Abnormal CTLA-4 function in T cells from patients with systemic lupus erythematosus

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Abstract

CTLA-4 is a critical gatekeeper of T-cell activation and immunological tolerance and has been implicated in patients with a variety of autoimmune diseases through genetic association. Since T cells from patients with the autoimmune disease systemic lupus erythematosus (SLE) display a characteristic hyperactive phenotype, we investigated the function of CTLA-4 in SLE. Our results reveal increased CTLA-4 expression in FOXP3 responder T cells from patients with SLE compared with other autoimmune rheumatic diseases and healthy controls. However, CTLA-4 was unable to regulate T-cell proliferation, lipid microdomain formation and phosphorylation of TCR-f following CD3/CD28 costimulation, in contrast to healthy T cells. Although lupus T cells responded *in vitro* to CD3/CD28 co-stimulation, there was no parallel increase in CTLA-4 expression, which would normally provide a break on T-cell proliferation. These defects were associated with exclusion of CTLA-4 from lipid microdomains providing an anatomical basis for its loss of function. Collectively our data identify CTLA-4 dysfunction as a potential cause for abnormal T-cell activation in patients with SLE, which could be targeted for therapy.

Key words

CTLA-4, Systemic lupus erythematosus, T-cell proliferation

Abbreviations

CTB: cholera toxin subunit B GM1: glycosphingolipid, PsA: psoriatic arthritis RA: rheumatoid arthritis SLE: systemic lupus erythematosus

Introduction

The immune response to antigen is controlled by potent negative regulators of TCR signalling, including the CD28 homologue, CTLA-4. The importance of CTLA-4 in the down-modulation of immune responses is demonstrated in CTLA-4-deficient mice that develop a lethal lymphoproliferative syndrome with features of autoimmunity [1, 2]. It is worth noting that by 3–5 wk, CTLA-4-deficient mice suffer from a severe lupus-like autoimmune syndrome, which is more rapid in onset than other mouse models of systemic lupus erythematosus (SLE) [3].

Several mechanisms have been proposed to explain the molecular basis for CTLA-4 inhibition including a dampening of T-cell signalling responses and alterations in T-cell motility [4, 5]. The location and interaction of CTLA-4 within membrane lipid microdomains also influences its inhibitory function. In healthy activated T cells, co-ligation of CTLA-4 during TCR stimulation strongly inhibits the upregulation of membrane lipid microdomains enriched with proximal signalling molecules [6, 7]. Moreover, although the surface expression of CTLA-4 is tightly regulated, during T-cell activation a pool of cell surface CTLA-4 is concentrated in lipid microdomains and associates with the TCR-ζ chain [7, 8]. This suggests that CTLA-4 controls TCR accumulation/retention in membrane microdomains thereby blocking formation of a stable immunological synapse and downstream signalling events. In addition, CTLA-4 is constitutively expressed on the majority of Treg, and plays an important role in their function [9–12]. The relative contribution of CTLA-4 to the intrinsic regulation of responder T-cell activation and Treg function remain unclear.

We and others have shown that lupus T cells display signaling patterns characteristic of hyper-responsiveness and altered expression of molecules regulated by CTLA-4, such as unregulated TCRζ phosphorylation and increased expression of lipid

microdomain-associated glycosphingolipid (GM1) [13–16]. Furthermore lupus T cells demonstrate more rapid formation of a stable immunological synapse [17, 18], which is normally inhibited by CTLA-4 [19]. We reasoned, therefore, that CTLA-4 maybe defective in its ability to inhibit activation in lupus T cells. Genetic studies linking CTLA-4 with SLE provide an additional rationale to investigate the role of CTLA-4 in the pathogenesis of this disease. Indeed, an association between CTLA-4 polymorphisms and lupus has been found in numerous studies around the world [20–27]. In addition, increased CTLA-4 expression has been noted in T cells from patients with SLE [28]. Here we demonstrate that despite the significantly increased expression of CTLA-4 in T responder cells from patients with SLE compared with healthy controls, its ability to regulate lupus T-cell signalling and proliferation following CD3/CD28 co-stimulation was impaired. This defect was associated with exclusion of CTLA-4 from lipid microdomains, which could account for its loss of function in T cells from patients with SLE. These data, identifying dysfunctional CTLA-4 in responder T cells indicate a disturbance in the immunoregulatory pathway of lupus patients, and provide a potential target for therapy in patients with SLE.

Results

CTLA-4 expression is significantly increased in T cells from patients with SLE

Patients with SLE show increased levels of membrane GM1 and cholesterol [14], molecules that are downregulated by the inhibitory co-receptor CTLA-4 [6]]. This encouraged us to investigate CTLA-4 expression in ex vivo PBMC from lupus patients compared with healthy controls by flow cytometry. In agreement with a previous report [28], a significant increase in the expression of total (t) CTLA-4 was seen in CD4+ T

cells from patients over healthy controls (Fig. 1A; %, p=0.0002; MFI, p=0.01). Although the change in the percentage of lupus T cells expressing CTLA-4 was small, this represented a twofold increase compared with T cells from healthy individuals. The more substantial shift in CTLA-4 expression as measured by MFI suggests that increased CTLA-4 expression affects a high proportion of lupus T cells. Surface (s)CTLA-4 expression was low in ex vivo T cells from healthy donors but significantly increased in lupus patients (p=0.01) (Fig. 1B). The elevation in CTLA-4 expression was independent of lupus disease activity status. No İncrease in tCTLA-4 expression was noted in T cells isolated from patients with other autoimmune rheumatic diseases, (rheumatoid arthritis, RA and psoriatic arthritis, PsA) (Fig. 1C).

