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Lys63-polyubiquitination by the E3 ligase casitas B-lineage lymphoma-b (Cbl-b) modulates peripheral regulatory T cell tolerance in patients with systemic lupus erythematosus

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Summary

T cells from systemic lupus erythematosus (SLE) patients display a wide array of anomalies in peripheral immune tolerance mechanisms. The role of ubiquitin ligases such as Cbl-b has been described recently in these phenomena. However, its role in resistance to suppression phenotype in SLE has not been characterized, which was the aim of the present study. Thirty SLE patients (20 with active disease and 10 with complete remission) and 30 age- and sex-matched healthy controls were recruited. Effector ($CD4^+CD25^-$) and regulatory ($CD4^+CD25^+$) T cells (T_{regs}) were purified from peripheral blood mononuclear cells (PBMCs) by magnetic selection. Suppression assays were performed in autologous and allogeneic co-cultures and analysed by a flow cytometry assay. Cbl-b expression and lysine-63 (K63)-specific polyubiquitination profile were assessed by Western blotting. We found a defective Cbl-b expression in T_{regs} from lupus patients in contrast to healthy controls ($1{\cdot}1 \pm 0{\cdot}9$ *versus* $2{\cdot}5 \pm 1{\cdot}8$, $P = 0{\cdot}003$), which was related with resistance to suppression ($r = 0{\cdot}633$, $P = 0{\cdot}039$). Moreover, this feature was associated with deficient K63 polyubiquitination substrates and enhanced expression of phosphorylated signal transducer and activation of transcription 3 (pSTAT-3) in T_{regs} from lupus patients. Our findings support that Cbl-b modulates resistance to suppression by regulating the K63 polyubiquitination profile in lupus T_{regs}. In addition, defective K63 polyubiquitination of STAT-3 is related to increased pSTAT-3 expression, and might promote the loss of suppressive capacity of T_{regs} in lupus patients.

Keywords: Cbl-b, K63-polyubiquitination, regulatory T cells, resistance to suppression, systemic lupus erythematosus

Introduction

Regulatory T cells (T_{regs}) in humans are characterized by the expression of diverse molecules such as interleukin 2 receptor α chain (IL-2Rα, CD25), forkhead box P3 transcription factor (FoxP3) and the absence or low expression of IL-7 receptor α chain (CD127) [1]. They play a crucial role in the regulation of the immune response and maintenance of peripheral tolerance, as they exert a suppressor effect on many cellular subpopulations, such as $CD4^+$ and $CD8^+$ T cells [2–4], through diverse mechanisms that include the production of suppressor cytokines such as IL-10 [5], IL-35 [6] and transforming growth factor (TGF)-β [7]. In murine models and humans, quantitative and functional alterations in T_{regs} have been related to the development and maintenance of many organ-specific and systemic autoimmune diseases [2,8–16]. In particular, defects in *FOXP3* gene, IL-2Rα signalling pathway and inhibitory markers, such as cytotoxic T lymphocyte-associated protein-4 (CTLA-4) or programmed cell death protein-1 (PD-1), as well as over-expression of T-bet or signal transducer and activator of transcription-3 (STAT-3), appear to be critical in the loss of suppressive function or stability in different T helper type 1 (Th1)- and Th17-dominated autoimmune settings, such as multiple sclerosis, type 1 diabetes, rheumatoid arthritis and systemic lupus erythematosus (SLE) [17].

Diverse post-translational modifications (PTM) that regulate immunity have been described; among these,

ubiquitination has gained recent focus. The conjugation of ubiquitin mainly to a lysine residue from a protein substrate involves three subsequential enzymatic reactions, including the action of activating enzyme (E1), conjugating enzyme (E2) and finally ligase (E3), which is the enzyme that provides specificity to the system. Current evidence supports the role of ubiquitination as a PTM whose outcome can be dependent or independent of proteosomal or lysosomal degradation [18–20]. In particular, in immune response regulation, proteolysis-independent mechanisms, such as inhibition of phosphorylation, have been described [21,22]. Currently, the role of ubiquitin ligases has been acknowledged as negative modulators of diverse immune responses [23]. Our group and others have characterized the role of the E3 ubiquitin ligase Casitas B lineage lymphoma b (Cbl-b) in the regulation of peripheral tolerance mechanisms through the interplay with multiple substrates associated with the T cell receptor (TCR) signalling pathway, such as protein kinase Cθ (PKCθ), phospholipase Cγ1, Vav-1, the p85 subunit of phosphatidylinositol 3-kinase (PI3K-p85) and phosphatase and tensin homologue deleted on chromosome 10 (Pten). In murine models, Cbl-b deficiency has been implicated in the development of lupus-like disease, secondary to impaired peripheral tolerance mechanisms, such as anergy and suppression by T_{regs} [19,24–30].

