Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4$^+$ CD25$^+$ regulatory T-cell-mediated suppression

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Summary

We have previously demonstrated that CD4$^+$ CD25$^+$ natural regulatory T cells (Treg cells) induce down-modulation of CD80 and CD86 (B7) molecules on dendritic cells (DCs) *in vitro*. In this report we show that the extent of down-modulation is functionally significant because Treg-cell conditioned DCs induced poor T-cell proliferation responses. Further, we report that down-modulation was induced rapidly and was inhibited by blocking cytotoxic T lymphocyte antigen-4 (CTLA-4), which is constitutively expressed by the Treg cells. Even though Treg cells have previously been reported to kill antigen-presenting cells, the down-modulation was not due to selective killing of DCs expressing high level of the costimulatory molecules. We propose that Treg cells down-modulate B7-molecules on DCs in a CTLA-4-dependent way, thereby enhancing suppression of T-cell activity.

Keywords: co-stimulation/costimulatory molecule; dendritic cells; regulatory T cells

Introduction

CD4$^+$ CD25$^+$ natural regulatory T cells (Treg cells) were first described by Sakaguchi and coworkers.[1] These cells have during the last few years been extensively studied both in the mouse and in man. Natural Treg cells were initially described as anergic cells that suppress T-cell proliferation *in vitro*. Recent evidence has shown, however, that natural Treg cells proliferate extensively *in vivo* (reviewed in 2) and also *in vitro* provided that interleukin-2 (IL-2) is present in culture.[3] The effector function of natural Treg cells is cell-contact dependent because these cells fail to suppress their target T cells when physically separated.[3,4] The observation that natural Treg cells fail to suppress B7-deficient T cells provides further evidence for the cell-contact dependency of suppression.[5]

The effector mechanism of natural Treg cells has remained elusive. Some data suggest that the regulatory function *in vitro* is transforming growth factor-β (TGF-β) dependent,[6] although this issue is controversial.[7] Another proposed function of the Treg cells is competition for IL-2,[8,9] which would be consistent both with their constitutive expression of the IL-2 receptor and the contact-dependence of their mechanism of suppression. However, the observation that Treg unresponsive to IL-2 effectively suppress T-cell proliferation indicates that competition for IL-2 is not essential for suppression.[10]

Early studies suggested that antigen-presenting cells (APC) would play only a minor role in suppression

Abbreviations: BM, bone marrow; GeoMFI, geometric mean fluorescence intensity; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; L-Nil, L-N^6-(1-iminoethyl)lysine hydrochloride; 1-MT, 1-methyl tryptophan; SA, streptavidin.

in vitro, because APCs could be fixed with aldehyde[3,4] and also be replaced by antibody-coated plastic beads.[11] However, more recent evidence indicates that natural Treg cells do communicate with APCs and in particular dendritic cells (DC). Thus, *in vitro* coculture of DC with natural Treg cells modulates DC function causing the induction of tolerogenic immune responses.[12–14] During coculture, natural Treg cells induce the expression of the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) in DCs, generating an immunosuppressive milieu that induces abortive immune responses.[12] The induction of IDO was shown to be cytotoxic T lymphocyte antigen-4 (CTLA-4) dependent[12] and may involve an interaction between CTLA-4 on the Treg cells and CD80-molecules on the DCs.[15] Further, DCs have been implicated in the expansion of natural Treg cells.[16,17] Recently it was shown that Treg-mediated suppression *in vivo* involves prolonged Treg/DC interactions but only transient Treg/T-cell interactions.[18] Studies performed in a mouse model for asthma also provided evidence suggesting an *in vivo* role of Treg as regulators of DC activation.[19] Thus, interaction with DCs is an emerging function of Treg in suppression *in vivo*.

Our previous work provided evidence that Treg cells may interfere with DC function.[20] We showed that natural Treg cells induced down-modulation of B7-molecules on cocultured DCs. In this report we have further investigated this mechanism. Our data show that down-modulation occurs rapidly, within the first hrs of coculture. Importantly, we show that this mechanism is CTLA-4-dependent indicating that the CTLA-4/B7 interaction between natural Treg cells and DCs apart from inducing IDO-activity also influences the costimulatory capacity of the DCs.

Materials and methods

Mice

BALB/c and C57BL/6 female mice were obtained from M & B, Ry, Denmark and were used for experiments at 6–12 weeks of age. *Cd28*$^{-/-}$ mice on C57BL/6 background were used for experiments at 6–8 weeks of age while *Ctla-4*$^{-/-}$ and wild type control mice on C57BL/6 background were used for experiments at 7–12 days of age.

Enrichment of cell populations

Single cell suspensions were prepared from pooled spleens and lymph nodes, the erythrocytes lysed and B cells were removed by panning on anti-mouse immunoglobulin-coated plates.[20] Two different protocols were used to enrich for $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells. 1) Total $CD4^+$ T cells were labelled with anti-CD4-fluoroscein isothiocyanate (FITC; Pharmingen, San Diego, CA) and were thereafter positively isolated using an anti-FITC Multisort Kit (magnetic-activated cell sorting; Miltenyi Biotec, Bergisch Gladbach, Germany). This procedure yielded >95% pure $CD4^+$ T cells. To further divide $CD4^+$ T cells into $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells, the isolated $CD4^+$ T cells were released from the beads. Subsequently the cells were incubated with biotin-conjugated CD25 antibodies (Pharmingen) followed by SA-microbeads (Miltenyi Biotec) and separated into $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells, yielding >95% pure $CD4^+$ $CD25^+$ T cells. 2) Alternatively, the pooled spleen and lymph node cells were directly incubated with biotin-conjugated CD25 antibodies (Pharmingen) followed by SA-microbeads (Miltenyi Biotec) and separated into $CD25^-$ cells and $CD4^+$ $CD25^+$ T cells as $CD25^+$ cells are almost exclusively found among $CD4^+$ T cells. $CD4^+$ $CD25^-$ T cells were subsequently isolated from the $CD25^-$ fraction using anti-CD4-conjugated magnetic microbeads (Miltenyi Biotec). We observed no functional differences between T cells prepared with these two protocols. To obtain more potent regulatory T cells purified $CD4^+$ $CD25^+$ T cells were cultured (1×10^6/ml) in 24-well plates (Falcon, BD) and stimulated by 10 μg/ml plate bound anti-CD3 (145.2C11) and 200 ng/ml recombinant mouse IL-2 (R & D Systems Inc., Minneapolis, MN) for 3 days.

