficient mice were capable of spontaneous tumor rejection as demonstrated through controlled tumor size (Figure 1a) and increased overall survival (Figure 1b). *Cbl-b* expression is generally found in the lymphocytic lineages, and T cells have been shown to play a particularly important role in this tumor model. To examine the role of Tregs in this model, we evaluated the proportion and markers expressed by this population of cells. Regulatory T cells were present in higher frequency in comparison to other lymphoid organs with a mean of 21% of CD3<sup>+</sup> cells expressing FoxP3 (Figure 1c). Furthermore, Treg cells from the tumor expressed highest percentage of CD25<sup>+</sup> compared to Tregs in the peripheral blood, spleen, draining and non-draining LN suggestive of enhanced Treg activation (Figure 1d). To evaluate whether Treg cells play a functional role in regulating

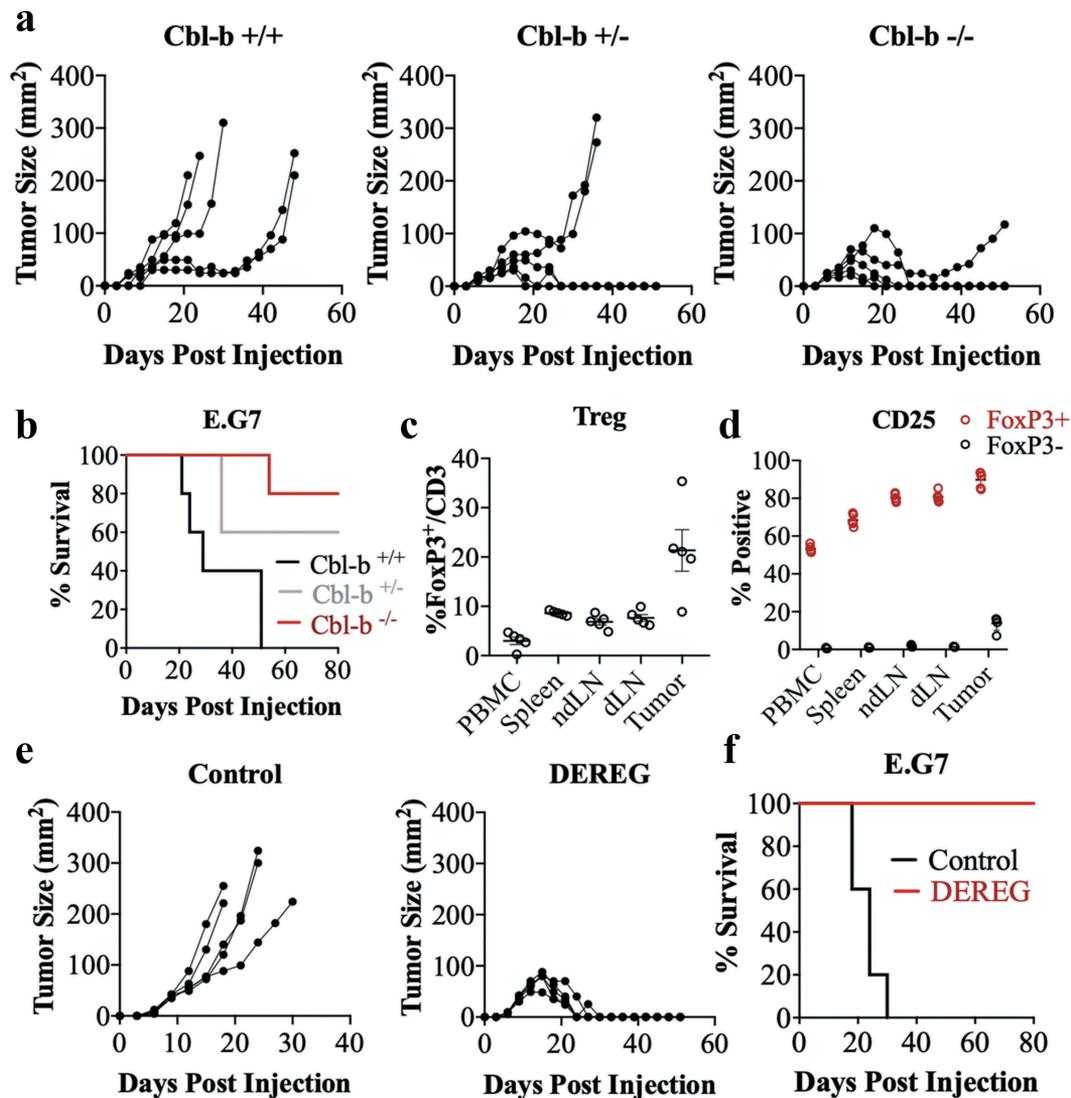
anti-tumor immunity against the E.G7 tumor, the transgenic DEREK mouse model was used which expresses the diphtheria toxin receptor under the control of FoxP3 promoter. Ablation of Treg cells by treatment with diphtheria toxin resulted in impaired tumor growth (Figure 1e), and 100% survival in mice (figure 1f). In summary, we demonstrate that Tregs play an important role in the E.G7 tumor model, and that the absence of *Cbl-b* can lead to the control of tumor growth.

### ***CD4*<sup>+</sup> T cells that are deficient in *Cbl-b* are resistant to inhibition by regulatory T cells**

Numerous studies have suggested that the absence of *Cbl-b* in T cells results in resistance to the inhibitory effects of regulatory T cells.<sup>28,30,47</sup> Due to the limitations of models available to precisely evaluate the impact of Treg cells on CD4<sup>+</sup> effector T cells, we examined the importance of *Cbl-b* in Treg-resistance through *in vitro* Treg suppression assays. The proliferative capacity and the ability of T cells to be suppressed by Tregs were quantified in *Cbl-b* KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells. Consistent with previous literature, *Cbl-b* KO CD4<sup>+</sup> effector T cells rapidly proliferated despite the presence of Treg cells (Figure 2a).<sup>28,30</sup> In contrast, the absence of *Cbl-b* in Treg cells did not impact the ability of Treg cells to suppress T cell proliferation (Figure 2a). As T cell proliferation may not necessarily indicate Treg resistance, we calculated a % suppression score. Division index (D.I) was quantified by averaging the number of cell divisions using FlowJo software, and % suppression was calculated by normalizing T cell proliferation in the presence of Tregs to intrinsic proliferative capacity of the T cells (% suppression = 100 - (D.I. with Tregs/D. I. without Tregs) x 100).<sup>48</sup> While *Cbl-b* KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells intrinsically possessed enhanced proliferative capacity, as quantified by an approximate 2-fold increase in its division index (Figure 2b), *Cbl-b* KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells still demonstrated significantly lower % suppression in any given ratio of T<sub>eff</sub> to Treg cells in comparison to C57BL/6 CD4<sup>+</sup>FoxP3<sup>-</sup> T cells (Figure 2c). Thus, CD4<sup>+</sup>FoxP3<sup>-</sup> T cells from *Cbl-b* KO mice were refractory to the inhibitory effects of Treg cells.