SLE constitutes the prototypical systemic autoimmune disease associated with a breach in peripheral tolerance. It has been well demonstrated that T cells from lupus patients present a wide variety of anomalies in peripheral immune tolerance mechanisms [31–33]. For instance, we have reported that $CD4^+$ T cells from SLE patients display resistance to the anergy phenotype, which is associated with a deficiency in Cbl-b expression and diverse phosphorylation defects in the TCR signalling pathway [19]. Moreover, resistance to suppression has been acknowledged as part of the abnormalities shown by lupus T cells [15,16,34].

Therefore, the primary objective of the present study was to assess Cbl-b protein expression in T_{regs} from lupus patients in contrast with healthy controls, as well as its relationship with resistance to the suppression phenotype and polyubiquitination profile in SLE.

Materials and methods

Patients and controls

Thirty patients with SLE diagnosis according to the American College of Rheumatology revised classification criteria were included, as well as 30 sex- and age-matched healthy controls [35]. Twenty-five of the patients were female, with a mean age of 34·4 (± 7·8) years. Ten patients were in complete remission, defined as an SLE Disease Activity Index-2000 (SLEDAI-2K) of 0 points in the absence of any treatment with corticosteroids or immunosuppressants during 12 months or more [36], and the mean duration of remission was 5·2 (± 8·9) years. The remaining 20 patients had active disease, with a mean SLEDAI-2K of 18·0 (± 8·9) points without no immunosuppressants, including corticosteroids, during the month prior to study entry (Table 1). The institutional ethics and research committees approved the study (Ref. 441) and all subjects signed the study informed consent prior to inclusion.

Table 1. Clinical and demographic features of SLE patients

Female gender, *n* (%)	25 (80·0)
Age (years), mean (s.d.)	34·3 (7·8)
Time since diagnosis (years), mean (s.d.)	5·8 (3·7)
Time in remission (years), mean (s.d.)	5·2 (3·4)
SLEDAI-2K (points), mean (s.d.)	18·0 (8·9)

SD, standard deviation; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index-2000.

Cell purification

All individuals provided a venous peripheral blood sample. $CD4^+$ T cells were purified from peripheral blood mononuclear cells (PBMCs) by negative selection with depletion column magnetic beads with a purity of $> 95\%$. For the isolation of T_{regs} we purified $CD25^+$ from this population by positive selection with magnetic beads, with a purity of 90% (MicroBeads $CD4^+CD25^+$ T_{reg} isolation kit; Miltenyi Biotec, San Diego, CA, USA). We considered effector T cells to be $CD4^+CD25^-$ and T_{regs} to be $CD4^+CD25^+$.

Cell cultures and proliferation assays

Before incubation, only the CD25-depleted cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE-DA; Biochemika-Fluka, Morris Plains, NJ, USA), according to the manufacturer's protocol. For the suppression assays, $CD4^+CD25^-$ effector and $CD4^+CD25^+$ T_{regs} were studied in autologous and allogenic co-cultures (effector : T_{regs} ratio, 1 : 1) in 24-well plates and were either left unstimulated (RPMI) or were activated using a combination of plate-bound anti-CD3 antibody (5 μg/ml) and soluble anti-CD28 antibody (2·5 μg/ml) for 48 h. Following this, cells were harvested for proliferation assays. Cell proliferation was evaluated by fluorescence activated cell sorter (FACS) (BD LSRII Fortessa; BD Biosciences, San Jose, CA, USA), according to the CFSE-DA dilution protocol. The gating strategy was performed according to the FlowJo software proliferation platform (version 10; Tree Star, Inc., Ashland, OR, USA) [15].

Western blotting analysis

Effector and T_{reg} lysates were obtained by using ice-cold erythrocytes lysis buffer (ELB) and immunoblotted as described previously [19]. Approximately 10–30 μg of

protein were required for each assay. We used the following antibodies: anti-Cbl-b (sc-8006) and β-actin (sc-47778) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-K63-linkage specific polyubiquitin (5621S), total STAT-3 (12640S) and pSTAT-3 (2145S) from Cell Signaling (Danvers, MA, USA). Densitometry was performed to determine protein expression using the Chemidoc MP image system (Biorad Laboratories, Berkeley, CA, USA) and ImageLab software. Values were normalized to β-actin.