DC were obtained by culturing total bone marrow (BM) cells in medium containing recombinant granulocyte–macrophage colony-stimulating factor, as previously described in detail.[20] DC were subsequently isolated with anti-CD11c conjugated magnetic microbeads (Miltenyi Biotec) yielding approximately 95% of $CD11c^+$ MHC II^+ (major histocompatibility complex II) cells, when analysed by flow cytometry. Alternatively, DCs were directly isolated from collagenase type IV (Sigma-Aldrich Inc., Sweden AB; 1·6 mg/ml, 30 min, 37°) treated spleens using anti-CD11c-conjugated microbeads (Miltenyi Biotec) and then resuspended in medium and left to adhere on plastic for 2 hr and after removal of non-adherent cells cultured for an additional 12 hr in medium or where indicated in the presence of 0·5 μg/ml of lipopolysaccharide (LPS) (Difco, Detroit, MI) to induce maturation. Alternatively, DCs were matured by 5 hr preculture *in vitro*. DCs that had been cocultured with either $CD4^+$ $CD25^-$ or $CD4^+$ $CD25^+$ T cells were purified using anti-CD11c-conjugated microbeads and then used for further cell cultures.

Cell cultures

Proliferation assays were performed in 200 μl cultures in round bottom 96-well plates (Costar, Cambridge, MA). T cells were polyclonally stimulated by the addition of 1 μg/ml of anti-CD3 antibodies (145·2C11) or 2·5 μg/ml concanavalin A (Con A; Amersham Pharmacia, Uppsala,

Sweden) to the cultures. Cells were cultured in RPMI medium (Gibco BRL, Grand Island, NY) supplemented with 50 μM 2-mercaptoethanol, antibiotics, 10% fetal calf serum, 1 mM sodium pyruvate and 10 mM HEPES buffer (all supplements from Gibco) at 37° and 5% CO_2. Thymidine incorporation was measured on day 3 of culture after a 4 hr pulse with 1 μCi [^{3}H]-thymidine (Amersham, Biosciences, Uppsala, Sweden). DCs were fixed with 1% paraformaldehyde. For flow cytometric analyses, DCs were cultured for the time indicated either in medium alone, or cocultured with anti-CD3 (1 μg/ml) stimulated $CD4^+$ $CD25^-$ and/or $CD4^+$ $CD25^+$ T cells, at the indicated ratios. IDO and inducible nitric oxide synthase (iNOS) activity was inhibited using 300 μM 1-methyl-D,L-tryptophan (1-MT; Sigma-Aldrich) and 300 μM L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL; Sigma-Aldrich), respectively.

Conjugation of fusion-proteins to latex beads

Latex beads (Interfacial Dynamics, Portland, OR) were conjugated with anti-CD3 (145.2C11) and CD80-Fc or CD86-Fc (both bought from R & D Systems Inc., Abingdon, UK) as previously described.[11] The beads were counted under a microscope before use in *in vitro* cell cultures.

Antibodies and flow cytometry

The following antibodies and fluorochrome-conjugated reagents were used for flow cytometry experiments: phycoerythrin (PE)-conjugated anti-CD86 (clone GL1, BD Biosciences, Pharmingen), biotin-conjugated anti-CD80 (clone 1610-A, Pharmingen) and PE-conjugated immunglobulin G1 (IgG1; Pharmingen), biotin-conjugated IgG1 (Pharmingen) as isotype controls, peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (clone RM4-5, Pharmingen), PE-conjugated anti-CD4 (clone L3T4, Pharmingen), Annexin V FITC (Molecular Probes, Leiden, Holland), 2 μg/ml 7-amino-actinomycin D (7AAD) (Sigma-Aldrich), FITC-conjugated anti-CD8α (clone YTS 169-41), Cy-5 conjugated anti-CD11c (clone N418), Cy-2 conjugated anti-CD4 (clone GK1.5), biotin-conjugated anti-D^d (clone HB102) (all prepared in our laboratory). Binding of biotin-conjugated CD80 was revealed in a second step using streptavidin (SA) Alexa Fluor 488 (Molecular Probes), SA-Cy5 (prepared in our laboratory), SA-Red 613 (Gibco) or SA-PE (Pharmingen). Intracellular staining of CTLA-4 was performed using fixation and permeabilization buffers, Cytofix/Cytoperm and Perm/Wash (Pharmingen), PE-conjugated anti-CTLA-4 (clone UC10-4F10-11, Pharmingen) and PE-conjugated IgG1 (Pharmingen) as isotype control. Stained cells were analysed with a FACSCalibur flow cytometer (Becton Dickinson, San José, CA). For cell culture experiments the following antibodies were used: anti-CD3 (145.2C11), anti-CD28 (37.51), anti-CD80 (1610-A), anti-CD86 (GL1) and 2.4G2 and all prepared in our laboratory. Purified anti-CTLA-4 (UC10-4F10-11) was purchased from Pharmingen.

Measurement of iNOS and IDO activity

NO production was measured as nitrite concentration using the Griess assay. Griess reagent was added (v/v) to cell culture supernatants or serial dilutions of standard ($NaNO_2$) in microplates. Plates were incubated for 10 min at room temperature and absorbance measured at 550 nm in an enzyme-linked immunosorbent assay reader. IDO activity was measured by quantifying tryptophan consumption and kynurenine production in tissue culture supernatants using a previously described method.[21]

Results

Rapid down-modulation of CD80 and CD86

We have previously shown that *ex vivo* Treg cells down-modulate the expression levels of both CD80 and CD86 on cocultured BM-derived DCs.[20] Similar down-modulation of CD80 and CD86 is also seen on splenic DCs in suppression cultures, where Treg cells in parallel inhibited the proliferation of $CD4^+CD25^-$ responder T cells (Fig. 1a). We next studied the kinetics of down-modulation by analysing the expression of B7-molecules on DCs at various time points of coculture with natural Treg cells. Down-modulation occurred within the first few hours of coculture and the reduced levels were maintained throughout the culture period (Fig. 1b). In contrast, B7-molecules were up-regulated on DCs cocultured with conventional $CD4^+$ $CD25^-$ T cells.

DCs conditioned by Treg cells are poor APC

Next we asked whether Treg cells influenced the functional capacity of DCs. To address this question, DCs were cocultured with natural Treg cells, purified and tested for their ability to induce proliferation of $CD4^+$ $CD25^-$ T cells in secondary cultures. However, when re-cultured with $CD4^+$ $CD25^-$ T cells in the absence of Treg cells the conditioned DCs regained their expression of the CD80 and CD86 molecules and effectively induced responder T-cell proliferation (data not shown). This may explain why Chai *et al.* failed to see any functional defect in DCs preconditioned with Treg cells *in vitro*.[22] Therefore, to preserve their 'conditioned' state we fixed the precultured DCs with paraformaldehyde before testing their ability to support anti-CD3 induced $CD4^+$ T-cell proliferation.