### ***Potential role for cytokines in mediating T cell resistance to regulatory cells***

Despite the inability of Tregs to suppress proliferation of *Cbl-b* KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells, the precise mechanism remains to be elucidated. Previous studies have highlighted the ability of *Cbl-b* deficient T cells to secrete higher levels of cytokines including IFN- $\gamma$  and IL-2.<sup>33,44,46,47,49</sup> Therefore, we extensively characterized soluble factors secreted by *Cbl-b* KO CD4<sup>+</sup> T cells in the context of Treg resistance. Cytokine production by *Cbl-b* KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells was characterized by cytometric bead array (CBA) in cultures that did not include Treg cells and was compared with C57BL/6 control T cells. The hyperactive *Cbl-b* KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells secreted increased levels of T<sub>H</sub>1 and T<sub>H</sub>2 cytokines including IFN- $\gamma$ , IL-2, IL-4 and TNF- $\alpha$ , as well as IL-10, IL-13 and IL-17A (Figure 2d). To examine which cytokines were elevated in the context of Treg suppression, we performed Treg suppression assays and analyzed the supernatant by CBA. *Cbl-b* KO



**Figure 1.** Cbl-b deficiency enhances anti-tumor immunity against E.G7 tumor.

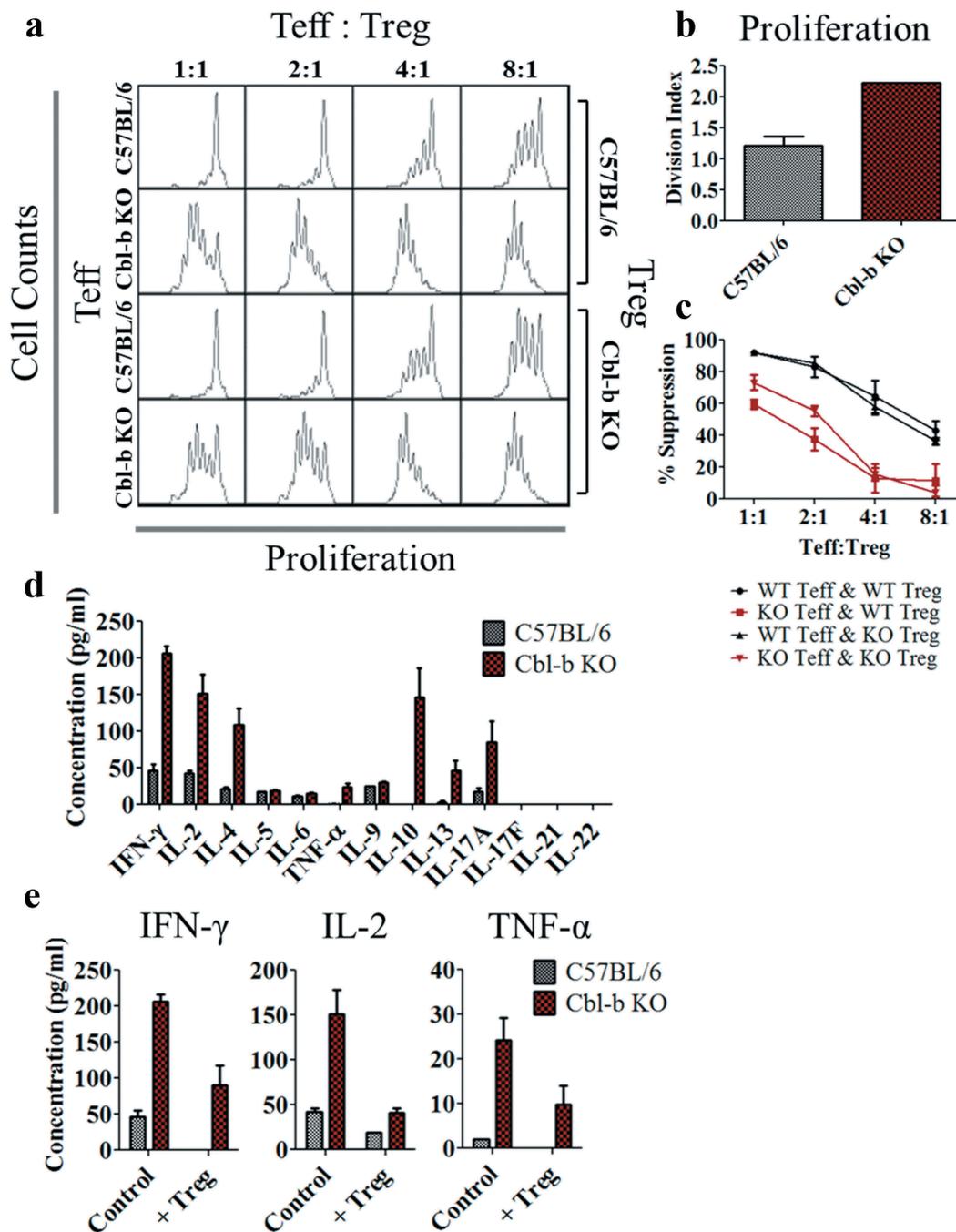
(a) Growth rates of inoculated E.G7 tumors and (b) survival of tumor-bearing Cbl-b deficient mice.  $1.0 \times 10^6$  E.G7-Ova tumor cells were subcutaneously injected into the left flanks of Cbl-b<sup>+/+</sup>, Cbl-b<sup>+/-</sup> and Cbl-b<sup>-/-</sup> mice (n = 5). Each curve represents one mouse, and tumor sizes were tracked every 3 days. (c,d). Evaluating the presence and activation state of Treg cells in E.G7 tumor. Tumor, non-draining lymph node (ndLN), draining lymph node (dLN), spleen and PBMC were isolated from tumor-bearing mice 17 days post-inoculation. Cells were stained with antibodies specific for CD4, FoxP3 and CD25 and analyzed by flow cytometry to acquire proportion of FoxP3<sup>+</sup> cells in T cell compartment (c) and expression of CD25 on Treg cells (d). (e,f). Growth rates of inoculated E.G7 tumor and survival of tumor-bearing DEREK mice. Tumor cells were inoculated into control and DEREK mice, diphtheria toxin was intraperitoneally administered on day 10 post inoculation, and tumor sizes were tracked every 3 days (n = 5) (e). Percentage of surviving mice is represented through Kaplan-Meier curve (f).

CD4<sup>+</sup> effector T cells cultured with C57BL/6 Treg cells secreted similar or higher levels of IFN- $\gamma$ , IL-2 and TNF- $\alpha$  compared to C57BL/6 CD4<sup>+</sup> effector T cells stimulated without Treg cells (Figure 2e), indicating the availability of these cytokines during Treg-mediated suppression. Therefore, Cbl-b KO CD4<sup>+</sup> T cells secrete a diverse array of cytokines including IFN- $\gamma$ , IL-2, and TNF- $\alpha$  that may potentially play a role in mediating Treg resistance.

