



Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



IL-10 suppresses CD2-mediated T cell activation via SHP-1

Alison Taylor^{a,*}, Johan Verhagen^{a,1}, Tunç Akkoç^{a,b}, Renate Wenig^c, Egbert Flory^c, Kurt Blaser^a, Mübeccel Akdis^a, Cezmi A. Akdis^a

^a Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland

^b Department of Pediatric Allergology, Marmara University, Istanbul, Turkey

^c Paul Ehrlich Institute, Langen, Germany

ARTICLE INFO

Article history:

Received 14 January 2008

Received in revised form 11 July 2008

Accepted 27 July 2008

Available online xxx

Keywords:

Suppression

CD4 T cells

SHP-1

IL-10

CD2

Tolerance

ABSTRACT

Interleukin (IL)-10 is an essential suppressive cytokine and plays a key role in peripheral T cell tolerance to allergens, autoantigens, transplantation antigens and tumor antigens. However, the molecular mechanisms of direct T cell suppression by IL-10 are not fully understood. Here, we demonstrate that IL-10 directly inhibits CD2 signaling in T cells. T cell stimulation via CD2 alone induces activation and proliferation, when endogenous IL-10 sources are eliminated from cultures. IL-10 utilizes the src-homology-2 domain containing tyrosine phosphatase (SHP-1) to directly suppress T cell activation. The role of SHP-1 in IL-10-mediated suppression of CD2 co-stimulation on T cells is demonstrated by using dominant-negative SHP-1 over-expressing T cells and silencing endogenous SHP-1 by small inhibitory RNA. Findings are confirmed using both SHP-1-deficient mice and IL-10-deficient mice. CD2-induced proliferation is suppressed by exogenous IL-10 in IL-10-deficient, but not SHP-1-deficient murine T cells. In conclusion, SHP-1-mediated inhibition of CD2 signaling represents a novel mechanism for direct T cell suppression by IL-10.

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1. Introduction

CD2 is a 50 kDa cell surface, transmembrane glycoprotein expressed on T cells, NK cells and thymocytes (Moingeon et al., 1989). CD2 plays an important role in regulating T cell responses and has two main functions; one in the promotion of cellular adhesion and the other in activation through the initiation of signal transduction events (Yang and Reinherz, 2001). Cross-linking CD2 with specific mAb or interaction with its ligand, leukocyte functioning antigen-3 (CD58) induces a major co-stimulatory signal that enhances T cell proliferation and cytokine production (Wingren et al., 1995). Various roles for CD2 in T cell activation have been proposed, as an adhesion molecule thereby reducing amounts of antigen required for T cell activation, as a co-stimulatory molecule, or as a direct promoter of T cell activation (Holter et al., 1996). However, T cells still function well in the absence of CD2, as demonstrated in CD2-deficient mice, which develop apparently normal T cell-dependent immune responses (Killeen et al., 1992). The failure of CD2-deficient mice to develop an obvious

abnormal phenotype and the observation that they can cope efficiently with viral infections, suggests that CD2 either does not play an essential role or that other receptors can compensate for its action in T cell activation. There is also the possibility that the phenotype is of a subtle nature, which has not yet been elucidated.

IL-10 is a suppressor of T cell proliferation and cytokine production (Moore et al., 2001) and has been demonstrated to play a major role in the induction of peripheral T cell tolerance to allergens, autoantigens, tumor antigens, transplantation antigens and in chronicity of infections (Akdis et al., 2004; Cong et al., 2002; Steidler et al., 2000). IL-10 has previously been shown to induce dephosphorylation of the co-stimulatory molecule CD28 and prevent its binding of phosphatidylinositol 3-kinase (PI3-K), thereby blocking further downstream signaling and hence inhibiting T cell proliferation (Akdis et al., 2000). IL-10 exerts its biological function through the activation of Jak1 and Tyk2, followed by Stat1 and Stat3 (Wehinger et al., 1996). SHP-1 is an intracellular protein tyrosine phosphatase, which has been demonstrated to form a complex with Tyk2 in T cells (Yetter et al., 1995). SHP-1 deficiency can lead to the development of autoimmune syndromes in association with severe combined immunodeficiency disorders as seen in the motheaten mouse, which completely lacks SHP-1 expression, hence termed SHP-1-deficient (Shultz et al., 1993).