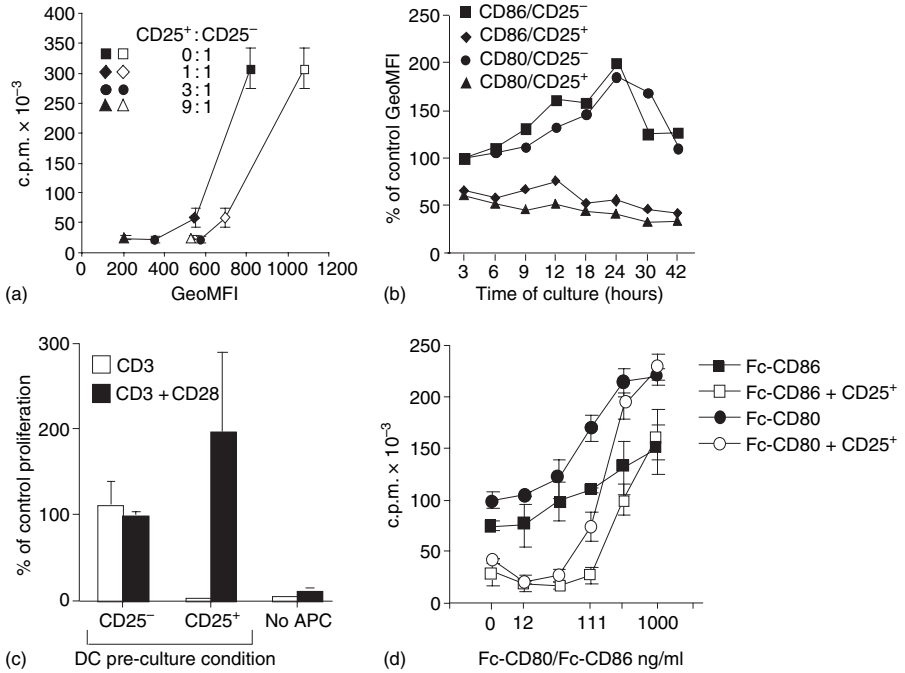

Figure 1. Correlation between suppression and down-modulation. (a) Splenic DCs were precultured for 5 hr *in vitro* in medium before re-culture ($2{\cdot}5 \times 10^4$/ml) with anti-CD3 and $CD4^+$ $CD25^-$ T cells (1×10^5/ml) in the absence or in the presence of $CD4^+$ $CD25^+$ T cells at the various ratios as indicated. T-cell proliferation was analysed after 72 hr and CD80 (open symbols) or CD86 (closed symbols) expression by $CD11c^+$ cells was analysed in parallel cultures after 12 hr. Rapid down-modulation of CD80 and CD86 on DCs. (b) BM-derived DCs (5×10^5/ml) were cultured either with $CD4^+$ $CD25^-$ T cells (1×10^6/ml) or $CD4^+$ $CD25^+$ T cells (2×10^6/ml) and stimulated with anti-CD3. The cocultures were harvested and the DCs analysed for cell surface expression of CD80 and CD86 at various time points as indicated. The results are presented as the percentage of the geometric mean fluorescence activity (GeoMFI; CD80 = 247 and CD86 = 816) obtained from analysis of DCs at the start of the experiment. Regulatory T cell-induced down-modulation of B7-molecules is functionally important. (c) BM-derived DCs ($0{\cdot}5 \times 10^6$/ml) were cultured for 48 hr in medium alone, or cocultured with anti-CD3-stimulated $CD4^+$ $CD25^-$ (1×10^6/ml) or $CD4^+$ $CD25^+$ T cells (2×10^6/ml). The DCs were thereafter isolated from the cultures, fixed with 1% paraformaldehyde and used as APCs (1×10^4/well) in triplicate cultures with fresh responder $CD4^+$ $CD25^-$ T cells ($2{\cdot}5 \times 10^4$/well) stimulated with anti-CD3 or with anti-CD3 + anti-CD28 (5 μg/ml). Proliferation was assayed after 72 hr of culture and the data are presented as the percentage of responder T cell proliferation induced by DCs precultured without T cells. Costimulation counteracts suppression. (d) $CD4^+$ $CD25^-$ T cells ($2{\cdot}5 \times 10^5$/ml) were cultured alone or in the presence of $CD4^+$ $CD25^+$ T cells ($8{\cdot}3 \times 10^4$/ml) in cultures containing an equal number ($2{\cdot}5 \times 10^5$/ml) of latex beads coated with various concentrations of Fc-CD80 or Fc-CD86 and anti-CD3 ($0{\cdot}5$ μg/ml) as indicated. Proliferation was assayed after 72 hr. Results from one experiment out of four (c) three (a and d) or two (b) with similar results are shown.

The fixed Treg-cell conditioned DCs induced poor $CD4^+$ T-cell proliferation (Fig. 1c). Importantly, addition of anti-CD28 antibodies restored the proliferation, suggesting that suboptimal CD28-signalling might be the cause for the poor proliferation. Co-culture with $CD4^+$ $CD25^-$ T cells increased the level of CD80 and CD86 on DCs (Fig. 1b) and DCs conditioned by these T cells, even after fixation, induced a robust proliferation response. These data demonstrate that DCs conditioned by Treg cells and $CD4^+$ $CD25^-$ T cells on a per cell basis are distinctly different in their ability to induce responder T-cell proliferation.

An increase in costimulatory signals circumvents suppression.[3,4,11,23] Consequently, reduced expression of costimulatory molecules on DCs would be expected to promote suppression. Fixation of the DCs prevents membrane movements but allows costimulation-dependent, albeit less efficient, induction of T-cell proliferation (data not shown). To mimic this situation, latex beads were coated with anti-CD3 and various concentrations of recombinant CD80 or CD86 proteins and used as surrogate APCs in suppression cultures.[11] As shown in Fig. 1(d), increased concentration of CD80 or CD86 resulted in increased responder T-cell proliferation. In suppression cultures, however, Treg cells inhibited responder T-cell proliferation most efficiently when stimulated with beads conjugated either with anti-CD3 alone or with anti-CD3 and low concentrations of CD80 or CD86. These data support the hypothesis that a reduction in B7-expression on Treg cell conditioned DCs is the cause for their poor capacity to stimulate T-cell proliferation (Fig. 1d).