Increased CTLA-4 in lupus is independent of FOXP3 but associated with increased T cell activation

Since CTLA-4 is an important marker of Treg, [9], we investigated whether its increased expression in lupus patients was associated with this population. However, the expression of CTLA-4 was increased significantly in the FOXP3– negative population from lupus patients compared with healthy controls

(p= 0.008) (Fig. 2A) indicating that the lupus-associated upregulation of CTLA-4 resides within the responder T-cell population. In contrast, there was no change in CTLA-4 expression in the FOXP3+ population from lupus patients compared with healthy controls (Fig. 2A).

Increased levels of CTLA-4 are also characteristic of activated and memory T cells associated with downmodulation of CD28 [4, 29, 30]. Almost all CTLA-4+ T cells expressed CD28, but the level of CD28 expression in the CTLA-4+ population in lupus

T cells was significantly reduced compared with healthy controls (p=0.0001) (Fig. 2B), consistent with their hyperactive phenotype. There was a significant population of CD28- T cells, which did not express CTLA-4, in lupus patients, which has been previously described [31, 32]. We found increased proliferation in lupus responder T cells using the proliferation marker Ki67.

The FOXP3- responder T cells showed a significant increase in Ki67 staining in lupus patients compared with controls (p=0.006) (Fig. 2C), indicating marked T-cell proliferation in lupus T cells. There was a substantial increase in CD4+FOXP3+ T cells in lupus patients (Fig. 2D), which has been suggested by others to reflect increased T-cell activation [33]. tCTLA-4 expression was increased in the FOXP3-Ki67+ population in lupus T cells compared with healthy controls (p=0.01) (Fig. 2E) indicating that CTLA-4 is upregulated on activated T cells in lupus patients. There was no difference in CLTA-4 expression in the CD4+FOXP3+Ki67+ population (Jury and Ehrenstein, unpublished data). Together, these results indicate that the elevated CTLA-4 expression is attributable to increased T-cell activation in the lupus patients and is associated with increased numbers of FOXP3+ T cells.

***In vitro* activation does not induce further CTLA-4 upregulation in lupus T cells**

To examine the effect of activation on CTLA-4 expression, CD4+CD25-CD127+ responder T cells from lupus patients and controls were activated *in vitro* using anti-CD3 and anti-CD28. CTLA-4 expression was maximally increased after 24 h in the T cells from healthy controls (p50.01) (Fig. 3A). However, CTLA-4 expression was not increased in T cells from lupus patients as a consequence of anti-CD3/CD28 stimulation. When CTLA-4 expression is increased in activated T cells its surface

expression is tightly regulated by a process of recycling to the plasma membrane surface [34], with the majority of CTLA-4 maintained in intracellular stores. To assess the recycling dynamics of CTLA-4, purified responder T cells were treated with PMA to induce its surface expression [34]. Confocal microscopy analysis revealed that *ex vivo* PMA-treated T cells from both healthy and lupus T cells show a similar pattern for CTLA-4 staining with large aggregates at or near the cell surface (Fig. 3B, left-hand panels). Upon 2 min incubation, however, a rapid change in staining pattern was seen. While T cells from healthy controls still presented areas of CTLA-4 aggregation, SLE responder T cells showed a more diffuse staining pattern, suggesting an increased internalisation rate (Fig. 3B, right-hand panels). This observation was supported by flow cytometry analysis (Fig. 3C) where the percentage internalisation rate was increased in *ex vivo* T cells from patients with SLE compared with healthy controls. In anti-CD3/CD28 activated cells the internalization and recycling rate of CTLA-4 in the healthy controls was increased to levels observed in the lupus T cells but no increase in CTLA-4 internalisation was noted in lupus T cells.These results suggest that the regulation of CTLA-4 at the cell surface is disrupted in patients with SLE.

Defective inhibition of T-cell activation by CTLA-4 in SLE

We next examined the function of CTLA-4 in the CD4+CD25-CD127+ responder T cells from patients with SLE. Initially, we noted that the proliferation of lupus responder T cells following CD3/CD28 activation was similar to their healthy counterparts (Fig. 4A). Cross-linking CTLA-4 significantly inhibited CD3/CD28 induced proliferation in healthy T-cells as expected. However, no reduction in anti-CD3/CD28 driven proliferation by CTLA-4 was observed in T cells from patients with SLE ($p=0.005$) (Fig.

4A and B). This lack of control by CTLA-4 on T-cell proliferation was accompanied by a failure to regulate anti-CD3/CD28 proximal signalling. Cross-linking CTLA-4 significantly reduced the CD3/CD28-induced phosphorylation of CD3-ζ (Fig. 4C and D) in responder T cells from healthy controls but not in lupus T cells. Taken together, the results suggest that CTLA-4-mediated inhibition of CD3/CD28 co-stimulation is lost in responder T cells from patients with SLE.

CTLA-4 is excluded from and fails to regulate membrane microdomains in lupus T cells