In the present study, we demonstrated the role of SHP-1 in IL-10-mediated suppression of CD2 signaling in T cells. IL-10 suppresses

* Corresponding author. Current address: Department of Pathology, University of Cambridge, CB2 1QP, UK. Tel.: +1223 333726; fax: +1223 764733.

E-mail address: at504@cam.ac.uk (A. Taylor).

¹ Current address: Department of Cellular and Molecular Medicine, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK.

the co-stimulatory effect of CD2 in T cells that have intact SHP-1, demonstrating an essential role of SHP-1 in IL-10-mediated direct T cell suppression.

2. Materials and methods

2.1. Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed three times with PBS and resuspended in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME (all from Life Technologies) and 10% heat-inactivated FCS (Bioconcept, Allschwill, Switzerland) (cRPMI) (Akdis et al., 1998; Jutel et al., 2001).

Human CD4⁺ T cells were isolated using magnetic activated cell sorting, according to the recommended protocol (Miltenyi Biotec, Bergish, Germany). CD4⁺ T cell purity was over 97%. Isolation of human CD45RO⁺ CD3⁺ T cells was carried out by negative selection using pan T cell kit and CD45RA microbeads, according to the recommended protocol (Miltenyi Biotec, Bergish, Germany). CD45RO⁺ cells were collected as the negative fraction and rested overnight in cRPMI at 37 °C.

Monocyte and NK cell depleted PBMC were obtained by depletion of CD14 and CD16-bearing cells by the same method. Monocyte and NK cell contamination as determined by the same mAbs and flow cytometry was less than 0.5%. 10⁵ cells were stimulated in 96-well plates by plate-bound anti-CD3 (clone: OKT3, ATCC, Maryland, USA), anti-CD28 (clone 15E8: CLB, Amsterdam, Netherlands), anti-CD2 (clones 4B2 and 6G4, CLB) or soluble combinations of these (10 µg/ml). No significant difference was seen between plate-bound and soluble combinations and therefore the latter was used in the case of multiple mAbs for experiment ease. Anti-CD2 mAbs were always used as a paired combination. In experiments with anti-CD2/3/28 stimulation equal amounts of isotype control mAbs (Pharmingen AG, St. Louis, MO) were used in control. IL-10 and IL-2 (Peprotech EC Ltd., London, UK) were added to cultures where indicated at 10 ng/ml. The doses of cytokines and mAbs were optimized in preliminary experiments. Plates were incubated at 37 °C for up to 5 days (dependent on optimum activation) and pulsed with 1 µCi [³H] thymidine (Hartmann Analytic, Braunschweig, Germany) for 8 h before cell harvesting. All donors gave their informed consent prior to the study. Experiments were further subject to assessment by the ethical committees of the Canton of Graubünden and the University of Zurich.

Mouse CD4⁺ T cells were isolated from the spleens of control C57BL/6J^{+/+} mice and C57BL/6J^{me/me}, which were of the motheaten phenotype (me) and therefore lacked SHP-1 expression (Shultz et al., 1993). Mouse CD4⁺ T cells were further isolated from the spleens of IL-10^{-/-} and Wt Balb/c mice (Jackson Laboratories, Bar Harbor, ME, USA), by using magnet activated cell sorting (Miltenyi Biotec). Cell purity was over 89% as analysed by flow cytometry. 10⁵ cells/well in 96-well flat-bottomed plates were stimulated in cRPMI medium (Pharmingen AG) with 10 µg/ml plate-bound anti-CD2 for 72 h and [³H] thymidine incorporation was measured.