Taken together, the present and the previous data[3,4,11] suggest some common denominators of live APCs, fixed APCs and antibody-coated beads. First, they provide a surface where regulatory and responder T cells can interact and receive T-cell receptor (via anti-CD3) and costimulatory signals (via CD80/CD86). Second, in all these cases suppression is more efficient when the responder T cells are exposed to a low level of costimulatory signals. In fact as suggested by previous reports[3,4,11] and supported by the data here (Fig. 1d), increasing the costimulatory signals inhibits the suppression, which in the effector phase involves direct regulatory/responder T-cell interactions.[24] We propose therefore that the main functional impact of down-modulation of the B7-molecules on APCs is to enhance suppression mediated by direct Treg cell/responder T-cell interactions.

Down-modulation is CTLA-4-dependent

Natural Treg cells, but not $CD4^+$ $CD25^-$ T cells, express CTLA-4 constitutively[25–27] and CTLA-4 has previously been implicated in the function of Treg cells.[26–28] Further, Treg cells induce IDO activity in DCs through a CTLA-4-dependent mechanism, implicating this molecule in Treg cell/DC interactions.[12] CTLA-4 therefore appeared as a likely candidate for a molecule mediating the rapid down-modulation of B7-molecules on the APCs.

To address this possibility we performed antibody-blocking experiments. As shown in Fig. 2(a), natural Treg cells either when cultured with DCs alone or when cocultured with responder T cells and DCs, decreased the expression of CD80 and CD86 on DCs. When a high concentration of anti-CTLA-4 antibody was added into such cultures, the down-modulation of both CD80 and CD86 was reversed. To avoid cross-linking by anti-CTLA-4, a saturating concentration of anti-Fc receptor antibody was added into the cultures.[29] Importantly, CTLA-4-blocking also interfered with suppression of proliferation, because the proliferation of responder T cells recovered to almost normal level in cocultures (Fig. 2b), confirming previous results.[27,28] It should be noted that CTLA-4-blocking did not significantly influence responder T-cell proliferation in the absence of natural Treg cells, indicating that the blocking indeed inhibited the performance of the Treg cells in the cocultures.

To address the involvement of CTLA-4 in the down-modulation in an independent way, we used $CD4^+$ $CD25^+$ T cells from *Ctla-4*-deficient mice. Because these mice develop a lymphoproliferative disease at about 2 weeks of age[30–32] we used cells from 7–12 day-old mice. Even though the $CD4^+$ $CD25^+$ T cells from *Ctla-4*-deficient mice express Foxp3[28] and were anergic (Fig. 2c), similar to Treg cells from age-matched control mice, they consistently failed to down-modulate CD80 and CD86 on cocultured APCs (Fig. 2d). This further supports the observation that down-modulation is CTLA-4 dependent. Natural Treg cells from *Cd28*-deficient and normal mice down-modulated B7-molecules to a similar extent.

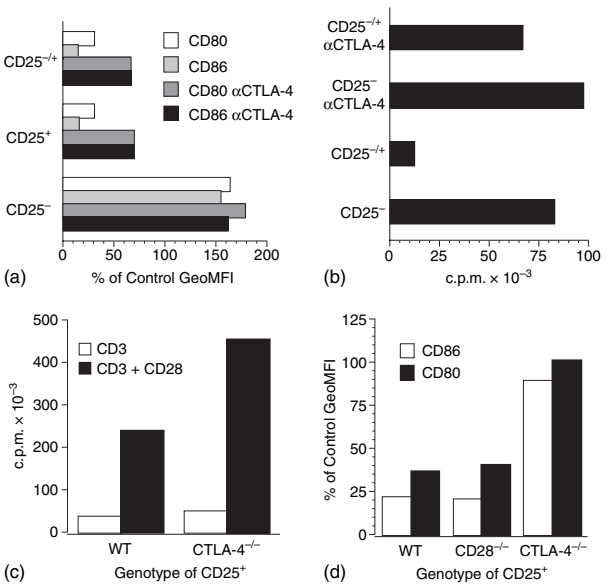

Figure 2. Down-modulation of B7-molecules and suppression of T-cell proliferation is CTLA-4-dependent. (a) BM-derived DCs ($0{\cdot}5 \times 10^6$/ml) were cultured either in medium alone, or with wild type $CD4^+$ $CD25^+$ T cells (2×10^6/ml) ($CD25^+$), $CD4^+$ $CD25^-$ T cells ($CD25^-$), or a 1 : 1 mixture of T cells ($CD25^{+/-}$) (2×10^6/ml) and Con A (2·5 μg/ml). Where indicated, anti-CTLA-4 (100 μg/ml) together with Fc-receptor blocking antibody 2.4G2 (2·5 μg/ml) were included in the cultures. The cultures were harvested and the DCs analysed for cell surface expression of CD80 and CD86 after 48 hr of culture. The results are presented as percentage of the GeoMFI (CD80 = 534 and CD86 = 581) obtained from analysis of DCs grown in the absence of T cells. (b) Parallel cultures to those in (a) were assayed for proliferation after 48 hr of culture. *Ctla-4*-deficient $CD4^+$ $CD25^+$ T cells are anergic but do not down-modulate B7-molecules on DCs. (c) $CD4^+$ $CD25^+$ T cells ($2{\cdot}5 \times 10^4$/well) from *Ctla-4*-deficient and normal (wild type) mice were cultured with DCs (1×10^4/well) and anti-CD3 (1 μg/ml) or anti-CD3 + anti-CD28 (5 μg/ml) and proliferation assayed after 72 hr (d) DCs ($0{\cdot}5 \times 10^6$/ml) were cultured for 48 hr either in medium alone or with anti-CD3 stimulated (1 μg/ml) $CD4^+$ $CD25^+$ T cells (2×10^6/ml) either from wild type, *Cd28*-deficient or *Ctla-4*-deficient mice. Cells were harvested, stained and $CD11c^+$ cells were analysed for CD80 and CD86 cell surface expression by flow cytometry. The results are presented as percentage of the GeoMFI (CD80 = 402 and CD86 = 2184) obtained from analysis of DCs grown in the absence of T cells. In (d) only age-matched wild type controls for the *Ctla-4*-deficient mice are shown, because the adult controls for the *Cd28*-deficient mice gave similar results. The data shown are representative of two to four independent experiments.