To investigate the established role of CTLA-4 as an inhibitor of membrane lipid microdomain formation during CD3/CD28 costimulation [6], FACS sorted responder T cells (CD4+CD25-CD127+) were stimulated in vitro using beads coated with anti-CD3/anti-CD287agonistic anti-CTLA-4 antibodies. After 72 h, a significant increase in membrane lipid GM1 (measured by binding to cholera toxin subunit B (CTB) [35] was noted in healthy T-responder cells stimulated via CD3/CD28 ($p=0.005$), which was downregulated by cross-linking CTLA-4 ($p=0.004$) as reported previously [6] (Fig. 5A upper panels). In contrast, ex vivo responder T cells from patients with SLE had a higher percentage of CTB1 cells, which increased upon CD3/CD28 co-stimulation ($p=0.04$). However, CTLA-4 cross-linking did not downregulate CTB binding in the lupus T cells (Fig. 5A bottom panels) supporting a disassociation between CTLA-4 and lipid-microdomain signalling in lupus T cells. T cells from patients with SLE are characterised by alterations in the localisation of membrane signalling molecule that can influence their function [14, 18, 36]. Therefore, we examined the location of CTLA-4 in relation to lipid microdomains by confocal microscopy. We reasoned that the

exclusion of CTLA-4 from membrane lipid microdomains could explain its failure to regulate CD3/CD28 co-stimulation. In activated healthy T-cells the majority of CTLA-4 was associated with lipid microdomains (Fig. 5B, purple colour in merged panel and Fig. 5C). In contrast, very little CTLA-4 (blue) and lipid raft (red) co-localisation was apparent in lupus T cells, indicating that the majority of CTLA-4 was excluded from membrane microdomains ($p=0.0006$) (Fig. 5B and C). These results demonstrate exclusion of CTLA-4 from membrane microdomains.

Discussion

The molecular mechanisms that underlie the aberrant T-cell activation in SLE have not been completely elucidated. Here we demonstrate abnormal CTLA-4 expression and function in lupus responder T cells, which could provide an explanation for their dysfunction in SLE. CTLA-4 was unable to inhibit lupus T-cell proliferation or regulate the induction of intracellular pathways induced by T-cell activation. CTLA-4 was displaced from membrane microdomains and unable to regulate either their expression or the intracellular signalling molecules triggered by T-cell activation. Given that T cells genetically engineered to lack CTLA-4 are resistant to tolerance induction [37], it is tempting to speculate that autoreactive lupus T cells are similarly affected due to deficiencies in CTLA-4 function. Thus the exaggerated T-cell responses characteristic of SLE could be attributed to CTLA-4 dysfunction. Although CTLA-4 can induce T-cell apoptosis [38] which might therefore be impaired in lupus due to CTLA-4 dysfunction, in fact lupus T cells display an increased rate of apoptosis compared with healthy T-cells [39–42]. It remains to be determined whether mechanisms

independent of CLTA-4 attempt to delete autoreactive T cells through increased apoptosis or their survival is impaired due to prolonged stimulation.

Lupus T cells exhibited a marked increase in proliferation as assessed by Ki67 staining supporting the notion that these cells have received substantial activation signals in vivo. CTLA-4 expression was elevated in the Ki671 responder T-cell population in both healthy controls and lupus patients, but the latter showed the highest expression consistent with their activated phenotype. This change in CLTA-4 expression in lupus responder T cells is likely to be a consequence rather than the cause of the abnormal T-cell activation in SLE. Although naïve and memory T cells are known to upregulate CTLA-4 upon activation *in vitro* with similar kinetics, the latter maintain elevated expression for longer time periods *in vitro* [30]. Therefore, alterations in the balance of naı¨ve and memory T cells may provide an explanation for the changes in CTLA-4 expression we have observed. However, it is unclear whether there is an increased ratio of memory to naïve T cells in lupus [14, 43, 44]. Intriguingly, although lupus T cells proliferated in response to CD3/CD28 co-stimulation in vitro, they were unable to upregulate CTLA-4 under the same conditions. This increased proliferation without associated increase in CTLA-4 expression may be partly responsible for the dysregulation characteristic of lupus T cells.

The increased expression of CLTA-4 ex vivo was irrespective of lupus disease activity perhaps suggesting an intrinsic, possibly genetic defect in CLTA-4 regulation. Importantly elevation in CLTA-4 is not a universal feature of peripheral T cells in patients with autoimmune rheumatic diseases since T cells from RA and PsA showed normal expression levels. Additional support for a role for CTLA-4 in the pathogenesis of lupus comes from genetic studies linking CTLA-4 with SLE [20–27]. One of these polymorphisms impairs the upregulation of CLTA-4 upon T-cell activation [45]. CTLA-

4 polymorphisms have been associated with a number of different autoimmune diseases, though the precise contribution of CTLA-4 to their pathogenesis may differ between diseases. Our observation that CTLA-4 was increased irrespective of disease activity would be consistent with a causal link rather than being as a consequence of disease.

The bulk of CTLA-4 is maintained in intracellular stores and its surface expression is tightly regulated by a process of exocytosis and continuous endocytosis [34]. As well as an elevation in CTLA-4 expression in lupus T cells, we noted that CLTA-4 internalisation was also increased ex vivo, which would tend to limit its expression.

However, whilst the rate of CTLA-4 recycling increased following *in vitro* activation of healthy T-cells as noted by others [46], the dynamics of CTLA-4 internalisation was unchanged upon activation of lupus T cells in vitro. CTLA-4 expression can be modified by increased transport to the cell surface. For instance, the T-cell receptor interacting molecule associates with CTLA-4 and promotes its delivery to the cell surface [47]. This results in increased CTLA-4 expression despite an increased rate of CTLA-4 internalisation. Therefore, if CTLA-4 is delivered to the cell surface at an accelerated rate in lupus T cells, its expression may remain elevated despite an increased rate of CTLA-4 internalisation. Surface expression of CTLA-4 is also governed by a motif within the cytoplasmic tail which when phosphorylated prevents its association with clathrin-associated AP-2 and hinders internalisation [48]. It is well established that proximal intracellular signalling is dysfunctional in lupus T cells and this may contribute to alterations in the kinetics of CTLA-4 recycling [49]. In addition, mutations in the amino acid sequence of the cytoplasmic tail of CTLA-4 can lead to its accumulation at the cell surface [50] although genetic studies have not indicated that mutations at this site predisposes to SLE. A soluble form of CLTA-4 has also been