#### **IL-2 hyper-secretion is correlated to CD25 overexpression in Cbl-b KO CD4<sup>+</sup> T cells**

One way that regulatory T cells have been reported to suppress effector T cells is via IL-2 consumption, leading to

cytokine deprivation-mediated cell death or blockade of differentiation.<sup>50-52</sup> We speculated that elevated secretion of IL-2 by Cbl-b KO CD4<sup>+</sup> T cells may be responsible for sustaining T cell proliferation and that upregulation of the IL-2 receptor by Cbl-b deficient effector cells may enhance the ability to outcompete Treg cells for IL-2, thereby providing resistance to suppression. Therefore, we further characterized the effect of Cbl-b on IL-2 secretion by performing an ELISA on the supernatant collected from Treg suppression assays. In addition, we evaluated IL-2Ra expression on C57BL/6 or Cbl-b KO CD4<sup>+</sup>CD25<sup>-</sup> T cells in the absence of Treg cells on day 1 and 3 post-stimulation. In agreement with previous findings, Cbl-b KO CD4<sup>+</sup> T cells hyper-secreted IL-2 in the absence (Figure 3a) or presence of Treg cells (Figure 3b), and



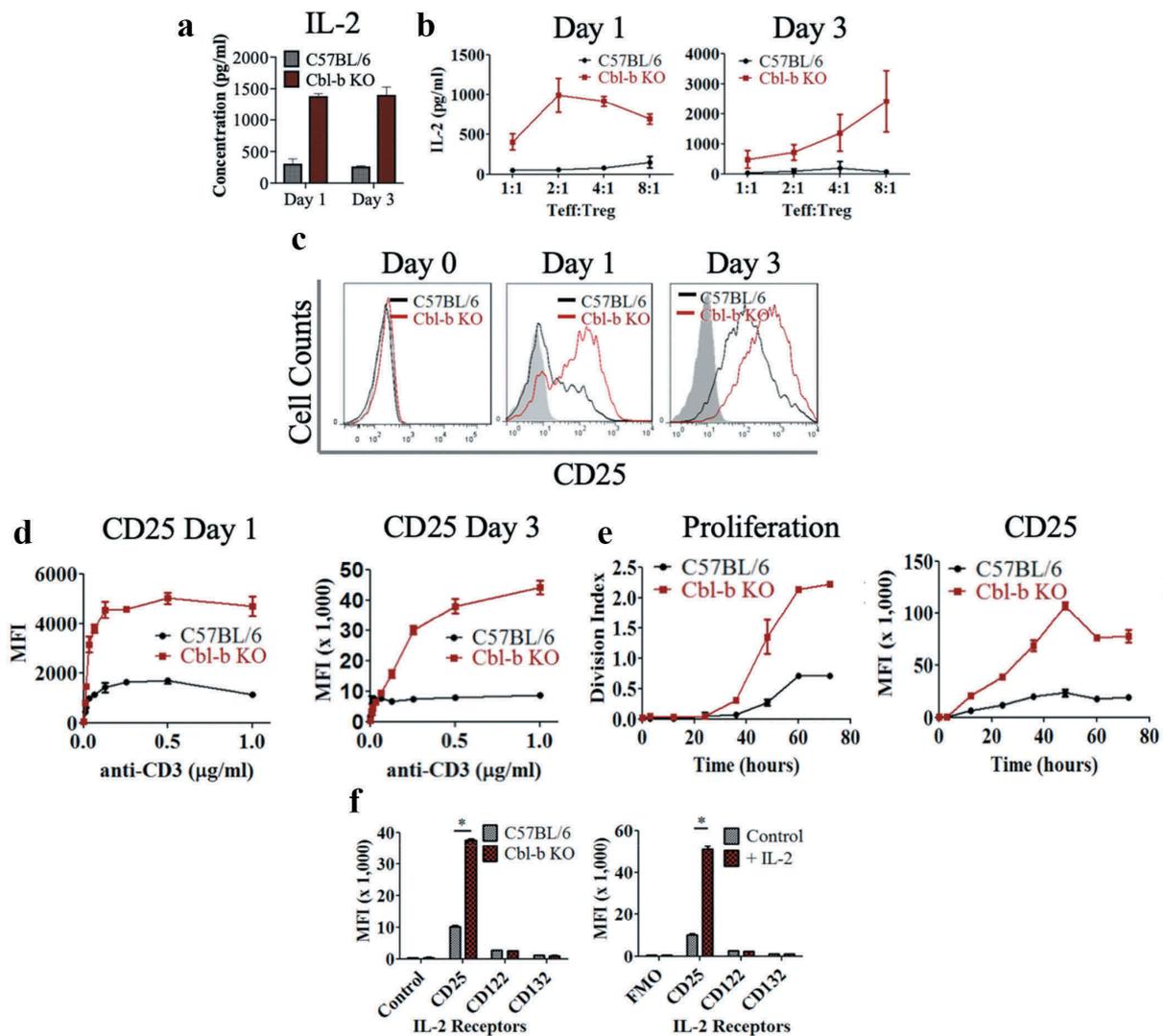
**Figure 2.** Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells display resistance to Treg-mediated suppression and enhanced cytokine secretion.

(a). Proliferation of C57BL/6 (WT) and Cbl-b KO CD4<sup>+</sup> effector T cells in the presence of WT or Cbl-b KO Treg cells, analyzed on day 3 post-stimulation. (b). Quantification of WT and Cbl-b KO CD4<sup>+</sup> T cell proliferation without Tregs using division index. (c). The % suppression of WT and Cbl-b KO Teff was determined at different Teff to Treg ratios, with Treg cells from WT or Cbl-b KO mice. The baseline proliferation of CD4<sup>+</sup> T cells without Tregs from (b) was used to normalize for the % suppression curve. (d). Cytokine secretion profile of WT or Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells. Supernatants from stimulated WT or Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells were collected on day 3 post-stimulation for cytokine measurements. (e). Cytokine secretion profile with or without Treg cells. IFN- $\gamma$ , IL-2 and TNF- $\alpha$  secretion levels by Teff in response to Treg cells were measured on day 3 post-stimulation.

displayed strikingly enhanced CD25 expression even at day 1 post-stimulation further suggesting that the T cells may have increased IL-2 uptake (Figure 3c).