As previously reported[27] we found that *Cd28*-deficient Treg cells also suppressed responder T-cell proliferation (data not shown). In our hands, the ability of

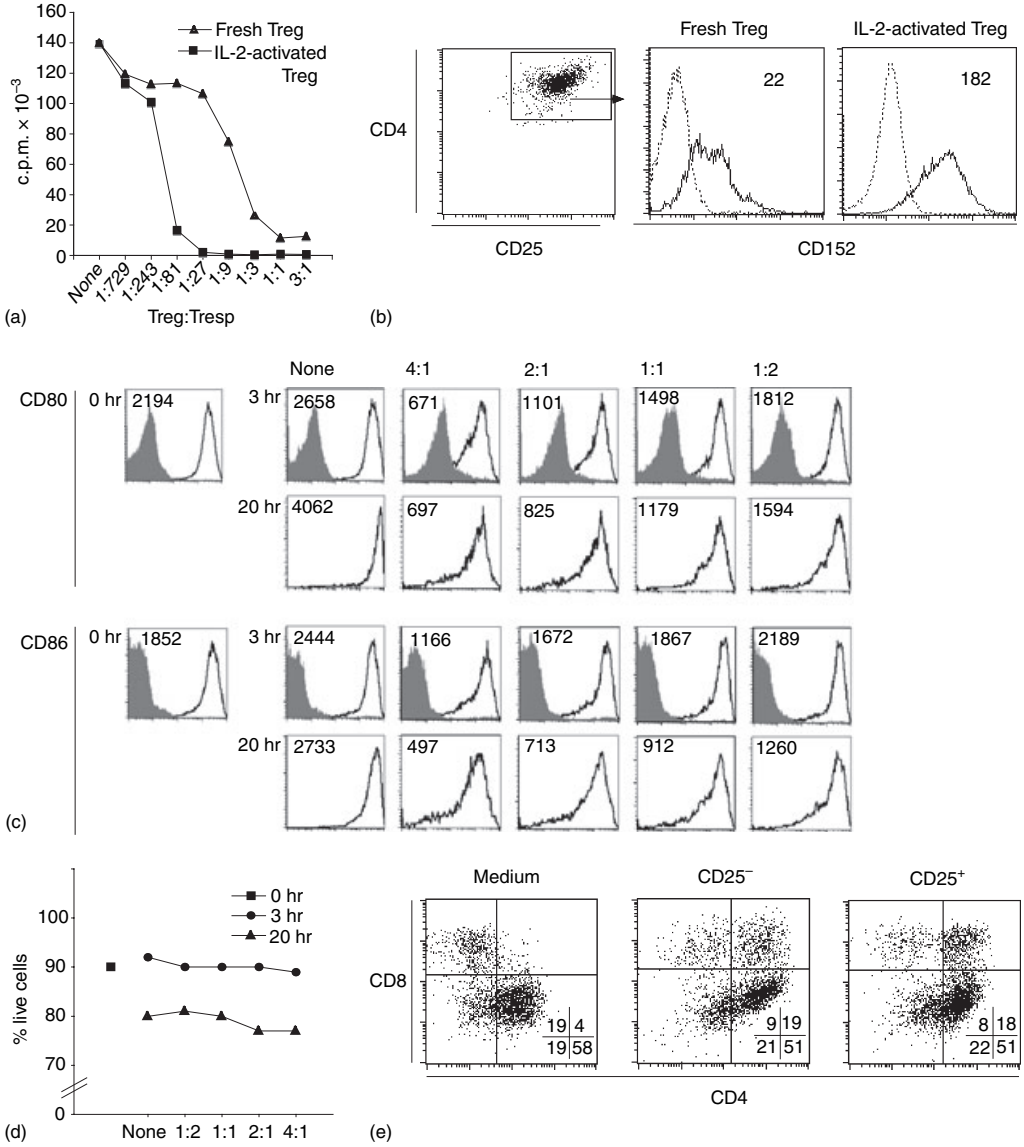

Figure 3. Activated Treg cells express elevated levels of CTLA-4 and are potent suppressor cells. (a) $CD4^+$ $CD25^-$ responder T cells were cultured ($2{\cdot}5 \times 10^5$/ml) either in medium alone (None), or at various ratios with either *ex vivo*, or IL-2-activated Treg cells, splenic DCs ($2{\cdot}5 \times 10^4$/ml) and anti-CD3 (1 μg/ml). Proliferation was assayed after 72 hr of culture. (b) *Ex vivo* or IL-2-activated Treg cells were stained with anti-CD4 and anti-CD25 and with anti-CTLA-4 (filled line) or isotype control antibody (dotted line) after permeabilizing the cells. GeoMFI of CTLA-4 is indicated in the histograms. Down-modulation of B7-molecules does not depend on apoptosis of, or selection for DC subpopulations. (c) Splenic DCs were cultured ($2{\cdot}5 \times 10^5$/ml) either in medium alone (None), or in the presence of activated Treg cells at the indicated ratios. Cells were stained with anti-CD11c and anti-CD80, anti-CD86 (or isotype controls for anti-CD80 and anti-CD86) and 7AAD and Annexin V both at the start of culture (0 hr) and at 3 hr and 20 hr of culture, respectively. CD80 and CD86 expression (open histograms; Geo MFI is indicated) or isotype controls (filled histograms) on $CD11c^+$ cells and (d) the percentage of $7AAD^-$ Annexin V^-, i.e. live cells among $CD11c^+$ cells was determined by fluorescence-activated cell sorting (FACS). Data from one of four experiments with similar results are shown. (e) Splenic DCs were cultured ($2{\cdot}5 \times 10^5$/ml) for 12 hr, either in medium alone, or with *ex vivo* $CD4^+$ $CD25^-$ T cells or IL-2-activated Treg cells and anti-CD3 (1 μg/ml), at 1 : 2 ratio between DCs and T cells. Thereafter cells were stained with anti-CD11c, anti-CD4 and anti-CD8 and analysed by FACS. Subset composition of $CD11c^+$ cells is indicated in percent in the plots. Data from one of two experiments with similar results are shown.

Ctla-4-deficient $CD4^+$ $CD25^+$ T cells to suppress responder T-cell proliferation[28,33] was variable. In some experiments these cells inhibited T-cell proliferation similarly to normal control Treg cells, while in other experiments suppression was bimodal with initial inhibition at low $CD4^+$ $CD25^+$/responder T-cell ratios followed by recovery of proliferation at high ratios (data not shown). Taken together, the results from both these approaches indicate

that down-modulation of B7-molecules on DC is CTLA-4 dependent.

Activated Treg cells potently down-modulate B7-expression but do not reduce DC survival

Natural Treg cells were initially described as anergic cells, but divide extensively *in vitro* when activated in the presence of IL-2.[8,34] Such *in vitro* activated Treg cells efficiently suppressed T-cell proliferation in secondary cultures (Fig. 3a) and expressed an increased level of CTLA-4 (Fig. 3b). Furthermore, the activated cells efficiently down–modulated B7-expression on DCs (Fig. 3c).