described, which is predominantly expressed on resting T-cells [51] and is increased in the serum of patients with SLE [52, 53]. The soluble form of CLTA-4 lacks the transmembrane domain of the CTLA-4 gene implying that alterations in its expression are not secondary to changes in the recycling of membrane CTLA-4. CTLA-4 is an important marker of Treg, but the increase in CTLA-4 expression in lupus T cells was confined to the FOXP3 population. Although we found a substantial increase in the number of CD4+FOXP3+ T cells from lupus patients in agreement with some recent reports [33], this could be a further indicator of abnormal T-cell activation. Indeed a number of groups have noted a reduction of CD4+CD25hi Treg in SLE [54, 55]. FOXP3 expression is known to be increased upon activation of human T cells [56–58]. However, the increased numbers of CD4+FOXP3+ T cells found in autoimmune inflammatory environments such as EAE or the rheumatoid joint [59–61] has been used to support the argument that Treg are expanded in inflammation but are unable to establish control [62]. Although defective CTLA-4 function and an expanded CD4+FOXP3+ population in lupus patients may not be connected, there is a striking parallel found in CTLA-4-deficient mice [1, 2]. It was originally noted that there is a marked increase in the number of CD41CD251 T cells in mice lacking CTLA-4. The most intuitive explanation is that this simply reflects widespread T-cell activation. However, these CD4+CD25+ T cells all stain brightly for FOXP3 suggesting an expansion of Treg, which attempt unsuccessfully to control the ensuing lymphoproliferation and autoimmunity [63]. Recently, using antibody blockade, CTLA-4 has been shown to regulate Treg numbers and homeostasis in intact mice [12]. Notwithstanding these observations, the precise significance of increased FOXP3+ T cells in lupus patients remains unclear. Defining the relationship between defective

CTLA-4 function and the expanded CD4+ FOXP3+ population in lupus patients could be key in re-establishing tolerance in this disease.

The exact mechanisms by which CTLA-4 is able to inhibit T-cell activation have been studied extensively; cross-linking CTLA-4 in the context of CD3/CD28 stimulation has been shown to inhibit T-cell proliferation via interaction with proximal intracellular signalling molecules such as TCR-ζ [7, 64, 65]. We show here that CTLA-4 fails to regulate proximal T-cell signalling and T-cell proliferation in lupus patients. This paradoxical association between increased CTLA-4 expression and loss of function has not been reported before in the context of autoimmune disease or inflammation.

One mechanism to explain the molecular basis of CTLA-4 inhibitory action includes the location of CTLA-4 within membrane lipid microdomains or lipid rafts. In healthy activated T-cells, co-ligation of CTLA-4 during TCR stimulation strongly inhibits the upregulation of membrane lipid microdomains enriched with proximal signalling molecules [6]. We show here that in lupus T cells CTLA-4 is excluded from GM1+ lipid microdomains and its ligation does not inhibit GM1 expression following CD3/CD28 costimulation. During T-cell activation a pool of cell surface CTLA-4 is concentrated in lipid microdomains and associates with the TCR-ζ chain [7, 8]. Thus the exclusion of CTLA-4 from membrane microdomains may account for the inability of CTLA-4 to inhibit phosphorylation of proximal signalling molecules and T-cell proliferation in T cells from patients with SLE compared with healthy controls.

In summary, our findings demonstrate abnormalities in T-cell CTLA-4 biology in lupus patients, and provide new insight into the function of CTLA-4 in a disease setting. It is possible that targeting CTLA-4 on responder T cells, or altering the membrane microdomains that exclude CTLA-4, could restore T-cell function in patients with SLE.

Materials and Methods

Patients and controls

Peripheral blood was obtained from healthy donors and patients with SLE, RA and PsA attending the Rheumatology clinic at University College Hospital, London. Patients fulfilling the revised classification criteria of the American College of Rheumatology for lupus were included in this study. Disease activity was assessed using British Isles Lupus Assessment Group Score. All patients studied were receiving less than 10mg prednisolone and no immunosuppression other than hydroxychloroquine for at least 1 month before analysis. Inactive patients were either untreated or received only hydroxychloroquine. Age and sex-matched healthy donors were used as controls. All RA patients had a disease activity score (DAS28) of >5.1. PsA patients had at least three swollen and tenderjoints. CD4+ lymphocytes were purified from PBMC by negative selection (Miltenyi Biotech). T responders (CD4+CD25-CD127+) were isolated using a cell sorter (FACS-Aria, BD). Approval was given by the Ethics Committee of the University College Hospital London, patients and control donors gave their informed consent.

Antibodies and reagents

Antibodies for flow cytometry and confocal microscopy: FITC-CTLA-4 (48815) from R & D Systems; PECy7-CD25 (BC96), PECy5-CD127 (eBioDR5), and Alexa-Fluor®-488-FOXP3 (PCH101) from eBioscience; APC-, PE- or FITC-CD4 (RPA-T4), Biotin- or PE-CTLA-4 (BNI3.1), Biotin-CD28, PE-Ki67, PECy7-and APC-streptavidin and fluorochrome-conjugated isotype controls from BD Bioscience. Alexa Fluor®-594-CTB and goat anti-rabbit Alexa Fluors-647 from Invitrogen, FITC-CTB from Sigma. For biological assays, purified antibodies to CD3 (HIT3a), CD28 (CD28.2), CTLA-4 (BNI3)

from BD Bioscience. Antibody-coated stimulating beads Dynabeadss M-280 Tosylactivated (Dynal Biotech) were coated with anti-CD3, anti-CD28 (10mg/mL, each) and anti-CTLA-4 (50 mg/mL) antibodies (or isotype control) following manufacturer's instructions. The following antibody was used for protein phosphorylation analysis by flow cytometry: Alexa-Fluors-488-phospho-Y142 CD3-ζ (K25-407.69) from BD Biosciences.