Immunoprecipitation assays

T_{reg} lysates (400 μg) were incubated with primary antibody to STAT-3 (12640S) from Cell Signaling (1 : 1000), and this solution (immunocomplex) was then incubated with Protein G Magnetic DynaBeads® from Invitrogen Thermo Fisher Scientific (Carlsbad, CA, USA), according to the manufacturer's protocol.

Statistical analysis

Outcomes are expressed as mean ± standard deviation (s.d.), unless noted otherwise. Differences between groups were analysed using independent-sample Student's *t*-tests. The strength of a linear association between percentage of suppression by T_{regs} in proliferation assays and Cbl-b protein levels was determined by Pearson's correlation coefficient. *P*-values less than 0·05 were considered statistically significant; SPSS software (version 21.0) was used for statistical analyses.

Results

Effector T cells from SLE patients display a resistance to suppression by T_{regs}

$CD4^+CD25^+$ T_{regs} and $CD4^+CD25^-$ effector T cells from SLE patients were tested for their ability to respond and/or suppress after stimulation *in vitro* with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody, as described in Material and methods. In autologous co-cultures, $CD4^+CD25^-$ effector T cells from SLE patients showed a decreased percentage of suppression compared with $CD4^+CD25^-$ effector T cells from healthy controls in the presence of T_{regs} (24 ± 3 *versus* 78 ± 15%, $P = 0{\cdot}002$). Conversely, in allogenic co-cultures $CD4^+CD25^-$ effector T cells from SLE patients showed an increased percentage of suppression in the presence of $CD4^+CD25^+$ T_{regs} from healthy controls, in contrast with $CD4^+CD25^-$ effector T cells from SLE patients in the presence of $CD4^+CD25^+$ T_{regs} from SLE patients (45 ± 12 *versus* 24 ± 3%, $P = 0{\cdot}035$). Furthermore, there was no statistically significant difference in the percentage of suppression of $CD4^+CD25^-$ effector T cells from healthy controls in the presence of $CD4^+CD25^+$ T_{regs} from SLE patients or healthy controls, respectively (Fig. 1). These results suggest a resistance to suppression by T_{regs} in SLE patients in contrast with healthy controls in proliferation assays.

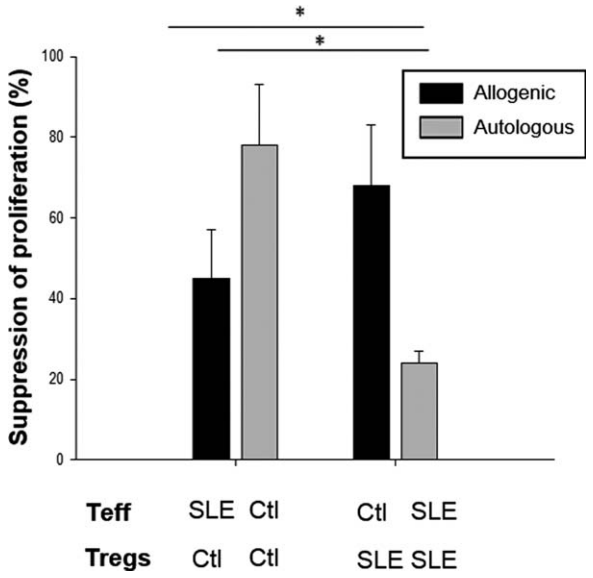

Fig. 1. Effector T cells from systemic lupus erythematosus (SLE) patients display a resistance to suppression by regulatory T cells (T_{regs}). $CD4^+CD25^+$ T_{regs} from SLE patients and healthy controls were tested for their ability to suppress $CD4^+CD25^-$ effector T cell proliferation *in vitro*. $CD4^+CD25^-$ effector and $CD4^+CD25^+$ T_{regs} were studied in autologous and allogenic co-cultures (effector : T_{regs} ratio, 1 : 1) stimulated previously with plate-bound anti-CD3 antibody (5 μg/ml) and soluble anti-CD28 antibody (2·5 μg/ml) for 48 h. Before incubation, only the CD25-depleted effector T cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE-DA; Biochemika-Fluka). Cells were then harvested for proliferation assays by flow cytometry according to the CFSE-DA dilution protocol. These are the pooled data from 30 SLE patients and 30 age- and sex-matched healthy control proliferation assays. Ctl = healthy controls; T_{eff} = effector T cells. $^*P < 0{\cdot}05$.