It has been reported that both human and mouse Treg cells can kill APCs such as DCs and B cells,[35,36] suggesting that the down-modulation of B7-molecules could be the consequence of selective death of DCs expressing high level of these molecules. To address this possibility, we determined the fraction of apoptotic DCs in cocultures with activated Treg cells. Down-modulation of B7-molecules was detectable in DC cocultured with Treg cells for 3 hr (Fig. 3c), confirming the data obtained using BM-derived DCs and *ex vivo* Treg cells (Fig. 1b). As shown in Fig. 3(d), there was no increase in apoptotic DCs at 3 hr of culture as compared to DCs analysed at the start of the culture or compared to control DCs cultured in the absence of Treg cells. The down-modulation was more pronounced at 20 hr of coculture. The reduced survival observed in these DCs, was Treg-independent since it was also observed in DCs grown in their absence. Co-culture with the activated Treg cells did not change the composition of the DC population (Fig. 3e). The increased frequency of $CD4^+$ $CD8\alpha^+$ DC observed in coculture with activated Treg cells as well as with $CD4^+$ $CD25^-$ T cells may represent uptake of T-cell membrane by the $CD8\alpha^+$ DCs, as previously demonstrated.[37] Thus, we could exclude that down-modulation was caused by selective survival of DCs expressing low level of B7-molecules.

Maintenance of Treg cells has been reported to be dependent on CD40-L/CD40 interactions[17,38] and activated $CD4^+$ T cells induce expression of iNOS in DCs via CD40-L/CD40 interactions.[39] Further, Treg cells induce IDO expression in DCs via CTLA-4/B7 interactions. We therefore investigated whether these pathways might be involved in the Treg cell-mediated down-modulation of B7 molecules on DCs. Addition of pharmacological inhibitors of these pathways to cocultures of activated Treg cells and DCs did not reduce the down-modulation (Fig. 4). Control experiments confirmed that the inhibitors inhibited LPS-induced NO-production by peritoneal cells and kynurenine production by LPS + interferon-γ-activated spleen-derived DCs (data not shown). We conclude therefore that the iNOS and IDO pathways are not involved in the down-modulation mechanism.

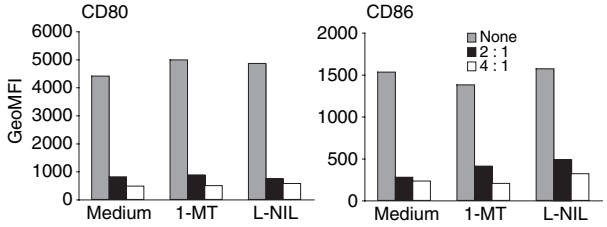

Figure 4. Down-modulation of B7-molecules is independent of IDO activity and NO production. (a) Splenic DCs were cultured ($2{\cdot}5 \times 10^5$/ml) for 20 hr in medium alone (None) or at the indicated ratios with IL-2-activated Treg cells and anti-CD3 (1 μg/ml), either in the presence of the IDO inhibitor 1-MT (300 μM) or of the NO inhibitor L-NIL (300 μM). Cells were thereafter stained with anti-CD11c, anti-CD80 and anti-CD86 and the GeoMFI of CD80 and CD86 on $CD11c^+$ cells was determined by FACS. Data from one of three experiments with similar results are shown.

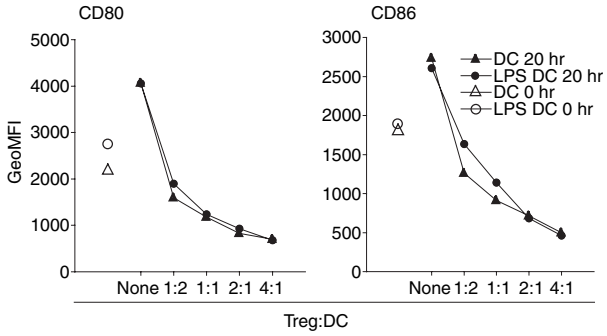

Figure 5. LPS does not render DCs refractory to modulation by regulatory T cells. Splenic DCs were precultured for 12 hr (2×10^6/ml) in medium or in the presence of LPS (0·5 μg/ml) and re-cultured ($2{\cdot}5 \times 10^5$/ml) for 20 hr either in medium alone (None), or at the indicated ratios with IL-2-activated Treg cells and anti-CD3 (1 μg/ml). Cells were stained with anti-CD11c, anti-CD80 and anti-CD86 and the GeoMFI of CD80 and CD86 on $CD11c^+$ cells was determined by FACS at the start of the second culture (0 hr) and at 20 hr of culture. Data from one of three experiments with similar results are shown.

The above data demonstrated that Treg cells down-modulated B7-molecules on both bone marrow-derived DCs and splenic DCs. It is well established that activation of DCs with bacterial components induces both functional and phenotypic changes in these cells. However, activated Treg cells down-modulated B7-expression equally efficiently on DCs matured *in vitro* in the presence or absence of LPS (Fig. 5) further supporting the generality of the mechanism.

Discussion

We have previously reported that *ex vivo* Treg cells down-modulate B7-molecules on cocultured DCs in a cell-contact dependent way.[20] In this report we have

addressed the functional significance and the mechanism responsible for the down-modulation. We show that the down-modulation occurred within the first few hours of *in vitro* culture and that it was functionally significant because Treg-cell conditioned DCs induced poor T-cell proliferation responses compared to DCs conditioned by conventional $CD4^+$ T cells. We provide evidence supporting the view that reduced expression of B7-molecules causes that deficiency. The mechanism is selective for some membrane molecules as MHC II molecules were not down-modulated in the same way.[40] Certain anergic Treg cells on the other hand can by an unknown mechanism down-modulate both B7- and MHC II molecules.[41]

Activation of Treg cells in the presence of exogenous IL-2 increases their regulatory function.[42] Our previous data demonstrated that *in vivo* superantigen-activated Treg cells, as compared to *ex vivo* Treg cells, were more efficient both in down-modulating B7-molecules on DCs and in inhibiting T cell proliferation.[40] We show here that *in vitro* activated Treg cells did not selectively kill DCs expressing high level of B7-molecules nor did they detectably select for certain DC populations. Thus, the down-modulation is most likely caused by a direct effect on the cocultured DCs and not caused by changing the composition of the DC population.