Flow cytometry

Membrane staining for phenotyping was carried out with the relevant antibodies using 1% FBS-PBS for 30 min at room temperature. Intracellular staining was carried out after fixation/permeabilisation (eBioscience). Cell phenotyping was read using a BD-LSR I flow cytometer and analysed with FlowJo 6.4.7.

CTLA-4 recycling

FACS-sorted Treg were rested for 1 h at 371C, and then stimulated for 1 h at 37ºC with PMA (10 ng/mL). After two washes, cells were membrane-stained with FITC-CTLA-4 for 1 h in ice. Following staining, cells were incubated at 37ºC for different time periods and then fixed and mounted for confocal microscopy analysis. Alternatively, following staining for CTLA-4 and incubation at 37ºC, cells were acid-washed with PBS (pH 2.0) in order to remove non-internalised Ab-labelled CTLA-4. Cells were fixed with 2% PFA and analysed by flow cytometry. CTLA-4 internalisation rate was calculated as the ratio of MFI acid wash/MFI PBS wash x100.

Cell activation

For cell signalling analyses, FACS-sorted responder T cell were rested for 1 h in RPMI with 0.5% human serum. Cells were incubated with anti-CD3/CD28 antibodies (1 mg/10^6 cells, each) on ice for 20 min, washed with ice-cold PBS and anti-CD3/CD28 antibodies cross-linked by goat anti-mouse IgG F(ab')2 (2 mg/10^6cells) on ice for 20min. After washing, cells were resuspended in warm-PBS and incubated for 10min at 37ºC. Cells were fixed/ permeabilised with 2x Fix/Perm buffer (eBioscience) and stained with the appropriate antibody. Analysis of GM1 expression was measured in T-responder cells following 72 h stimulation beads coated with anti-CD3/CD287anti-CTLA-4 or isotype control and staining with CTB-FITC. For proliferation assays, T responders were incubated in the presence of anti-CD3/CD287anti-CTLA-4 F(ab')2 for 3 days and then pulsed with [3H]thymidine, incubated for 16 h before harvesting, and analysed in a scintillation counter.

Confocal microscopy

Images were taken with a 63x objective in a Leica DMIRE2 microscope. Analysis of co-localisation in composite merged RGB images was carried out with ImageJ software (NHS, UK).

Statistical analysis

All values are expressed as means±SEM. Analysis of significance was by the unpaired or paired two-tailed Student's t-test with Welch's correction for two groups.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends.

Figure 1. Increased expression of surface and intracellular CTLA-4 in T cells from patients with SLE. Representative FACS plots and cumulative data for percentage CD4+ T cells expressing (A) total (t) and (B) surface (s) CTLA-4. (C) Cumulative data of total CTLA-4 expression in CD4+ T-cells from healthy donors compared with patients with active and inactive lupus disease and patients with RA and PsA as disease controls. Active lupus defined by global British Isles Lupus Assessment Group scores of46. Data from 37 healthy donors and 40 patients with SLE. In (C), data from active eight patients with RA and eight patients with active PsA are included. Mean±SEM, *p=0.01, **p=0.008, ***p=0.0002.

Figure 2. Increased CTLA-4 is independent of FOXP3 expression but associated with increased T-cell activation in lupus patients. (A) Representative FACS plots and cumulative data of tCTLA-4 expression in CD4+FOXP3- and CD4+FOXP3+ T cells, **p=0.008. (B) CD28 and tCTLA-4 expression in CD41T cells, cumulative data shows CD28 MFI and % CD28+ in the CTLA-4+ population p=0.0001, (C) FOXP3 and Ki67 expression in CD4+ T cells, cumulative data shows Ki67 expression in the CD4+ FOXP3- subset **p=0.006. (D) PBMC from 33 healthy controls and 35 patients with SLE were stained for CD4 and FOXP3. The cumulative data indicates the percentage of CD4+FOXP3+ T cells, ***p=0.0001. (E) Representative histograms and cumulative data of tCTLA-4 expression in the CD41FOXP3 subset defined according to Ki67 expression, *p=0.01. Results from ten healthy donors and ten patients with SLE, mean±SEM.

Figure 3. Abnormal kinetics of CTLA-4 expression in lupus T cells following in vitro activation. T responder cells (CD4+CD25-CD127+) were isolated by FACS-sorting. (A) Cells were cultured in medium alone or with soluble antibodies to anti-CD3/CD28 for 24 h before staining for tCTLA-4. Cumulative results from five healthy and five patients with SLE, *p=0.01. (B) T-responder cells (CD4+CD25-CD127+) were treated with PMA to induce CTLA-4 accumulation at the cell membrane. PMA-treated cells were membrane-stained for CTLA-4 and then incubated to allow CTLA-4 internalisation. CTLA-4 internalisation was analysed by confocal microscopy (B) or flow cytometry (C). Microscopy analysis in (B) showed areas of CTLA-4 aggregation after 2 min in healthy T-cells (arrow) compared with a diffuse staining pattern seen in SLE T cells (arrow). Results are representative of six healthy controls and six patients with SLE. Results in (C) are expressed as the mean percentage of CTLA-internalisation±SEM *p=0.05.