The E3 ligase Cbl-b is decreased in T_{regs} from SLE patients and is associated with resistance to suppression

Cbl-b protein constitutes an anergy factor and adapter molecule for T cell proliferation and production of IL-2 [37,38]. In previous works, we have reported resistance to anergy phenotype in T cells from SLE patients, which is associated with a deficiency in Cbl-b expression [19]. Moreover, Cbl-b deficiency has been associated with a TGF-β-dependent resistance to suppression by T_{regs} in murine models [28–30]. Thus, we hypothesized that resistance to suppression would be related to a defect in this E3 ubiquitin ligase expression in SLE patients. We purified $CD4^+CD25^+$ T_{regs} from SLE patients and healthy controls and evaluated Cbl-b protein expression by Western blotting. As expected, it was decreased in lupus $CD4^+CD25^+$ T_{regs} in comparison to healthy controls (mean ± s.d. fold change: 1·1 ± 0·9 *versus* 2·5 ± 1·8; $P = 0{\cdot}003$). We did not

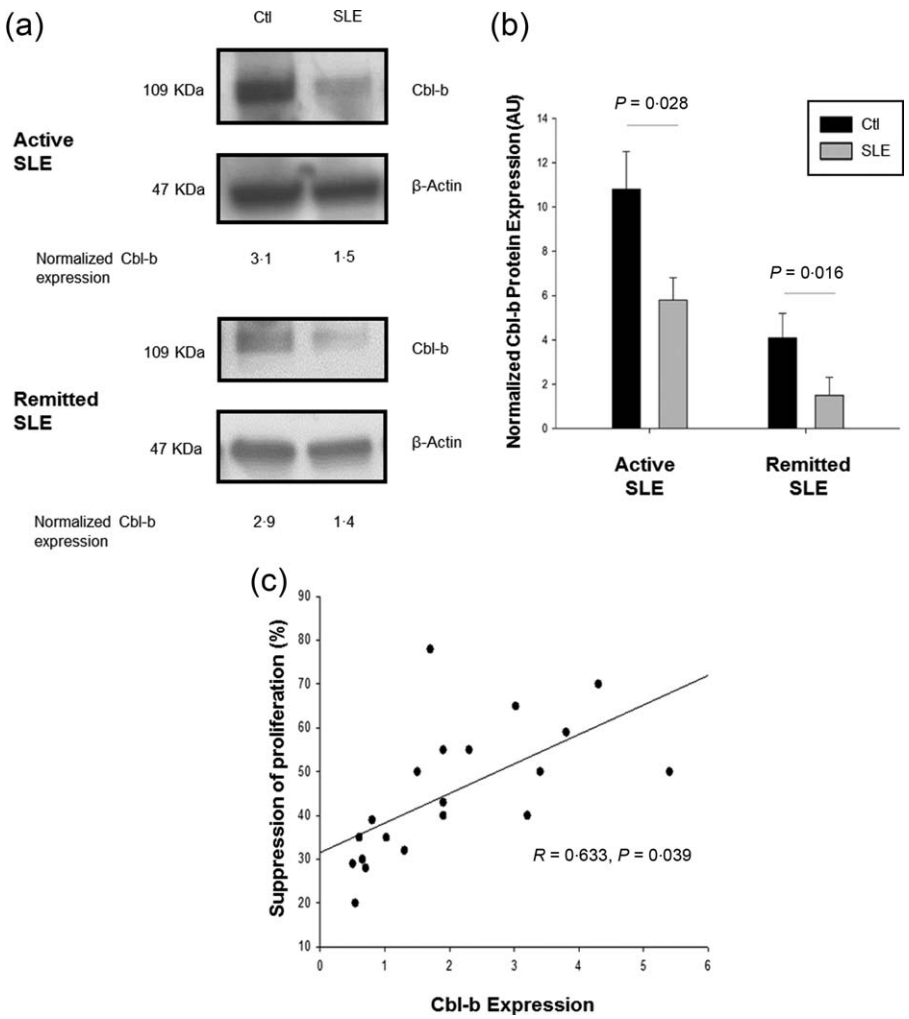

Fig. 2. Cbl-b expression is decreased in regulatory T cells (T_{regs}) from systemic lupus erythematosus (SLE) patients and is associated with resistance to suppression. Cbl-b protein expression was assessed in $CD4^+CD25^+$ T_{reg} lysates from SLE patients and healthy controls by Western blotting. A representative image of a Western blot for Cbl-b from an active and a remitted SLE patient *versus* healthy control is shown (a), as well as the pooled data from 30 SLE patients and 30 age- and sex-matched healthy controls normalized Cbl-b protein expression (b). There was a positive correlation between percentage of $CD4^+CD25^-$ effector T cell proliferation suppression and Cbl-b expression in T_{regs} from SLE patients (c). Ctl = healthy controls; AU = arbitrary units.