The *in vivo* superantigen-activated Treg cells in our previous report[40] and the *in vitro*-activated Treg cells used in the present report, both expressed high level of CTLA-4 as compared to *ex vivo* Treg cells. This protein has been implicated in Treg cell function both *in vivo* and *in vitro*,[12,26,27] although its role in Treg cell-mediated suppression of T-cell proliferation *in vitro* still is controversial.[27,28,33,42] It has been shown that *Ctla-4*-deficient Treg cells can suppress T-cell proliferation *in vitro*.[28,33] However, this suppression was partially TGF-β1 dependent and might represent a compensatory mechanism, as suppression by *Ctla-4*-sufficient Treg cells was shown to be TGF-β1 independent.[28]

In this report we present data indicating that the down-modulation of B7-molecules by Treg cells is CTLA-4-dependent. Addition of anti-CTLA-4 antibodies to *in vitro* cocultures of Treg cells and DCs significantly reduced the down-modulation of B7-molecules and also inhibited suppression of T-cell proliferation. Treg cells from *Ctla-4*-deficient mice did not down-modulate the B7-molecules of cocultured DCs, consistent with the antibody blocking data. In contrast to previous reports[28,33] *Ctla-4*-deficient Treg cells in our laboratory did not suppress T-cell proliferation as reproducibly as Treg cells from normal mice. Even though we used young mice in these experiments we believe that the variability might be caused by contamination of the Treg cell population by activated conventional $CD4^+$ $CD25^+$ T cells. Such cells might produce IL-2 and could therefore interfere with the suppression when present in sufficiently high numbers.

Down-modulation might be the result of internalization of B7-molecules by the DCs or by the Treg cells themselves. Indeed, there is evidence that T cells can acquire membrane molecules from APCs.[43] In either of these cases, CTLA-4 might either be directly involved in the internalization process or alternatively in transmitting signals leading to this event. Another possibility would be that CTLA-4 molecules, secreted or shed locally in the cell contact area, would block the costimulatory molecules. Putative soluble forms of the CTLA-4 molecule have been reported in both human and in the mouse.[44,45] Whatever the role of CTLA-4 might be, its role in down-modulation is specific for Treg cells, because activated conventional $CD4^+$ T cells, even though they up-regulate CTLA-4 expression upon activation[46] fail to down-modulate B7-expression on DC (Fig. 1b). We propose that CTLA-4 may have several functional roles in suppression. First, as shown here, it mediates down-modulation of B7-molecules on the DC. Second, it is involved in the activation of the tryptophan metabolism, at least in DC[12]. Third, cross-linking of CTLA-4 induces production of the immunosuppressive cytokine TGF-β1.[47]

Previous studies from other laboratories have suggested that the APC has only a passive role in the *in vitro* suppression of T-cell proliferation by Treg cells.[3,11,24,48] Further, antibody-coated latex beads can replace APCs in suppression.[11] Our present results and those of Ermann *et al.*[11] indicate that even when presented on a rigid matrix (latex beads) the level of B7-signals clearly influences suppression. Thus, at a given suppressor to responder T-cell ratio, suppression was only seen when the level of B7-signals was low[11] or in the absence of deliberate B7-signals, as shown here. We therefore propose that the essential role,[6] of both live and surrogate APCs in contact dependent suppression of $CD4^+$ T cells, is to bring the Treg cells and responder T cells in close physical contact and to activate the T cells. In fact, in parallel experiments using the same latex beads and cell concentration, we observed suppression in round-bottom microplates and enhanced proliferation in flat bottom microplates (C. Oderup, unpublished data). The fact that surrogate APCs can replace live APCs *in vitro* does, however, not exclude an active role of live APCs in suppression. This conclusion is supported by a substantial number of reports that indicate that both natural Treg cells and anergic Treg cells functionally interact with APCs.[12–14,41,49–51] Recent *in vivo* studies provided further evidence supporting that Treg may regulate DC activity *in vivo*.[18,19]

Originally natural Treg cells were described as anergic cells, exhibiting poor proliferation when activated *in vitro*. However, although these cells produce little IL-2, they proliferate extensively when exposed to exogenous IL-2 in culture.[8,42] Recent reports have demonstrated that both responder T cells and natural Treg cells divide in suppression cultures.[8,42] Treg cells efficiently bind and consume

IL-2 and IL-2 production by the responder T cells therefore becomes a critical component of *in vitro* suppression. Induction of T-cell proliferation and IL-2 production are both costimulation dependent. Consequently, the down-modulation mechanism could potentially interfere with T-cell activation and IL-2 production by activated responder T cells in the suppression cultures. In addition, the natural Treg cells, which constitutively express CTLA-4, would be expected to more efficiently engage remaining B7-molecules than the responder T cells, therefore promoting suppression rather than T-cell proliferation.

In conclusion, we propose that the role of the APC in suppression is twofold. First, it is required to establish Treg cells to responder T-cell contact and activation. Second, it promotes suppression by decreasing its expression of costimulatory molecules and by producing suppressive molecules. Because the effector phase of suppression only requires direct interaction between the Treg cells and its T-cell target, one can replace the APCs with various substitutes in *in vitro* settings, which fulfil the requirement of both activating the regulatory T cell and bringing the two T cells together.

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References

1 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**:1151–64.

2 von Boehmer H. Dynamics of suppressor T cells: in vivo veritas. *J Exp Med* 2003; **198**:845–9.

3 Takahashi T, Kuniyasu Y, Toda M *et al.* Immunologic self-tolerance maintained by $CD25^+$ $CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 1998; **10**:1969–80.

4 Thornton AM, Shevach EM. $CD4^+$ $CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 1998; **188**: 287–96.

5 Paust S, Lu L, McCarty N, Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. *Proc Natl Acad Sci USA* 2004; **101**:10398–403.

6 Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4 (+) CD25 (+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001; **194**:629–44.

7 Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, Mizuhara H, Shevach EM. CD4 (+) CD25 (+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 2002; **196**:237–46.

8 de la Rosa M, Rutz S, Dorninger H, Scheffold A. Interleukin-2 is essential for $CD4^+$ $CD25^+$ regulatory T cell function. *Eur J Immunol* 2004; **34**:2480–8.

9 Scheffold A, Huhn J, Hofer T. Regulation of CD4 (+) CD25 (+) regulatory T cell activity: it takes (IL-) two to tango. *Eur J Immunol* 2005; **35**:1336–41.

10 Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 2005; **6**:1142–51.

11 Ermann J, Szanya V, Ford GS, Paragas V, Fathman CG, Lejon K. CD4 (+) CD25 (+) T cells facilitate the induction of T cell anergy. *J Immunol* 2001; **167**:4271–5.

12 Fallarino F, Grohmann U, Hwang KW *et al.* Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 2003; **4**:1206–12.

13 Serra P, Amrani A, Yamanouchi J *et al.* CD40 ligation releases immature dendritic cells from the control of regulatory $CD4^+$ $CD25^+$ T cells. *Immunity* 2003; **19**:877–89.

14 Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Cutting edge. human $CD4^+$ $CD25^+$ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J Immunol* 2004; **172**:4676–80.