Figure 4. Defective inhibition of lupus T-cell activation and proximal signalling by CTLA-4. (A) Purified healthy and lupus T responder cells (CD4+CD25-CD127+) were activated with anti-CD3/CD287anti-CTLA-4. Data from a representative experiment with unstimulated T cells shown as a control and (**p=0.005). (B) Cumulative data of the proliferation in cells stimulated with anti-CD3/CD28 compared with anti-CD3/CD28/CTLA-4 stimulated cells expressed as a percentage. Results from ten healthy donors and six patients with SLE, ***p=0.003. (C) FACS-sorted T-responder cells (CD4+CD25-CD127+) were stimulated with anti-CD3, anti CD28± anti-CTLA-4 antibodies (Materials and methods). Phosphorylation of signalling molecules was evaluated by flow cytometry after intracellular staining with phospho-specific

antibodies. (D) Cumulative data of the percentage change in levels of TCR-ζ tyrosine-phosphorylation in stimulated compared with non-stimulated purified responder T cells. Results are from five healthy controls and five lupus patients, *p=0.04.

Figure 5. Altered membrane localisation of CTLA-4 in lupus T cells. (A) Representative histograms and cumulative data showing lipid raft (CTB/GM1) expression in purified T-responder cells after culture with anti-CD3/CD287anti-CTLA-4 antibodies or isotype control (72 h). Data from six healthy controls and seven patients with active SLE. **p=0.005. (B) Purified responder T cells were labelled with CTB (red) followed by crosslinking with anti-CTB antibodies in order to patch the areas of lipid rafts in the membrane. Cells were fixed and stained for intracellular CTLA-4 (blue) and analysed by confocal microscopy. Colocalisation of CTLA-4 and CTB is indicated by pink colour. (C) Analysis of co-localisation of membrane microdomains and CTLA-4. Representative dot plots indicate the distribution of red (CTB) and blue (CTLA-4) pixels. Cumulative data showing the percentage of cells showing CTLA-4/CTB co-localisation. Representative images of at least 25 cells from 6 healthy controls and 6 patients with SLE. Bar=5 μM, ***p=0.0006.

Figures

Figure 1

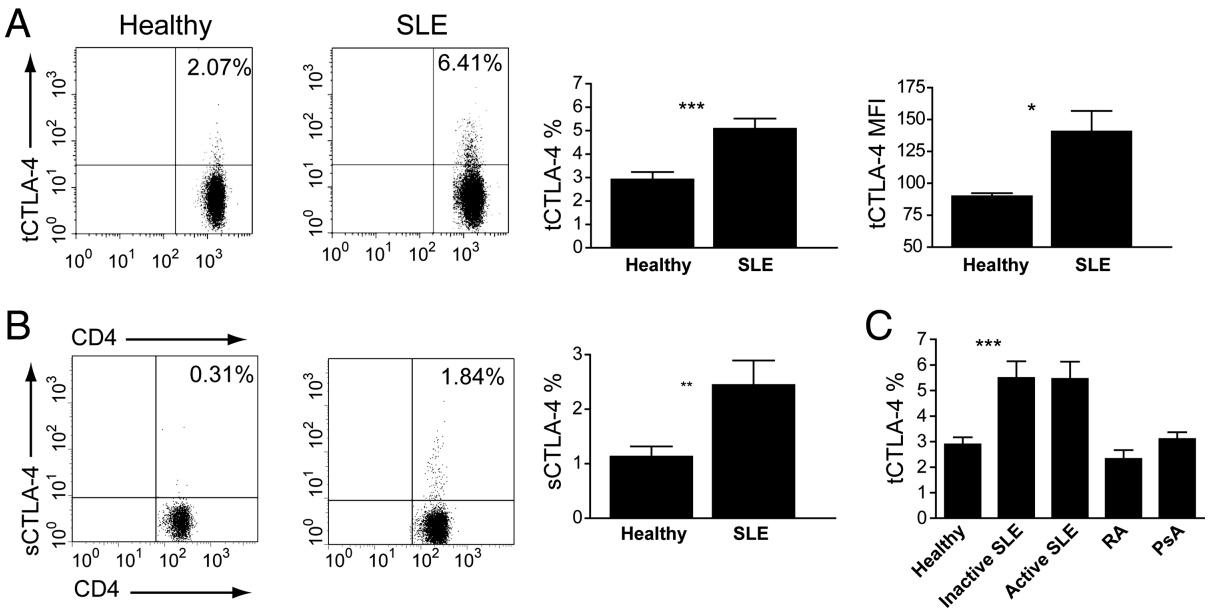
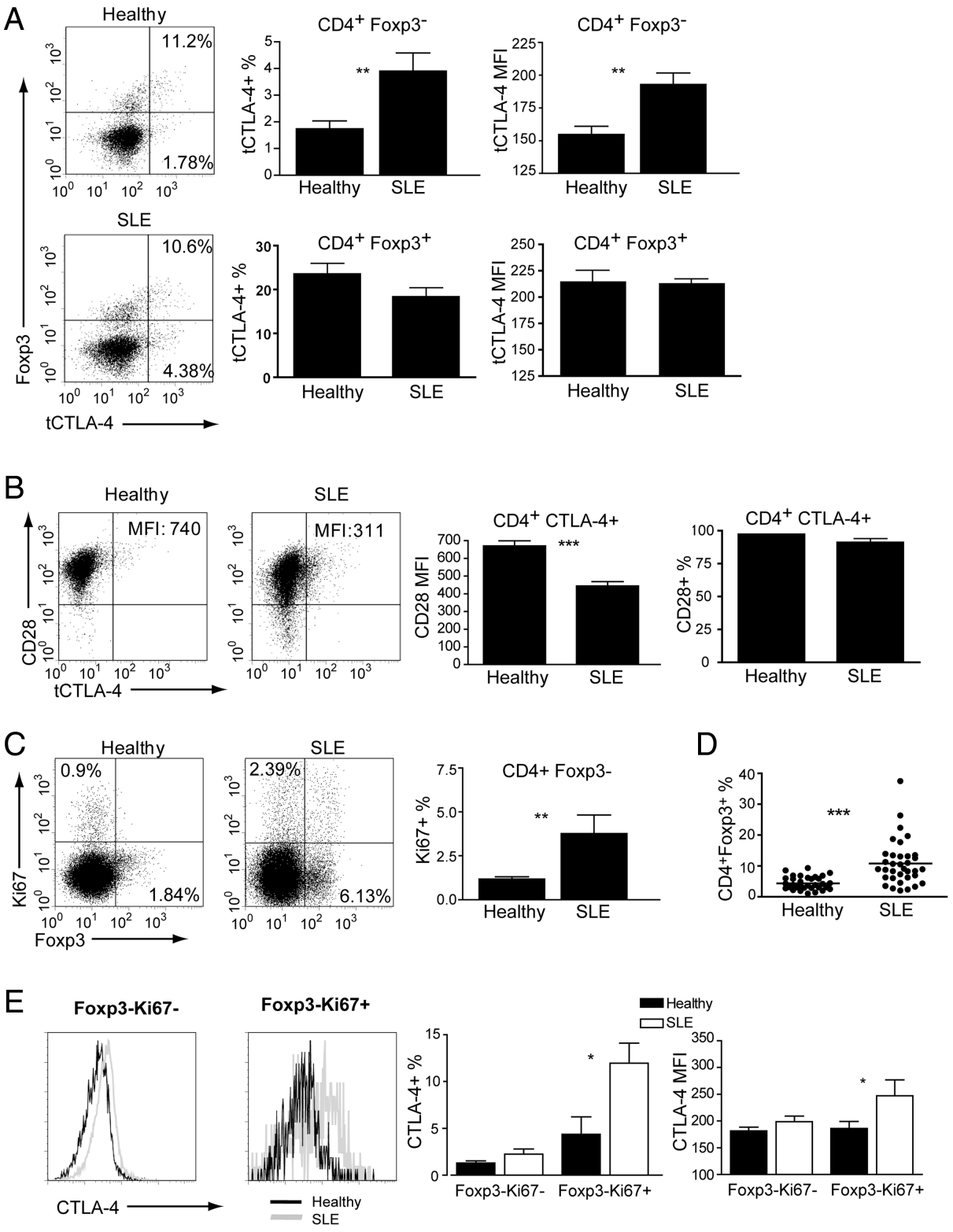