Previous reports suggested that TCR signaling is enhanced in the absence of Cbl-b, and that T cell activation is independent of CD28 signaling.<sup>38,44,45,53</sup> This augmented TCR signaling may

explain the higher expression of CD25 on T cells. Therefore, we examined whether the upregulation of CD25 expression was relative to the strength of the TCR signal. Overall, a substantial increase in CD25 expression is observed by increasing TCR signaling using graded anti-CD3 concentrations or increased duration of stimulation (Figure 3d,e). However, Cbl-b KO



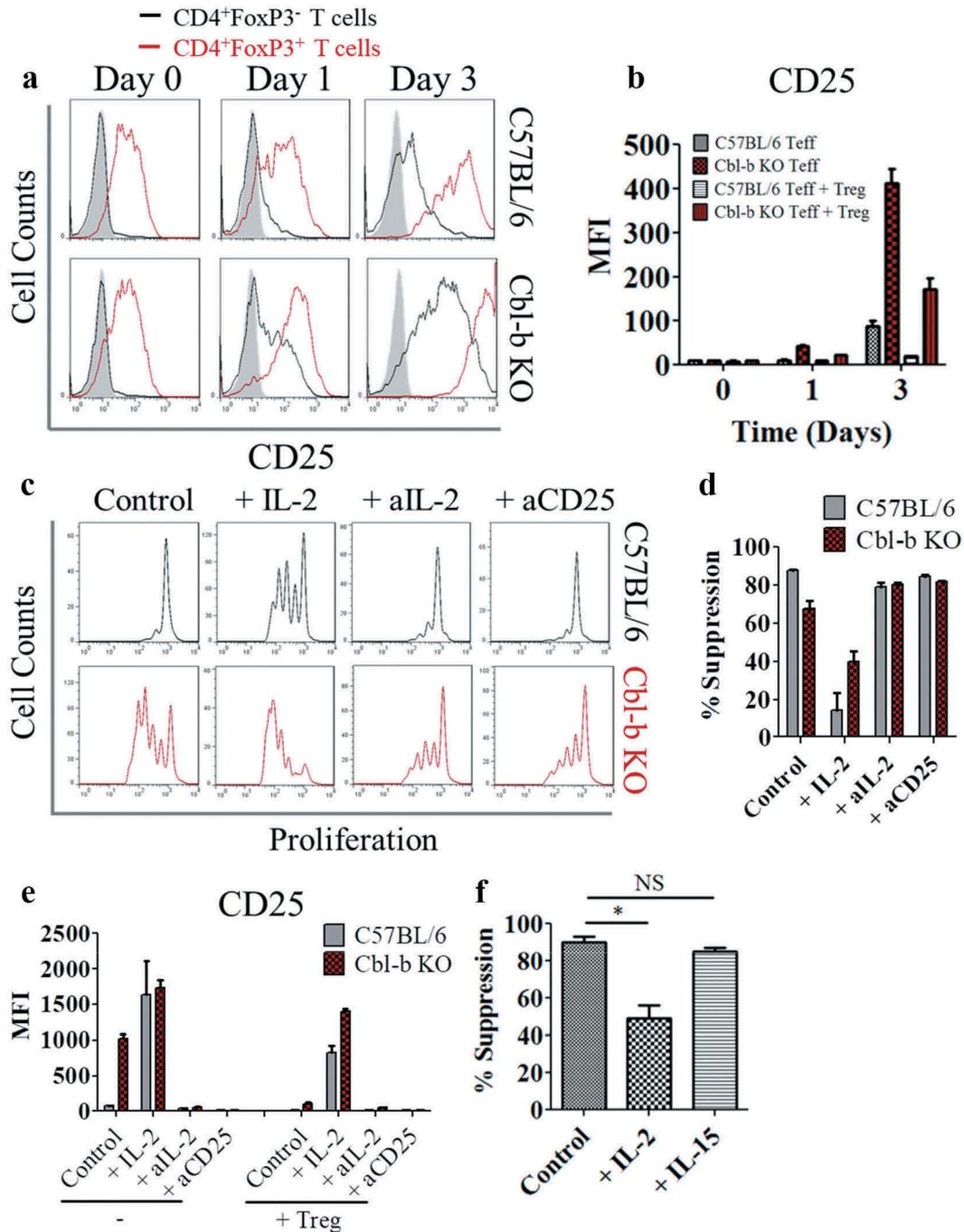
**Figure 3.** Cbl-b deficient  $CD4^+$  T cells upregulate CD25 levels and IL-2 production.

IL-2 secretion by Cbl-b KO  $CD4^+FoxP3^-$  T cells was measured without (a) or with Treg cells (b). Supernatants were collected from a Treg suppression assay on day 1 and 3 post-stimulation, and ELISA was performed to quantitate IL-2 levels. (c). CD25 expression on stimulated WT and Cbl-b KO  $CD4^+FoxP3^-$  T cells.  $CD4^+CD25^-$  T cells were stimulated with anti-CD3 and irradiated APCs, and CD25 expression was evaluated on day 0, 1 and 3 post-stimulation by flow cytometry. (d). CD25 expression on WT and Cbl-b KO  $CD4^+FoxP3^-$  T cells in response to different concentrations of anti-CD3. (e). Following  $CD4^+$  Teff cell stimulation, proliferation and CD25 expression was measured using flow cytometry every 12 hours for 3 days. (f). CD25, CD122 and CD132 expression on WT or Cbl-b KO  $CD4^+$  effector T cells, or IL-2 supplemented (20 ng/ml)  $CD4^+FoxP3^-$  T cells were measured on day 3 post-stimulation.

$CD4^+$  T cells required less TCR signal to induce a high level of CD25 expression, suggesting a link with TCR sensitivity and upregulation of CD25 (Figure 3d,e). Lastly, when stimulated without Treg cells, Cbl-b deficiency or exogenous IL-2 supplementation specifically enhanced CD25 expression while CD122 and CD132 surface expression were kept at basal expression (figure 3f). This observation can be explained by previous studies demonstrating that high-affinity IL-2 receptors (consisting of CD25, CD122 and CD132) are internalized upon IL-2 ligation, whereas CD122 and CD132 are degraded while CD25 is recycled back to the plasma membrane.<sup>54–56</sup> In summary, hyper-secretion of IL-2 is correlated with the upregulation of IL-2 receptors on Cbl-b KO  $CD4^+$  T cells and may play an important role in rendering T cells refractory to Treg suppression.

### **IL-2 signaling is critical for the ability of Cbl-b KO $CD4^+FoxP3^-$ T cells to counteract the suppressive effects of Treg cells**

To further explore the possibility that Cbl-b KO  $CD4^+$  T cells overcome Treg suppression through increased IL-2 production and CD25 expression, we directly examined CD25 expression on T cells and Treg cells at different time-points throughout the suppression assay. First, the levels of CD25 expression on  $CD4^+FoxP3^+$  and  $CD4^+FoxP3^-$  T cells were compared on day 0, 1 and 3 post-stimulation. Even in the presence of Treg cells, Cbl-b KO  $CD4^+$  T cells expressed a markedly higher level of CD25 on day 1 and 3 post-stimulation, but were still lower in comparison to Treg cells



**Figure 4.** IL-2 signaling in Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells mediate Treg cell resistance.

(a). CD25 expression comparison between Teff and Treg cells. CD25 expression on WT Tregs (CD4<sup>+</sup>FoxP3<sup>+</sup>) and WT or Cbl-b KO Teff (CD4<sup>+</sup>FoxP3<sup>-</sup>) was measured on days 0, 1 and 3 post-stimulation. (b). CD25 expression comparison between Teff controls and suppressed CD4<sup>+</sup> T cells. (c,d). Treg suppression assay using exogenous IL-2 or IL-2 blockers. (c) Treg and Teff co-cultures (at 1:1 ratio) were supplemented with IL-2 (20 ng/mL), anti-IL2 (100 μg/mL) or anti-CD25 (25 μg/mL) on day 0 and T cell proliferation was measured on day 3 post-stimulation. (d) Division index calculations by the FlowJo software were subsequently used to generate % suppression measurement. (e). Evaluation of CD25 expression on CD4<sup>+</sup>FoxP3<sup>-</sup> T cells: Treg suppression assay using exogenous IL-2 or IL-2 signaling blockers. (f). IL-15 does not influence T cell proliferation in the Treg suppression assay. Exogenous IL-2 (20 ng/mL) or IL-15 (20 ng/mL) was added to the co-culture, and % suppression was quantified based on day 3 proliferation.