find any significant differences in Cbl-b expression in $CD4^+CD25^+$ T_{regs} from patients with active SLE compared with those with remitted disease (Fig. 2a,b). Moreover, we found a positive correlation between percentage of suppression by T_{regs} in proliferation assays and Cbl-b protein levels ($r = 0{\cdot}633$, $P = 0{\cdot}039$) (Fig. 2c). Although we also found decreased protein levels of Cbl-b in lupus effector T cells, it did not correlate with the percentage of suppression by T_{regs}. Thus, Cbl-b expression is decreased in $CD4^+CD25^+$ T_{regs} from SLE patients and is associated with resistance to the suppression phenomenon.

T_{regs} from SLE patients are deficient in K63 ubiquitinated substrates

Lysine-63-specific protein ubiquitination has been implicated in the regulation of signal transduction in immune receptors [39–45], which is mediated by the E2 ubiquitin-conjugating enzyme Ubc13, as shown by multiple studies, and directs conjugated proteins to lysosomes [44]. As Ubc13 inducible knock-out mice specific for T_{regs} display a reminiscent pathological phenotype of the T_{reg}-deficient mice [46], we hypothesized that Cbl-b deficiency would modify the ubiquitin profile of T_{regs} from SLE patients in a K63-dependent manner, which could contribute to resistance to suppression. We analysed Lys63 (K63) expression by Western blotting in $CD4^+CD25^+$ T_{reg} lysates from SLE patients compared to healthy controls. We observed a differential polyubiquitination profile distinguished by a decreased expression of K63 polyubiquitin chains in $CD4^+CD25^+$ T_{regs} from SLE patients related to healthy controls ($1{\cdot}47 \pm 0{\cdot}03$ *versus* $4{\cdot}31 \pm 0{\cdot}28$, $P < 0{\cdot}0001$) (Fig. 3a,b). This abnormal K63 polyubiquitination profile was not present in $CD4^+CD25^-$ effector T cells from SLE patients.

Deficient Cbl-b expression and K63 polyubiquitination are associated with higher levels of activated STAT-3 in T_{regs} from SLE patients

We evaluated various signalling molecules known to be prone to K63 regulation (p85, Vav1). Previous studies have proposed that resistance to suppression by T_{regs} is dependent upon the STAT-3 signalling pathway, as inhibition of