Experiments were performed in vitro on frozen splenocytes and therefore did not involve licensed procedures and could be executed in a non-licensed facility (SIAF). Animals were kept at the Washington University School of Medicine, St. Louis, MO, USA and under assessment of the local ethical committee.

2.2. Flow cytometric analysis

The percentage of proliferating cells in response to stimulation was measured by carboxyfluorescein succinimidyl ester (CFSE) dilution. 2 × 10⁷ cells were labeled with CFSE (5 µM, Molecular Probes Europe, BV, Leiden, The Netherlands) and washed two times with medium before being subjected to the anti-CD2 mAbs (10 µg/ml) (Sigma Chem. Co., St. Louis, MO, USA) stimulation in the presence or absence of IL-10 (10 ng/ml). Ethidium bromide was used during analysis to exclude dead cells.

2.3. Immunoprecipitations and immunoblots

PBMC and CD45RO⁺ T cells (2–5 × 10⁶) were treated with IL-10 (50 ng/ml) for 10 min at 37 °C and the reaction stopped on ice, before lysing cells in 5% Triton X-100 containing 500 mM EDTA, 500 mM iodoacetamine, 10 mg/ml leupeptin, 5 mg/ml aprotinin (all from Sigma Chem. Co., St. Louis, MO, USA) and 100 mM Na orthovanadate (Fluka Chemie AG, Buchs, Switzerland). Cell lysates were incubated with primary antibody (50 µg/ml) in excess, 2 h at 4 °C. This was followed by the addition of 20 µl of protein A/G beads (Santa Cruz biotechnology Inc., California) for 1 h. Immunoprecipitations were performed with anti-CD2 mAbs (clones 4B2 and 6G4, CLB, Amsterdam, Netherlands), anti-SHP-1 (BD Transduction laboratories, St. Louis, MO) and anti-PI3-K (clone AB6, MBL, Naka-ku Nagoya, Japan).

Cell lysates were washed three times in lysis buffer and boiled in Laemmli sample buffer before electrophoresis. Immunoprecipitates were electrophoresed and proteins were transferred as described. Immunoblots were probed with primary antibodies; anti-phosphotyrosine HRP-labelled (clone 4G10, Upstate Biotechnology Inc., Lake Placid, NY), anti-PI3-K (clone AB6, MBL) and anti-SHP-1 (BD Transduction Laboratories), followed by appropriate secondary antibodies (HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG, respectively). Western blots were developed with the chemiluminescence system (Amersham).

2.4. Nuclear transfection

1.0 µg SHP-1 siRNA was added to 7 × 10⁶ PBMC that had been washed in PBS and resuspended in 100 µl of Nucleofector™ solution for T cells (Amara Biosystems, Cologne, Germany). Cells were transferred into a cuvette and electroporated using program U-15 of the Nucleofector™ (Amara Biosystems), and then immediately transferred into prewarmed cRPMI medium. SHP-1 and GL-3 luciferase specific siRNA were synthesized by Dharmacon Research, Lafayette, USA. Control cells were transfected with 1.0 µg luciferase siRNA using the same protocol. Transfected cells were rested 48 h, before proliferation assays commenced. The coding strand of the RNA oligonucleotide duplexes directed to the human SHP-1 messenger RNA was 5'-CCAGGGUGACUUCUCGCUC-3', with a 3' dTdT overhang.

2.5. Retroviral transduction

PBMC were cultured in 1 ml cRPMI medium in 24-well flat-bottom cell culture plates, previously coated with 10 µg/ml anti-CD3 and anti-CD28 mAb at 37 °C for 48 h. Cells were washed with medium and resuspended in 500 µl cRPMI supplemented with 20 ng/ml IL-2, in 0.1 mg/ml retronectin (Bio Whittaker, Europe) coated 24-well plates. 1 ml of either [MLV-GALV(SHPC453S-NGFR)] or [MLV-GALV(GFP-NGFR)] vector supernatant was added to each well. Plates were centrifuged 200 × g at 32 °C for 90 min and incubated at 37 °C for 3 h (Steidl et al., 2002). After exchange to fresh medium, cells were rested for 2 days before analysis. Cells were