(Figure 4a). It was worth noting that on day 3, CD25 expression of Cbl-b KO CD4<sup>+</sup> effector T cells suppressed by Treg cells were still higher than that of C57BL/6 T cells stimulated without Tregs (Figure 4b), demonstrating an enhanced potential for Cbl-b KO CD4<sup>+</sup> T cells to capture IL-2 even in the presence of Tregs.

To examine the direct role of IL-2 on the Treg-resistant phenotype observed in Cbl-b KO CD4<sup>+</sup> T cells, a Treg suppression assay was performed with the addition of exogenous IL-2, anti-IL-2 or anti-CD25. The addition of IL-2 induced C57BL/6 CD4<sup>+</sup> T cell proliferation in the presence of Treg cells, and the addition of anti-IL-2 or anti-CD25 on Cbl-b KO

CD4<sup>+</sup> effector T cells reversed its hyper-proliferative state (Figure 4c). When quantified for Treg cell resistance, addition of IL-2 to C57BL/6 T cells induced an approximately 5-fold decrease in % suppression. In contrast, the addition of anti-IL-2 or anti-CD25 on Cbl-b KO CD4<sup>+</sup> T cells resulted in an increase in % suppression (Figure 4d). As expected, the addition of IL-2 led to an increased CD25 expression on T cells despite the presence of Tregs, whereas blocking IL-2 or CD25 showed the opposite effect (Figure 4e), strengthening the link between the presence of IL-2 and CD25 expression.

To demonstrate the importance of CD25 on the Treg-resistant phenotype, Treg suppression assays were performed on C57BL/6 T cells with either exogenous IL-2 or IL-15 (interacting with CD122 and CD132, but lacking CD25 interaction). IL-15 did not decrease % suppression of C57BL/6 CD4<sup>+</sup>FoxP3<sup>-</sup> T cells co-cultured with Treg cells (figure 4f). In summary, elevated secretion of IL-2 by Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells and the increased CD25 surface-expression by Cbl-b KO T cells serves as an important mechanism that alters the ability of CD4<sup>+</sup> T cells to be suppressed by Tregs.

### **Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells mediate Treg resistance despite Treg expansion**

While the overproduction of IL-2 and increased CD25 expression by Cbl-b KO CD4<sup>+</sup> T cells played an important role in rendering these T cells refractory to regulation, the overall effect on Treg cells remained to be explored. As such, Treg suppression assays were performed using either C57BL/6 or Cbl-b KO CD4<sup>+</sup>CD25<sup>-</sup> T cells with C57BL/6 Treg cells. By gating on CD4<sup>+</sup> cells, a higher proportion of CD25<sup>+</sup>FoxP3<sup>+</sup> population was present on day 1 post-stimulation and only a 2-fold decrease in % FoxP3<sup>+</sup> T cells were observed with Cbl-b KO CD4<sup>+</sup> T cells on day 3 post-stimulation (Figure 5a). Despite the inability for C57BL/6 Tregs to proliferate *in vitro*, Cbl-b KO CD4<sup>+</sup> T cells were able to promote the expansion of Treg cells in the suppression assay (Figure 5b). Three days post-stimulation, co-culture of C57BL/6 Treg cells with Cbl-b KO CD4<sup>+</sup> T cells led to increased levels of CD25, FoxP3 and CTLA-4 on Treg cells (Figure 5c–e) which are correlated with enhanced Treg function. Finally, we performed Treg suppression assays with either IL-2 or IL-2 signaling blockers (anti-IL-2 or anti-CD25) and monitored the phenotype of Treg cells. The addition of IL-2 also increased CD25 expression on FoxP3<sup>+</sup> T cells, and the use of IL-2 blockers decreased CD25 expression on Treg cells (figure 5f). Treg suppression assay using IL-2 pre-stimulated wild-type Treg cells demonstrated comparable suppressive function to pre-stimulated Treg cells without IL-2 (Figure 5g). Despite an IL-2-induced Treg expansion during the co-culture, Cbl-b KO CD4<sup>+</sup> T cells were still capable of hyper-proliferating. These assays showed that the effect of Cbl-b deficient CD4<sup>+</sup> T cells was primarily exerted on T cells without disrupting the function of Treg cells.

### **IL-2 serves as the central cytokine in mediating Treg cell-resistance**

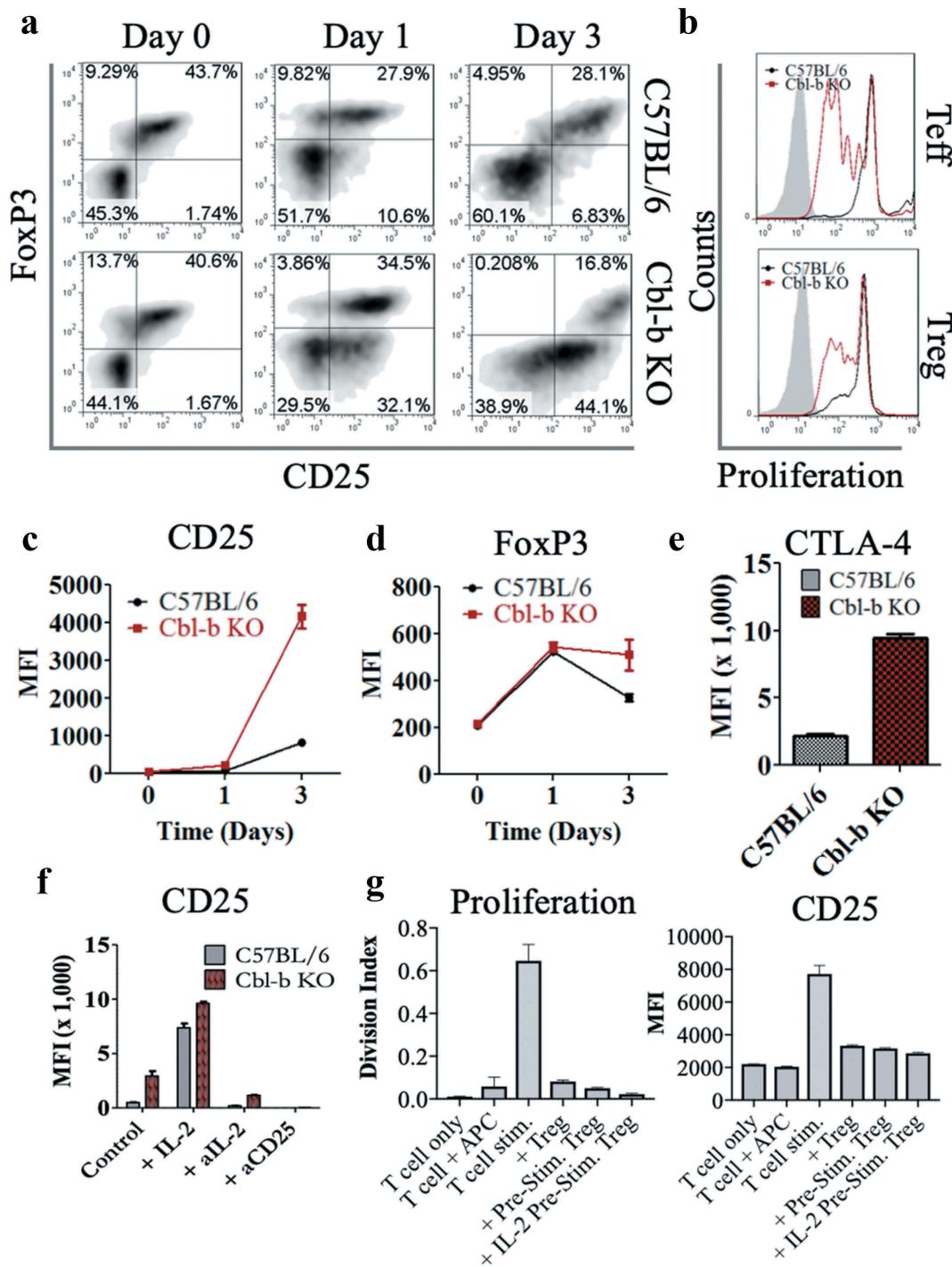
In addition to IL-2, Cbl-b KO CD4<sup>+</sup> T cells secreted other cytokines including IFN- $\gamma$ , IL-4, IL-17A, and IL-10 (Figure 2d). Since