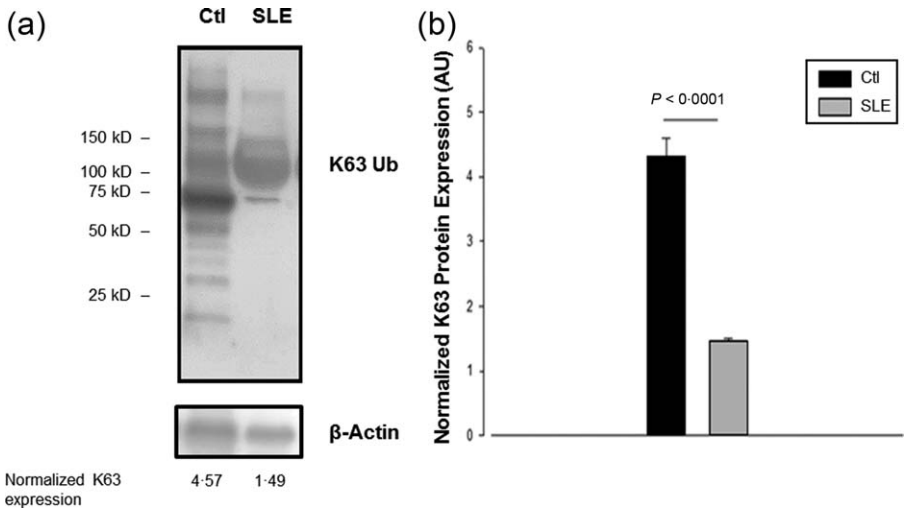

Fig. 3. Regulatory T cells (T_{regs}) from systemic lupus erythematosus (SLE) patients are deficient in K63 ubiquitinated substrates. Lys63 (K63) protein expression was assessed in $CD4^+CD25^+$ T_{reg} lysates from SLE patients and healthy controls by Western blotting. A representative image of a Western blot for K63 from an SLE patient *versus* healthy control is shown (a), as well as the pooled data from 30 SLE patients and 30 age- and sex-matched healthy controls normalized K63 protein expression (b). Ctl = healthy controls; AU = arbitrary units.

this molecule reverts the resistance to suppression phenotype by T_{regs} stimulated previously with IL-6 [47]. Moreover, the role of different ligases, such as tumour necrosis factor-associated factor 6 (TRAF-6), in the ubiquitination of STAT-3 to promote degradation has been well demonstrated [48]. Thus, we hypothesized that deficiency of Cbl-b and K63 polyubiquitination in T_{regs} from SLE patients could be associated with an increase in the STAT-3 signalling pathway. We then immunoprecipitated total STAT-3 protein from SLE patients and healthy controls $CD4^+CD25^+$ T_{reg} lysates and evaluated phosphorylated STAT-3 (pSTAT-3) expression by Western blotting. As expected, we observed an increased expression of pSTAT-3 in lupus T_{regs} when compared to healthy controls (Fig. 4). Our findings suggest that deficient Cbl-b expression and K63 polyubiquitination, primarily of STAT-3, are related to the increased pSTAT-3 expression in T_{regs} from SLE patients.

Discussion

Deficiency in E3 ubiquitin ligases has been related to spontaneous systemic autoimmunity secondary to diverse abnormalities in peripheral tolerance mechanisms. In particular, we have demonstrated that, in patients with SLE, $CD4^+$ T cells display a resistance to the anergy phenotype, which is associated with an abnormal E3 ubiquitin ligase Cbl-b expression [19]. However, the role of ubiquitination in resistance to suppression phenotype in SLE has not been defined completely. Although many studies have suggested that resistance to suppression in SLE is due to an effector T cell resistance and not to an abnormal regulatory function [15,16], it has been demonstrated that this subpopulation displays some molecular defects which could be associated with the acquisition of an effector phenotype [46,47].

Consistent with previous works, autologous co-cultures of effector and T_{regs} from lupus patients after mitogenic stimulation showed resistance to suppression, as demonstrated by less inhibition of cell proliferation when compared with healthy controls. When effector T cells from SLE patients were studied in the presence of T_{regs} from healthy controls, the percentage of suppression increased almost twice with statistical significance. Even though we found no difference in the percentage of suppression of effector T cells from healthy controls co-cultured with T_{regs} from healthy controls or from SLE patients, our data do not support an intact suppressive capacity of T_{regs} from SLE patients, as described previously [15,16].

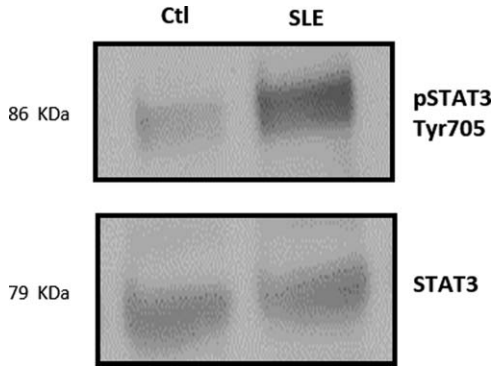

Fig. 4. Deficient Cbl-b expression and K63 polyubiquitination are associated with higher levels of activated signal transducer and activator of transcription 3 (STAT-3) in regulatory T cells (T_{regs}) from systemic lupus erythematosus (SLE) patients. The $CD4^+CD25^+$ T_{reg} lysates from SLE patients and healthy controls were immunoprecipitated with anti-STAT-3 antibody and then subjected to Western blotting with the anti-STAT-3 or anti-pSTAT-3 antibodies. This is a representative image of a Western blot for pSTAT-3 from an SLE patient *versus* healthy control. Ctl = healthy controls.