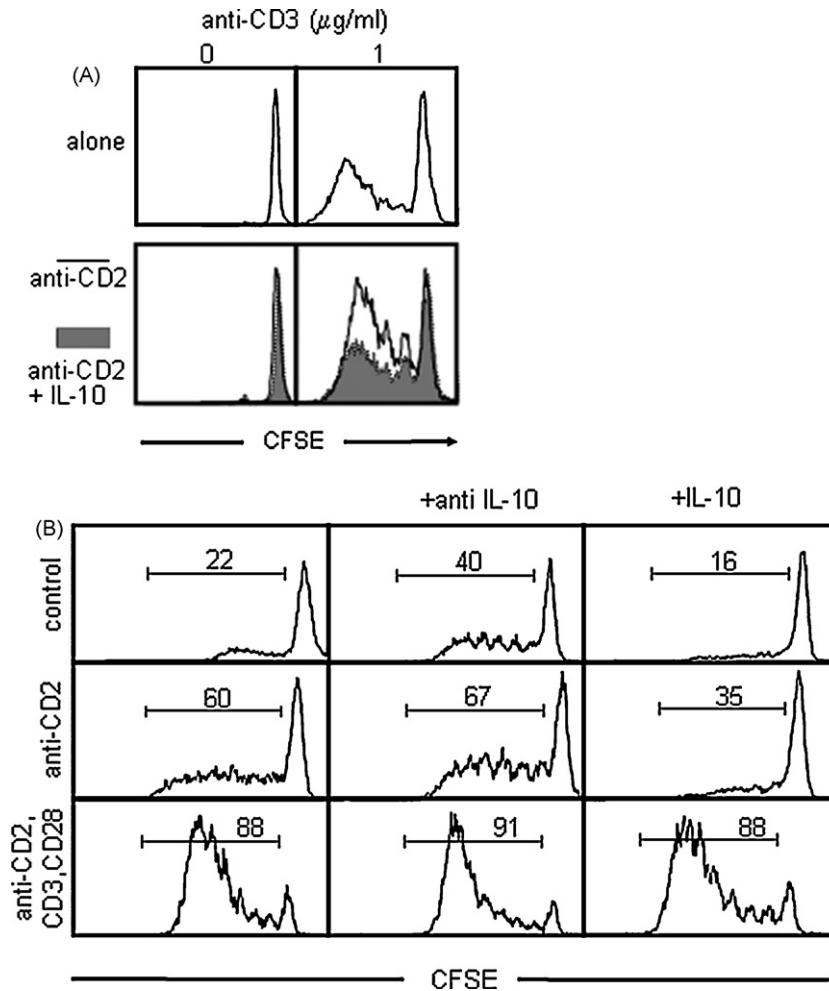


Fig. 1. IL-10 suppresses T cell proliferation induced by CD2 stimulation. (A) PBMC were labeled with CFSE and subjected to anti-CD2 mAb (10 µg/ml) stimulation with or without anti-CD3 mAb in the presence or absence of IL-10 (10 ng/ml). CD3 positive T cells were gated and proliferation assessed. The lower right histogram indicates T cell suppression by IL-10, approximately 30%. (B) NK cell and monocyte depleted PBMC were labeled with CFSE and subjected to anti-CD2 or a combination of anti-CD2, -CD28 and -CD3 mAb stimulation in the presence or absence of IL-10 and anti-IL-10 Ab. CD3⁺ T cells were gated and cell division was analysed. Percentages of proliferating T cells are shown. (A and B) The same results were observed in at least three-independent experiments ($p < 0.0001$, for suppressive effect of IL-10 and co-stimulatory role of anti-CD2 mAbs). Appropriate isotype control mAbs were used in control conditions in all experiments.

kept in culture up to 6 months, with IL-2 (20 ng/ml) supplemented RPMI and 2-week-cycles of restimulation with PHA and irradiated PBMC as feeder cells. Proliferating cells were expanded with IL-2.