several reports have highlighted the role of cytokines such as IL-6 in rendering T cells refractory to the suppressive function of Tregs,<sup>16,57–59</sup> we explored whether other cytokines could also modulate Treg resistance. First, co-cultures were set up including C57BL/6 CD4<sup>+</sup> T cells and C57BL/6 Treg cells, supplemented with 14 independent cytokines to determine whether these cytokines could inhibit Treg cell mediated suppression. The cytokines used for this Treg suppression assay were selected from those that were hyper-secreted by Cbl-b KO CD4<sup>+</sup> T cells, or were previously implicated in Treg resistance. Along with % suppression, CD4<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>FoxP3<sup>-</sup> cells from each condition were analyzed for expression of activation markers (Figure 6a). The addition of IL-2 or IL-4 to the co-culture demonstrated the strongest capacity to block the ability of Tregs to inhibit proliferation, as well as the highest expression of CD25 and GITR on effector T cells (Figure 6a). Beyond IL-2 and IL-4, other cytokines that reduced % suppression by approximately 2-fold or more included: IFN- $\alpha$ , IL-6, IL-7, IL-10 and IL-18 (Figure 6b). It is important to note that most of these cytokines had a stimulatory effect on the CD4<sup>+</sup> effector T cells (Figure 6a), potentially inducing higher IL-2 secretion for subsequent Treg resistance. To test whether these cytokines mediated Treg resistance through indirect IL-2 upregulation by T cells, we collected supernatants from the Treg suppression assay performed in Figure 6a,b. We then correlated the influence of each exogenous cytokine on % suppression with either IL-2 secretion or CD25 surface expression by effector T cells. Percent suppression achieved by individual cytokines negatively correlated with IL-2 secretion by T cells, where cytokines such as IL-6 induced high levels of IL-2 secretion (Figure 6c). Similarly, % suppression quantified for individual cytokines also negatively correlated with CD25 expression on effector T cells (Figure 6d). These data suggest that cytokine-induced Treg cell resistance may likely converge on the amplification of IL-2 production and signaling for its *in vitro* phenotype.

To directly confirm whether these cytokines depend on IL-2 signaling to mediate the observed phenotype, naïve C57BL/6 or CD25 KO CD4<sup>+</sup> T cells were stimulated with the cytokines used in Figure 6a. In the absence of CD25 expression, majority of the cytokines were insufficient to induce CD4<sup>+</sup> T cell proliferation (Figure 6e). When stained with viability dye on day 3 post-stimulation without exogenous cytokines, C57BL/6 CD4<sup>+</sup> effector T cells had a higher viability of 78%, while approximately 48% of CD25 KO CD4<sup>+</sup> T cells were still viable (data not shown). Altogether, these data suggest that while other cytokines could be used to confer resistance against regulatory T cells, the cytokine networks tested here relied on IL-2 as the central molecule to mediate Treg resistance.

## **Discussion**

Recently, a genome-wide CRISPR screen of primary human T cells has identified Cbl-b as the top candidate to enhance T cell activation, proliferation and tumor cell killing *in vitro*.<sup>60</sup> This is consistent with previous findings which identify Cbl-b as an important gene regulating autoimmunity and anti-tumor immunity in mice.<sup>46,47,53</sup> In this study, we focused on CD4<sup>+</sup> T cells and explored the mechanism of Cbl-b in mediating T cell resistance to Treg cells. We show that hyper-secretion of IL-2 by