Figure 2


A
Healthy
11.2%
1.78%
SLE
10.6%
4.38%
Foxp3
tCTLA-4
CD4+ Foxp3-
tCTLA-4+ %
Healthy
SLE
CD4+ Foxp3-
tCTLA-4 MFI
CD4+ Foxp3+
tCTLA-4+ %
CD4+ Foxp3+
tCTLA-4 MFI
B
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MFI: 740
SLE
MFI:311
CD28
tCTLA-4
CD4+ CTLA-4+
CD28 MFI
CD4+ CTLA-4+
CD28+ %
C
Healthy
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1.84%
SLE
2.39%
6.13%
Ki67
Foxp3
CD4+ Foxp3-
Ki67+ %
D
CD4+Foxp3+ %
Healthy
SLE
E
Foxp3-Ki67-
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Healthy
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CTLA-4+ %
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CTLA-4 MFI

Figure 3

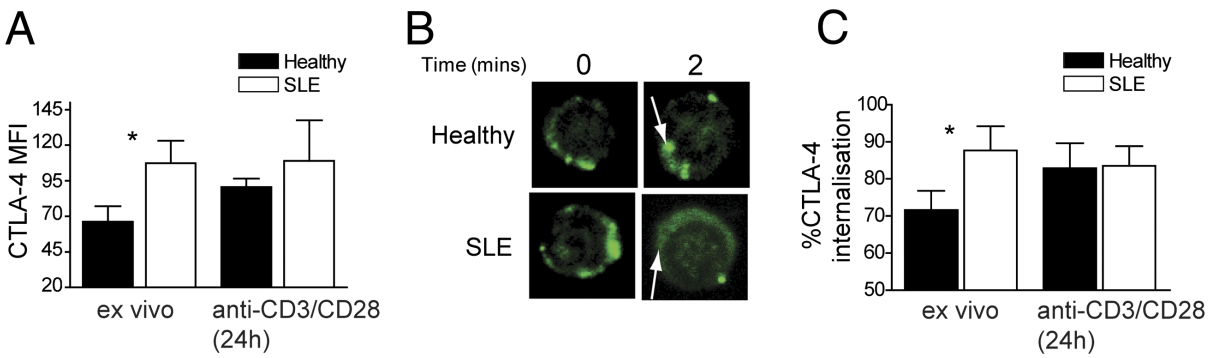


Figure 4

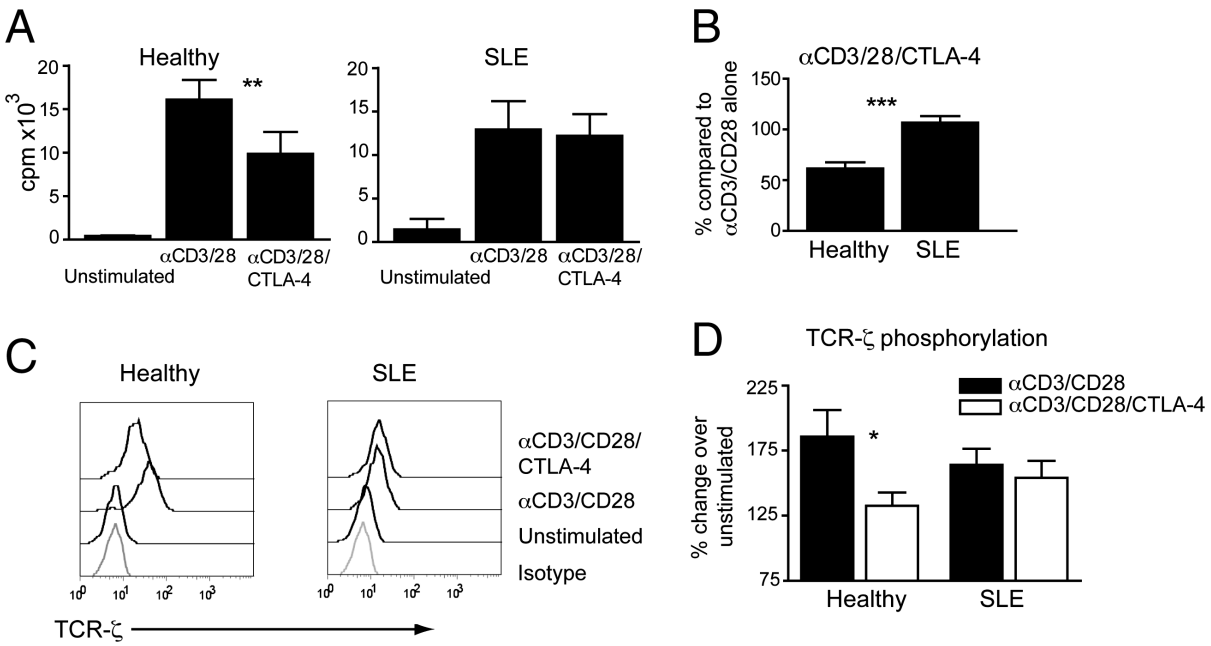
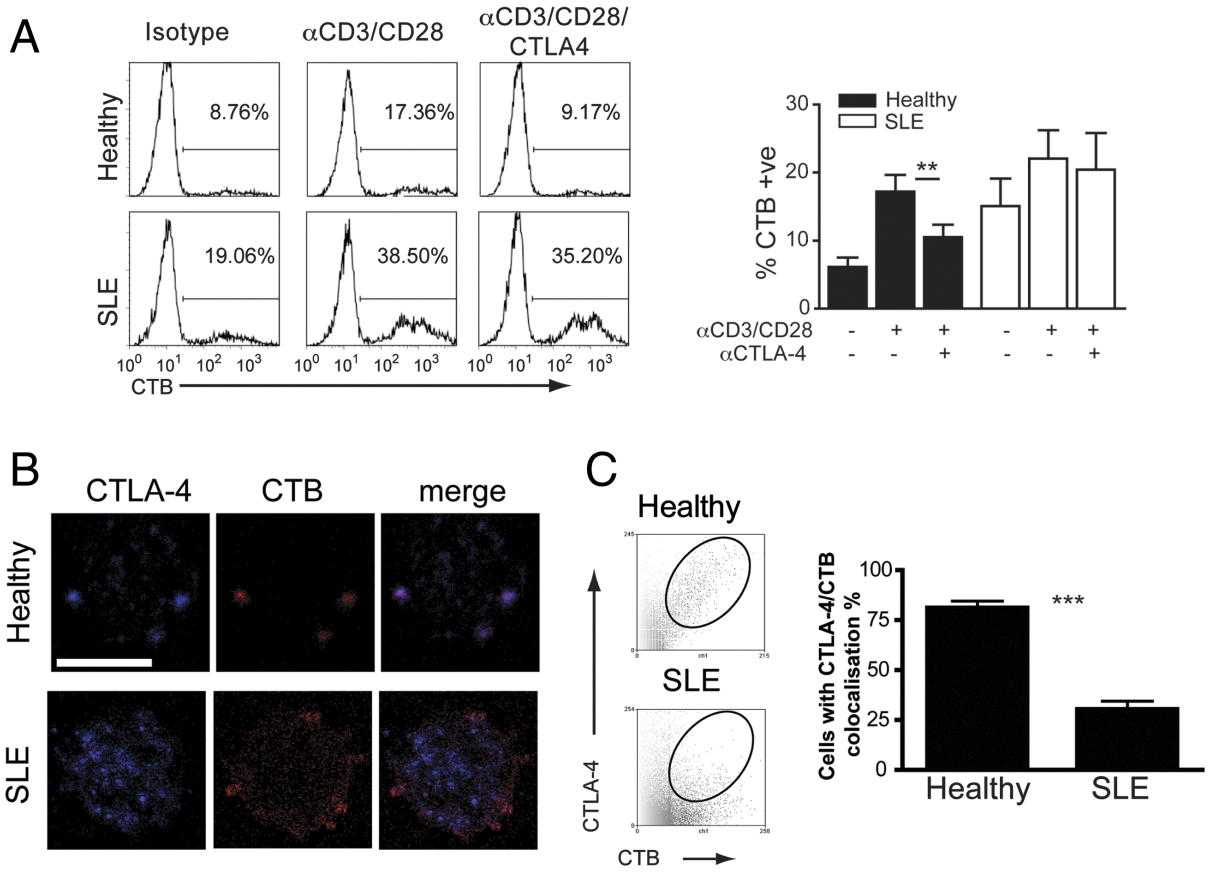


Figure 5


A
Isotype
αCD3/CD28
αCD3/CD28/
CTLA4
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8.76%
17.36%
9.17%
19.06%
38.50%
35.20%
CTB
% CTB +ve
Healthy
SLE
**
αCD3/CD28
αCTLA-4
B
CTLA-4
CTB
merge
C
Healthy
SLE
CTLA-4
CTB
Cells with CTLA-4/CTB
colocalisation %
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