2.6. Statistical analysis

All data are expressed as means $\pm$ S.D. Statistical analysis was performed using the paired Student's *t*-test and Mann–Whitney *U*-test for samples $n < 6$ ($*p < 0.05$).

3. Results

3.1. IL-10 suppresses T cell proliferation induced by CD2 stimulation

The CD2 pathway provides co-stimulatory signals and therefore acts as a means of amplification of T cell responses (Bierer et al., 1988; Yang et al., 1986). To investigate the role of IL-10 on CD2 co-stimulation, PBMC were subjected to stimulation by anti-CD3 and/or anti-CD2 mAb in the presence or absence of IL-10 (Fig. 1A). Anti-CD2 stimulation alone did not induce proliferation of human T cells within PBMC. However, the addition of anti-CD2 mAbs to

conditions stimulated with low amounts of anti-CD3 mAb significantly enhanced proliferation. IL-10 could abrogate the additional proliferation induced by anti-CD2, but not the proliferation induced by anti-CD3 alone. Cell viability was analysed by flow cytometry, insuring that there was no increased cell death in these cultures.

We then investigated how T cells behave in anti-CD2 stimulation when endogenous cellular sources of IL-10 are eliminated. PBMC depleted of NK cells and monocytes were subjected to anti-CD2 mAb or combined anti-CD2, -CD28 and -CD3 mAb stimulation (Fig. 1B). Here, when monocytes and NK cells were depleted, T cells showed a significant proliferation induced by anti-CD2 mAbs alone, which was significantly suppressed by IL-10. In contrast, when proliferation was induced with a combination of anti-CD2, -CD3 and -CD28 mAbs, this was not suppressed by IL-10. As NK cells and monocytes are not the only sources of endogenous IL-10, neutralising anti-IL-10 Abs were also used, giving rise to very similar results.

3.2. IL-10 suppresses proliferation induced by CD2 stimulation in the presence of IL-2

We went on to investigate if this suppressive effect of IL-10 on CD2 was still observed in the presence of IL-2. PBMC were labeled