We have shown for the first time, to our knowledge, that resistance to the suppression phenomenon in $CD4^+$ T cells from patients with SLE is associated with a Cbl-b deficiency at the protein level. Our results are consistent with those from Wohlfert and colleagues, who demonstrated that Cbl-b deficiency is associated with TGF-β-dependent resistance to suppression by T_{regs} in murine models [28], even though the loss of Cbl-b is not as complete in lupus T cells as those from Cbl-$b^{-/-}$ mice. Cbl-b seems to be a critical mediator of T_{res} differentiation and suppressive function through different mechanisms. For instance, Cbl-b promotes TGF-β-mediated induced T_{regs} (iT_{regs}) conversion by tuning the threshold of T cell activation via an Akt-2 (protein kinase B)-dependent mechanism [49]. Moreover, mothers against decapentaplegic homologue 7 (SMAD7), a negative regulator of TGF-β signalling, has been identified as a crucial target of Cbl-b, thus regulating sensitivity towards TGF-β effects [50]. Thus, iT_{reg} differentiation could be compromised in SLE, as well as $CD4^+$ T cell sensitivity to a TGF-β suppressive effect. It is important to note that even though we also found a defective Cbl-b expression in effector lupus T cells, as described previously, it did not correlate with the data from the suppression assays.

As prior studies have illustrated numerous roles of Lys63-linked (K63) polyubiquitylation in immune responses [39–45], as well as its relationship with Cbl-b [27], we determined if the deficiency in this E3 ubiquitin ligase could be associated with an abnormal polyubiquitin profile of T_{regs} from SLE patients in a K63-dependent manner. According to our hypothesis, we observed a differential polyubiquitination profile distinguished by a decreased expression of K63 substrates in lupus T_{regs}, but not in effector T cells. This is in agreement with Chang and colleagues, who stated that E2 ubiquitin-conjugating enzyme Ube2n, which is the only enzyme that specifically promotes K63-polyubiquitination [40], seems to be crucial for stability and immunosuppressive *in-vivo* function of T_{regs} in murine models [46]. Taken together, there is strong evidence to support that there are several defects in the ubiquitination profile of T_{regs} from patients with SLE which contribute to resistance to suppression, a phenomenon that is not confined to effector T cell abnormalities. Cbl-b seems to be necessary for the correct inhibitory function of CTLA-4 [51] and PD-1 [52]. Moreover, other E3 ubiquitin ligases have been implicated in resistance to suppression by T_{regs}, such as stress-induced phosphoprotein 1 (STIP1) homology and Ubox-containing protein-1 (Stub1), which interacts with FoxP3 to promote its K48-linked (K48) polyubiquitination [53]. However, it has not been evaluated in SLE.

Recent studies support that the key distinctive feature of T_{reg} polarization to an effector phenotype is the STAT-3 signalling pathway, which favours a Th17-like effector phenotype through RAR-related orphan receptor gamma (RORγt) and IL-23 receptor expression. Specifically, Goodman and colleagues demonstrated that inhibition of STAT-3 restores immunosuppressive function by T_{regs} stimulated previously with IL-6 [47]. Our results are consistent with this notion, and imply that increased STAT-3 phosphorylation in T_{regs} is the hallmark of resistance to suppression in SLE. Moreover, in Ubc13-deficient T_{regs} there is a reduced expression of suppressor of cytokine signalling-1 (SOCS1), which contributes to T_{reg} stability by repressing the output of proinflammatory cytokine receptors, and particularly favours STAT molecule degradation [46]. Finally, it is well known that Ubc13 expression is down-regulated by STAT-3 operating as a transcriptional repressor of the *Ube2n* gene and modulating nuclear factor kappa B (NF-κB) activity, which seems to be crucial for T_{reg} stability and prevention of their conversion into effector-like T cells [54]. Thus, Cbl-b deficiency could favour STAT-3 over-expression by a defect in its degradation in T_{regs}, and at the same time interferes with the subpopulation stability mediated by the Ubc13-dependent IKK-NF-κB signalling pathway.

In summary, our findings suggest that E3 ubiquitin ligase Cbl-b modulates the interplay between effector and T_{regs}, particularly resistance to the suppression phenotype by regulating the K63 polyubiquitination profile in lupus T_{regs}. Moreover, defective K63 polyubiquitination primarily of STAT-3 is related to increased pSTAT-3 expression in this subpopulation and might promote the loss of suppressive capacity of T_{regs} in SLE, as reported previously for other autoimmune diseases.

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Disclosure

The authors have no conflicts of interest to declare.

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