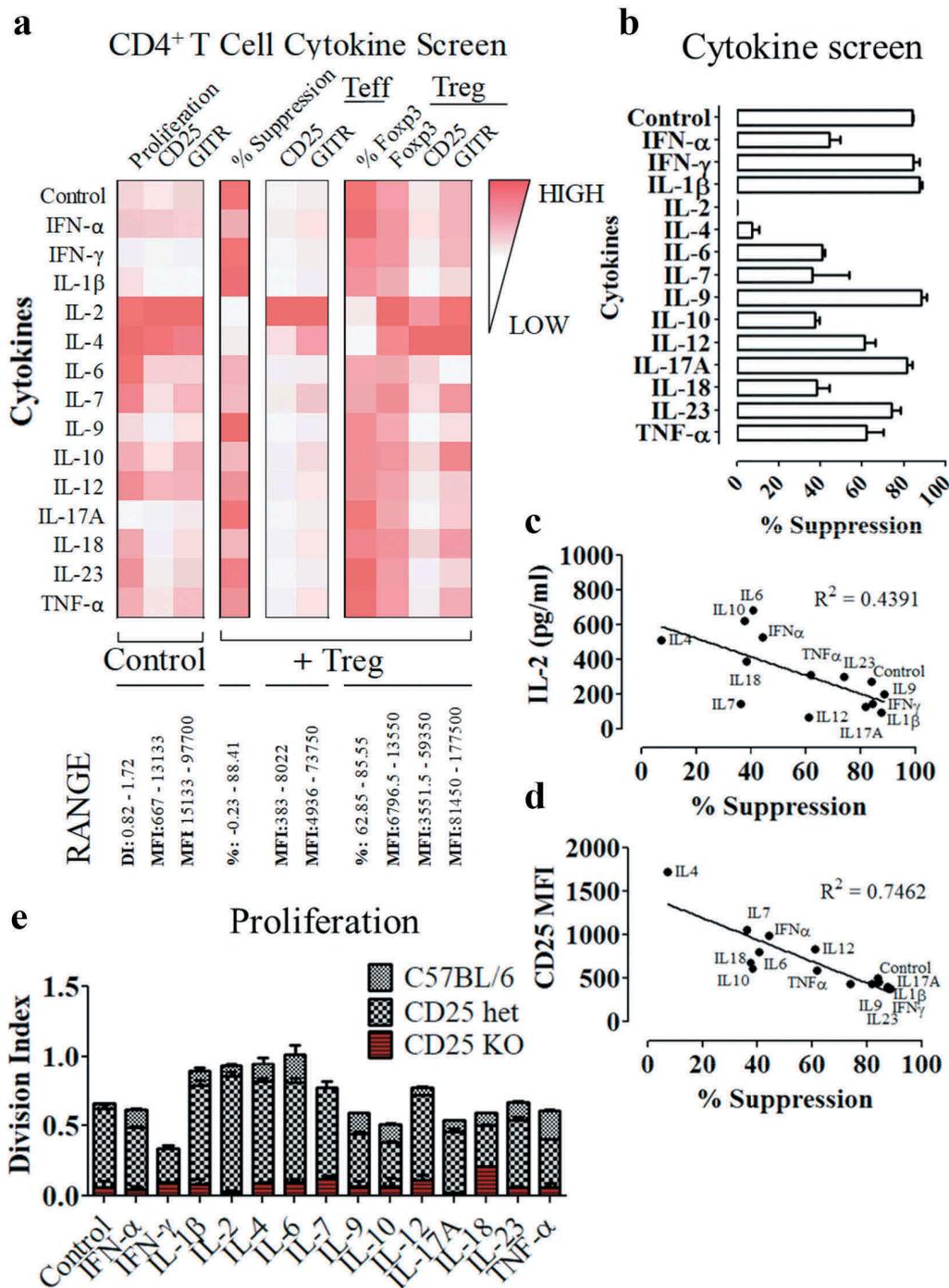


**Figure 5.** IL-2 secretion by Cbl-b KO CD4<sup>+</sup>FoxP3<sup>-</sup> T cells positively contribute to Treg activities.

(a). Quantification of the Treg and Teff populations found in Treg suppression assay. The effect of WT or Cbl-b KO CD4<sup>+</sup> effector T cells on the proportion of WT CD4<sup>+</sup>FoxP3<sup>+</sup> T cells were analyzed after gating on the CD4<sup>+</sup> population. (b). Proliferation of Treg vs. Teff cells co-cultured at a 1:1 ratio. Both Treg and Teff cells were stained with proliferation dye to compare the effects of WT or Cbl-b KO effector T cells on WT Treg cells. (c–e). The effect of WT or Cbl-b KO CD4<sup>+</sup> effector T cells on functional markers expressed by WT Tregs. Expression of CD25 (c) and FoxP3 (d) on Tregs were quantified on day 0, 1 and 3 post-stimulation. (e) Intracellular CTLA-4 staining and analysis were performed on WT Treg cells 3 days post-stimulation. (f). The role of IL-2 signaling on WT Treg expression of CD25. WT Treg cells were co-cultured either WT or Cbl-b KO CD4<sup>+</sup> T cells, and exogenous IL-2 (20 ng/mL), anti-IL-2 (100 µg/mL) or anti-CD25 (25 µg/mL). CD25 expressed by CD4<sup>+</sup>FoxP3<sup>+</sup> T cells was quantified on day 3 post-stimulation. **G**. The role of IL-2 on Treg cell function. Congenically labeled CD45.1 CD4<sup>+</sup>CD25<sup>-</sup> T cells were stimulated in the presence of purified naïve CD45.2 Treg cells or Treg cells that were pre-stimulated with or without IL-2 (10 ng/mL) for 24 hours. Proliferation and CD25 expression of labeled effector CD4<sup>+</sup> T cells were quantified.

Cbl-b KO CD4<sup>+</sup> T cells and upregulation of CD25 increase effector T cell exposure and sensitivity to IL-2 (Figure 7a,b). Increased exposure to IL-2 signaling then counteracts the

previously established ability of Treg cells in sequestering IL-2 required for effector T cell proliferation and survival.<sup>15,61</sup> Furthermore, while other cytokines can mediate Treg cell



**Figure 6.** IL-2 serves as a central cytokine responsible for CD4<sup>+</sup>FoxP3<sup>+</sup> T cell resistance against Tregs.

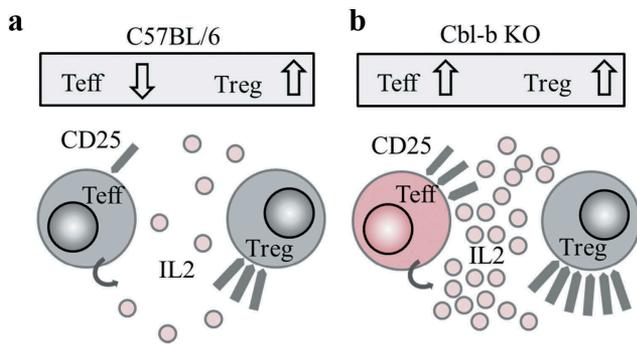
(a). Summary of cytokine screening using the Treg suppression assay. Naïve WT CD4<sup>+</sup>FoxP3<sup>+</sup> T cells were stimulated with anti-CD3, irradiated APCs, and independently co-cultured with 14 independent cytokines (10 ng/mL), with or without Tregs for 3 days. Effector T cells and Tregs were evaluated for CD25, GITR and FoxP3 expression. In addition, % suppression was quantified and the data was presented using a heat map. (b). Quantification of CD4<sup>+</sup> T cell resistance to Treg cells in response to different cytokines (expanded from A). (c,d). Correlation between the ability of each cytokine to induce either IL-2 or CD25 and Teff resistance to Tregs. (c) IL-2 production by T cells, measured through ELISA, in response to different cytokine stimulation was plotted against its % suppression. (d) CD25 expression on CD4<sup>+</sup>FoxP3<sup>+</sup> Teff cells co-cultured with Tregs in response to different cytokine stimulation was directly plotted against % suppression. (e). TCR and cytokine-induced stimulation of naïve WT, CD25 het or CD25 KO CD4<sup>+</sup> T cells. WT, CD25 het or CD25 KO CD4<sup>+</sup> T cells were stimulated using anti-CD3, irradiated APCs and 14 independent cytokines. Proliferation was quantified on day 3 post-stimulation.

resistance, we found that IL-2 served as the central molecule essential for cytokine-driven Treg resistance.