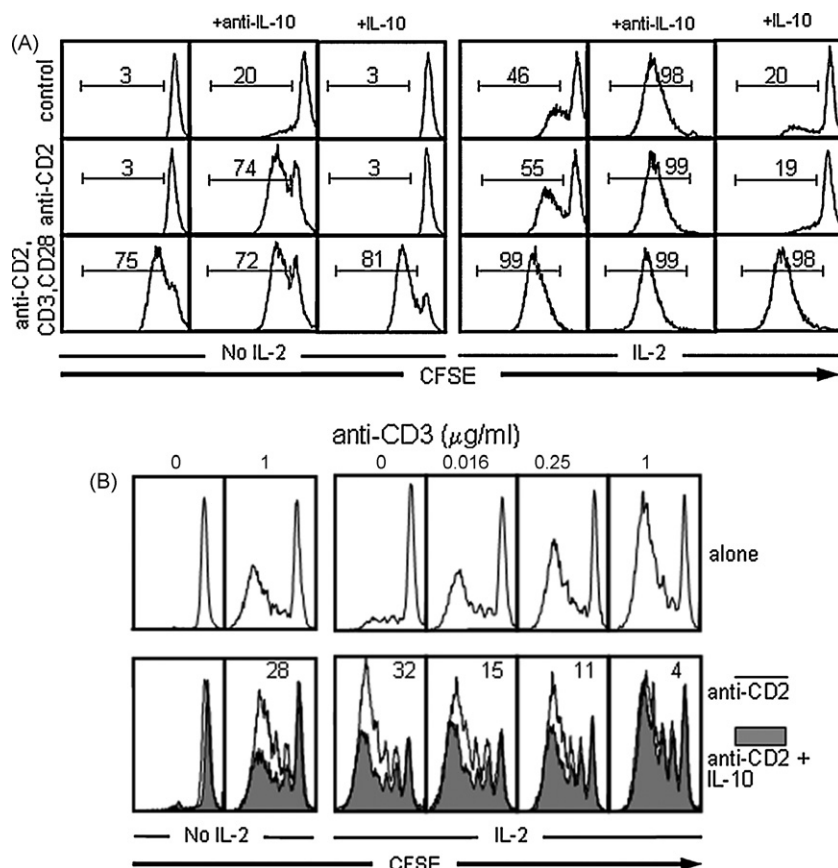


Fig. 2. T cell proliferation induced by CD2 stimulation and by IL-2, is suppressed by IL-10. (A) PBMC were labeled with CFSE and subjected to stimulation by either anti-CD2 mAbs alone, or a combination of anti-CD2 and IL-2, with or without IL-10 and anti-IL-10 Ab. CD3⁺ T cells were gated and cell division was analysed at day 5. Anti-CD2 mAb induced proliferation was apparent in the presence of IL-2 and was fully suppressed by IL-10. Percentage of T cells in certain number of cell divisions are shown in each histogram. (B) Human CD4⁺ T cells were labeled with CFSE and stimulated with anti-CD2 mAb (10 µg/ml) and increasing doses of anti-CD3 mAb in the presence or absence of IL-10 and/or IL-2. After 5 days, CD3⁺ T cells were gated and cell division was assessed by flow cytometry. The numbers indicate the percentage of dividing cells suppressed by IL-10. (A and B) The same results were observed in at least three-independent experiments ($p < 0.0001$, for suppressive effect of IL-10 and co-stimulatory role of anti-CD2 mAbs). Appropriate isotype control mAbs were used in control conditions in all experiments.

with CFSE and stimulated with anti-CD2 mAbs in the presence or absence of IL-2, with or without IL-10 (Fig. 2A). IL-2 alone induced proliferation, which was enhanced by anti-CD2 mAbs. IL-10 significantly suppressed T cell proliferation induced by IL-2 alone and in combination with anti-CD2 mAbs. The proliferation induced by anti-CD2 and IL-2 is only marginally higher than that induced by IL-2 alone. This shows that high levels of IL-2 can lead to T cell proliferation without a requirement for co-stimulation (e.g. CD2). It should be noted that endogenous IL-10 produced by PBMC will also be present in these conditions but its suppressive effect is not observed. This may be because the additional cells activated by anti-CD2 already receive a strong activation signal and the endogenous level of IL-10 in this case is not sufficient to prevent activation. Also, the secretion of IL-10 by NK cells and monocytes leads to IL-10 production by T cells in a STAT3-dependent manner. Others have previously shown that tolerised (IL-10 secreting) T cells temporarily lose their ability to secrete IL-10 in the presence of IL-2 (Anderson et al., 2005). This IL-10 secreted by T cells themselves, may be crucial for the suppression observed. Addition of exogenous IL-10 seems to restore the required levels for immunosuppression.

Human CD4⁺ T cells subjected to anti-CD2 mAb and IL-2 stimulation with increasing doses of anti-CD3 mAb in the presence or absence of IL-10 demonstrated the co-stimulatory effect of CD2 particularly at low levels of anti-CD3 mAb stimulation (Fig. 2B). T cell proliferation induced by this CD2 co-stimulation was suppressed by

IL-10, whereas there was no effect on anti-CD3- and IL-2-induced T cell proliferation. T cell proliferation induced by high levels of anti-CD3 mAb stimulation neither required CD2 co-stimulation nor was it inhibited by IL-10. There was no suppressive effect of IL-10 on anti-CD3 mAb stimulations alone, with or without IL-2 (data not shown).