15 Zheng Y, Manzotti CN, Liu M, Burke F, Mead KI, Sansom DM. CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells. *J Immunol* 2004; **172**:2778–84.

16 Fehervari Z, Sakaguchi S. Control of $Foxp3^+$ $CD25^+$ $CD4^+$ regulatory cell activation and function by dendritic cells. *Int Immunol* 2004; **16**:1769–80.

17 Guiducci C, Valzasina B, Dislich H, Colombo MP. CD40/CD40L interaction regulates $CD4^+$ $CD25^+$ T reg homeostasis through dendritic cell-produced IL-2. *Eur J Immunol* 2005; **35**:557–67.

18 Tang Q, Adams JY, Tooley AJ *et al.* Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 2006; **7**:83–92.

19 Lewkowich IP, Herman NS, Schleifer KW *et al.* $CD4^+$ $CD25^+$ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med* 2005; **202**:1549–61.

20 Cederbom L, Hall H, Ivars F. $CD4^+$ $CD25^+$ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* 2000; **30**:1538–43.

21 Laich A, Neurauter G, Widner B, Fuchs D. More rapid method for simultaneous measurement of tryptophan and kynurenine by HPLC. *Clin Chem* 2002; **48**:579–81.

22 Chai JG, Tsang JY, Lechler R, Simpson E, Dyson J, Scott D. $CD4^+$ $CD25^+$ T cells as immunoregulatory T cells *in vitro*. *Eur J Immunol* 2002; **32**:2365–75.

23 George TC, Bilsborough J, Viney JL, Norment AM. High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression in vitro. *Eur J Immunol* 2003; **33**:502–11.

24 Piccirillo CA, Shevach EM. Cutting edge: control of CD8 (+) T cell activation by CD4 (+) CD25 (+) immunoregulatory cells. *J Immunol* 2001; **167**:1137–40.

25 Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+$ $CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 2000; **12**:431–40.

26 Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25 (+) CD4 (+) regulatory cells that control intestinal inflammation. *J Exp Med* 2000; **192**:295–302.

27 Takahashi T, Tagami T, Yamazaki S *et al.* Immunologic self-tolerance maintained by CD25 (+) CD4 (+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 2000; **192**:303–10.

28 Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-beta in $CD4^+$ $CD25^+$ regulatory T cell function. *Eur J Immunol* 2004; **34**:2996–3005.

29 Walunas TL, Lenschow DJ, Bakker CY *et al.* CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994; **1**:405–13.

30 Waterhouse P, Penninger JM, Timms E *et al.* Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4 [see comments]. *Science* 1995; **270**:985–8.

31 Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 1995; **3**:541–7.

32 Chambers CA, Cado D, Truong T, Allison JP. Thymocyte development is normal in CTLA-4-deficient mice. *Proc Natl Acad Sci USA* 1997; **94**:9296–301.

33 Kataoka H, Takahashi S, Takase K, Yamasaki S, Yokosuka T, Koike T, Saito T. CD25 (+) CD4 (+) regulatory T cells exert *in vitro* suppressive activity independent of CTLA-4. *Int Immunol* 2005; **17**:421–7.

34 Thornton AM, Donovan EE, Piccirillo CA, Shevach EM. Cutting edge. IL-2 is critically required for the in vitro activation of $CD4^+$ $CD25^+$ T cell suppressor function. *J Immunol* 2004; **172**:6519–23.

35 Janssens W, Carlier V, Wu B, VanderElst L, Jacquemin MG, Saint-Remy JM. $CD4^+$ $CD25^+$ T cells lyse antigen-presenting B cells by Fas–Fas ligand interaction in an epitope-specific manner. *J Immunol* 2003; **171**:4604–12.

36 Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 2004; **21**:589–601.

37 Nolte-'t Hoen EN, Wagenaar-Hilbers JP, Peters PJ, Gadella BM, van Eden W, Wauben MH. Uptake of membrane molecules from T cells endows antigen-presenting cells with novel functional properties. *Eur J Immunol* 2004; **34**:3115–25.

38 Kumanogoh A, Wang X, Lee I *et al.* Increased T cell autoreactivity in the absence of CD40–CD40 ligand interactions: a role of CD40 in regulatory T cell development. *J Immunol* 2001; **166**:353–60.

39 Lu L, Bonham CA, Chambers FG, Watkins SC, Hoffman RA, Simmons RL, Thomson AW. Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis. *J Immunol* 1996; **157**:3577–86.

40 Grundstrom S, Cederbom L, Sundstedt A, Scheipers P, Ivars F. Superantigen-induced regulatory T cells display different suppressive functions in the presence or absence of natural $CD4^+$ $CD25^+$ regulatory T cells in vivo. *J Immunol* 2003; **170**:5008–17.

41 Vendetti S, Chai JG, Dyson J, Simpson E, Lombardi G, Lechler R. Anergic T cells inhibit the antigen-presenting function of dendritic cells. *J Immunol* 2000; **165**:1175–81.

42 Thornton AM, Piccirillo CA, Shevach EM. Activation requirements for the induction of $CD4^+$ $CD25^+$ T cell suppressor function. *Eur J Immunol* 2004; **34**:366–76.

43 Hwang I, Huang JF, Kishimoto H *et al.* T cells can use either T cell receptor or CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells [in process citation]. *J Exp Med* 2000; **191**:1137–48.

44 Magistrelli G, Jeannin P, Herbault N, Benoit De Coignac A, Gauchat JF, Bonnefoy JY, Delneste Y. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. *Eur J Immunol* 1999; **29**:3596–602.

45 Oaks MK, Hallett KM, Penwell RT, Stauber EC, Warren SJ, Tector AJ. A native soluble form of CTLA-4. *Cell Immunol* 2000; **201**:144–53.

46 Lindsten T, Lee KP, Harris ES *et al.* Characterization of CTLA-4 structure and expression on human T cells. *J Immunol* 1993; **151**:3489–99.

47 Chen W, Jin W, Wahl SM. Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor beta (TGF-beta) production by murine CD4 (+) T cells. *J Exp Med* 1998; **188**:1849–57.

48 Thornton AM, Shevach EM. Suppressor effector function of $CD4^+$ $CD25^+$ immunoregulatory T cells is antigen nonspecific. *J Immunol* 2000; **164**:183–90.

49 Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells *in vitro* [see comments]. *Science* 1994; **264**:1587–9.

50 Frasca L, Carmichael P, Lechler R, Lombardi G. Anergic T cells effect linked suppression. *Eur J Immunol* 1997; **27**:3191–7.

51 Taams LS, van Rensen AJ, Poelen MC *et al.* Anergic T cells actively suppress T cell responses via the antigen-presenting cell. *Eur J Immunol* 1998; **28**:2902–12.