Previous studies have highlighted the importance of other T cell activating cytokines including IL-4, IL-6, and IL-7 in

generating resistance to Treg-mediated suppression.<sup>16,19,21,23</sup>

It is possible that many of these cytokines can induce resistance to the suppressive effects of Treg cells (Figure 6b), because these cytokines also share overlapping signaling



**Figure 7.** Proposed mechanism: Cbl-b KO CD4<sup>+</sup> T cells promote Treg resistance through enhanced IL-2 production.

(a). Interaction between WT Tregs and WT Teff. When WT CD4<sup>+</sup> T cells are stimulated in the presence of Tregs, IL-2 produced by the CD4<sup>+</sup> T cells are immediately acquired by regulatory T cells which express a higher level of IL-2 receptors. This scenario favors T cell suppression. (b). Interaction between WT Treg and Cbl-b KO Teff. Cbl-b KO CD4<sup>+</sup> T cells hyper-secrete IL-2 which provides a sufficient amount of IL-2 for T cell activation and survival as well as Treg expansion. Cbl-b KO CD4<sup>+</sup> T cells also upregulate IL-2 receptors which increase the potential for T cells to acquire IL-2. Together, Cbl-b KO CD4<sup>+</sup> T cells override the ability of Tregs to restrict T cell activation.

pathways with IL-2 such the JAK/STAT and PI3 K/Akt pathways. While the additional exposure to T cell activating cytokines such as IL-6 was sufficient in mediating a robust T cell response, as well as Treg resistance, they also triggered the release of IL-2 and were unable to induce proliferation in the absence of CD25 (Figure 6c,e). Although these cytokines will likely exert distinct effects on T cell activation, differentiation and survival, their mechanism of resistance to Tregs depend on the ability of CD4<sup>+</sup> T cells to process IL-2 signaling. The precise signaling pathway responsible for this phenotype is currently unknown, but it has been proposed that the PI3 K/Akt signaling could serve as a convergence point of numerous cytokine signaling pathways to mediate Treg resistance.<sup>27</sup> However, the cytokines implicated in Treg resistance such as IL-4, IL-6, and IL-7, which were also capable of inducing PI3 K signaling, were heavily dependent on IL-2 (Figure 6e). Thus, the PI3 K/Akt signaling pathway itself may not be sufficient to explain the effects of IL-2, and may act in conjunction with other components of the IL-2 signaling pathway to mediate Treg resistance. Here, we highlight the importance of IL-2 as a crucial cytokine required to induce CD4<sup>+</sup> T cell resistance to Treg cells.

While Cbl-b deficiency renders T cells resistant to Treg-mediated suppression, we found that Treg activities were also enhanced (Figure 5b–e). Our observation that Treg expansion occurred under conditions for Treg resistance is novel, but perhaps not unexpected. The role of IL-2 in Treg expansion has been reported in numerous studies, where systemic administration of IL-2 increased Treg activities.<sup>62–64</sup> Because IL-2R signaling is crucial for STAT5-driven Treg cell function,<sup>52,65</sup> and Cbl-b KO CD4<sup>+</sup> T cells confer resistance through overproduction of IL-2, Treg cell expansion is indeed inevitable. Consistent with our model, when TC1 or EL4 exogenous tumor cells were transplanted into the flank of C57BL/6 or Cbl-b KO mice, Cbl-b deficient mice displayed higher tumor-infiltrating Tregs despite the increased anti-tumor immune response and overall survival.<sup>46,47</sup> Although enhanced quality

and quantity of Treg cells are worth noting, the overall immunological phenotype is dependent on the balance between inhibitory and activating mechanisms, not strictly based on the number of cells.

Clinically, IL-2 served as the first effective immune therapy against human cancer.<sup>66</sup> Earlier studies involving systemic administration of IL-2 demonstrated promising clinical responses in patients with metastatic melanoma and renal cancer, resulting in durable, complete and curative regressions.<sup>66–68</sup> Some of these initial studies stimulated substantial activity evaluating the therapeutic potential of IL-2, despite the known toxicity.<sup>66</sup> To date, IL-2 has been investigated for diverse applications including monotherapy, combination therapy, and *ex vivo* expansion of T cells for cell therapy.<sup>66,69,70</sup>

However, the role of IL-2 as therapy in disease has been controversial. Numerous studies have shown that low dose IL-2 preferentially expand Treg cells for the treatment of autoimmune diseases,<sup>70–72</sup> whereas high dose IL-2 expands Tregs while also enabling anti-tumor immunity.<sup>69,73,74</sup> Sim et al. (2014) demonstrated that patients with metastatic melanoma treated with high-dose recombinant IL-2 (720,000 IU/kg; Proleukin, Novartis) elevates the number of ICOS<sup>+</sup> Treg cells for both responders and non-responders. In the study, those with higher frequency of ICOS<sup>+</sup> Treg cells remaining in blood post high-dose IL-2 therapy had worse clinical outcomes than those with lower percentage of ICOS<sup>+</sup> Treg cells.<sup>75</sup> Similarly, Yao et al. (2012) performed nonmyeloablative chemotherapy followed by tumor infiltrating lymphocyte (TIL) transfer and IL-2 administration, and suggest that the proportion of reconstituted CD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells in blood after the treatment negatively correlated to responsiveness to the therapy.<sup>76</sup> Adoptive T cell therapies using TILs routinely include IL-2.<sup>77–83</sup> However, controversies surrounding its ability to promote T cell survival versus the detrimental effects of Treg cell expansion has been unclear. Our studies suggest that if patients are treated with TIL and high dose IL-2, it may lead to the expansion of Treg cells due to the presence of high dose IL-2 but this may not necessarily be detrimental if it occurs together with the development of resistance to the inhibitory effects of Treg cells.

Alternatively, recently proposed strategies involve preferential expansion of T cells over Treg cells using IL-2-antibody complexes or modified IL-2 biologics.<sup>15,84–88</sup> For example, Bempegaldesleukin (NKTR-214), which consists of an IL-2 core conjugated to 6 releasable polyethylene glycol chains, preferentially bind to IL-2R $\beta$  over IL-2R $\alpha$  which induce greater T cell to Treg cell ratio and a more robust anti-tumor immunity in comparison to Aldesleukin.<sup>87</sup> Similarly, conjugating anti-IL-2 with IL-2 selectively enhance T cell performance to induce stronger anti-tumor immunity.<sup>86,88</sup> These strategies may be more effective in comparison to conventional IL-2 usage, however most of these still require validation in a clinical setting.

In summary, our studies have defined a mechanism to render T cells refractory to the inhibitory effects of Tregs. We also highlight the potential in promoting an immune response by IL-2 overproduction through targeting Cbl-b or other related signaling pathways within CD4<sup>+</sup> T cells. Such a strategy could potentially be incorporated in many treatment modalities including the Chimeric Antigen Receptor

(CAR) -T cell therapy or TCR-engineering based immunotherapy to generate robust effector T cell responses and subsequent anti-tumor immunity.

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## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## Author Contributions

SH conceived and performed experiments, managed the research project and wrote the manuscript. DCC, MSP, ZQL and CGB performed experiments. ARE provided reagents. CWT, LC and PSO provided expertise and feedback. PSO secured funding and supervised the project.

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