3.3. IL-10 suppresses phosphorylation and PI3-K binding

Our previous study demonstrated IL-10-mediated inhibition of CD28 and ICOS co-stimulation via SHP-1 (Taylor et al., 2007). This was due to the reversal of phosphorylation of YxxM motifs in the cytoplasmic tails of CD28 and ICOS thus preventing PI3-K binding. In the current study, a similar mechanism was proposed. T cells were subjected to anti-CD2 mAbs stimulation in the presence and absence of IL-10, these conditions were then lysed, immunoprecipitated with anti-CD2 and then analysed by western blot (Fig. 3A). Phosphorylation was detected in the anti-CD2 mAbs stimulated conditions but was not apparent with the addition of IL-10. Furthermore, the presence of PI3-K associating with CD2 was found, but again not in the presence of IL-10. Although CD2 itself does not contain a phosphotyrosine or PI3-K binding motif, the use of an adaptor molecule, as seen in previous CD2 signaling studies, could provide both of these requirements (Huber et al., 2003; Nishizawa et al., 1998; Tibaldi and Reinherz, 2003; Umehara et al., 1997). The involvement of SHP-1 in CD2 signaling was revealed in

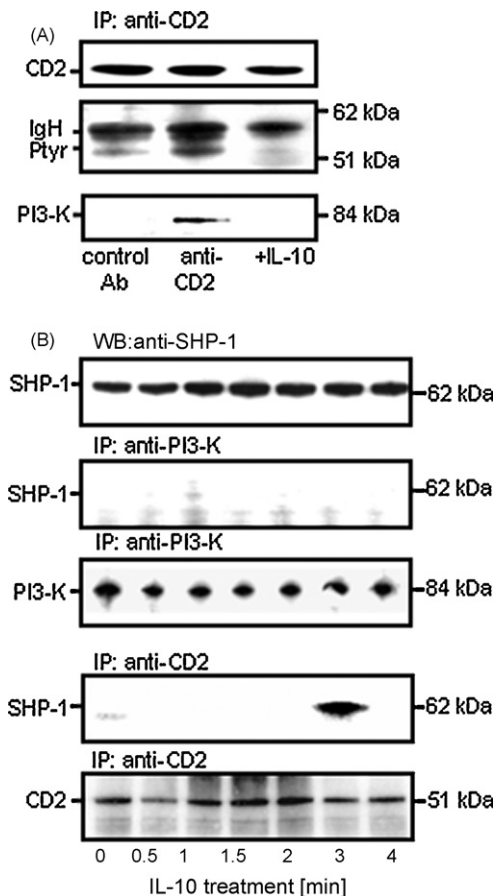


Fig. 3. IL-10 inhibits phosphorylation and PI3-K binding in CD2 stimulated CD4⁺ T cells. (A) Human CD4⁺ T cell lysates were immunoprecipitated with anti-CD2 mAbs and then probed with anti-CD2 mAbs (top), phosphotyrosine Ab (middle) or PI3-K Ab (bottom). (B) Human CD4⁺ T cells were treated with IL-10, lysed and probed with SHP-1 or immunoprecipitated with either PI3-K Ab or CD2-Abs. Immunoprecipitations of CD2 or PI3-K were probed with anti-SHP-1 and their corresponding Abs, anti-CD2 and anti-PI3-K, respectively. Ptyr: tyrosine phosphorylation, IP: immunoprecipitation.

immunoblots, detecting a rapid association and disassociation of SHP-1 with CD2 after 3 min of IL-10 treatment (Fig. 3B).

3.4. SHP-1 is essential for T cell suppression by IL-10

To demonstrate the molecular mechanism of IL-10-mediated suppression of CD2 co-stimulation, SHP-1 was silenced with small inhibitory RNA (siRNA) of SHP-1 by nuclear transfection of human CD4⁺ T cells (Fig. 4). SHP-1 expression was significantly decreased with 1 μ g of SHP-1 siRNA, but not control siRNA-transfected cells as detected by western blots (Fig. 4A). Luciferase (control) siRNA- and SHP-1 siRNA-transfected cells were stimulated with anti-CD2 mAbs alone or in combination with anti-CD28, with or without anti-CD3 mAbs and anti-CD3 mAb alone. IL-10 successfully suppressed proliferation induced with anti-CD2 mAbs in control transfected CD4⁺ T cells (Fig. 4B). In contrast, IL-10-mediated suppression was not observed in SHP-1-silenced cells. Furthermore, IL-10 suppressed T cell proliferation induced by a combination of mAbs against the co-stimulatory molecules, CD2 and CD28 in control, but not SHP-1 siRNA-transfected cells. Moreover, as previously demonstrated (Akdis et al., 2000), the combination of these two mAbs with anti-CD3 mAb not only gave an enhanced proliferative response, but also could not be abrogated by IL-10 in both SHP-1 siRNA or control siRNA-transfected cells.

To further demonstrate the role of SHP-1 in IL-10-mediated suppression of CD2 co-stimulation, human T cells over-expressing a dominant negative (DN) form of SHP-1 were generated. For the stable and efficient retroviral transduction of human CD4⁺ T cells, the moloney murine leukemia virus (MLV) transfer vector encoding either the green fluorescent protein (GFP) or SHP-1 mutant (C453S) was pseudotyped with variant envelope glycoprotein of the Gibbon ape leukaemia virus (GALV). Human T cells retained the transduced genes in cultures for many months and proliferation assays were carried out (Fig. 4C). Similar to findings with SHP-1 siRNA-transfected T cells, SHP-1 DN-expressing T cells showed a proliferative response to anti-CD2 mAbs alone, which could not be suppressed by IL-10. In contrast, T cell proliferation due to the same stimulation was inhibited by IL-10 in control cells. IL-10 also suppressed proliferation induced by a combination of anti-CD2 and anti-CD28 mAbs in control cells, but not in SHP-1 DN cells. Again, IL-10 had no effect on either control or SHP-1 DN T cells stimulated by anti-CD3 mAb alone or in combination with anti-CD2 and anti-CD28 mAbs.

3.5. IL-10 and SHP-1-deficient mouse T cells demonstrate the suppressor role of IL-10 and SHP-1

IL-10-deficient and homozygous motheaten (me/me, SHP-1-deficient) (Shultz et al., 1993) mice provided suitable tools for the analysis of IL-10-mediated suppression of T cell proliferation. Splenic CD4⁺ T cells were isolated and stimulated with anti-CD2 mAbs in the presence or absence of IL-10. IL-10-deficient, but not control mouse CD4⁺ T cells displayed enhanced proliferation in response to anti-CD2 stimulation alone, which was suppressed by IL-10 (Fig. 5A). Similarly, SHP-1-deficient T cells showed a significantly increased proliferative response to stimulation by anti-CD2 mAbs alone compared to control T cells. However, the suppressive effect of IL-10 was fully abolished in the SHP-1-deficient T cells (Fig. 5B). Together, these data demonstrate that in the absence of IL-10 signaling and its main mediator SHP-1, T cells can proliferate in response to stimulation via CD2 alone. Differences are observed between the CD2-induced proliferations of control cells, the reason for this could be strain-dependent.

4. Discussion

The role of CD2 in signal transduction and T cell activation has been under debate. It was thought that CD2 had some association with TCR signals as signaling occurred whilst the TCR associated ζ -chain was expressed on the cell surface. However, it was then found that CD2 signaling could occur without ζ -chain (Steeg et al., 1997) and that unique pairs of CD2-specific mAbs can induce T cell proliferation in the absence of engagement of the CD3–TCR complex (Meuer et al., 1984). Further to this, as also observed in the present study, signaling through CD2 has been shown to lower the threshold for T cell activation through the TCR/CD3 complex (Bierer et al., 1988). As in the CD28 co-stimulation pathway, it was thought that PI3-K binds to CD2 and that this initiates downstream signaling events leading to T cell proliferation, IL-2 production and activation. However, CD2 contains neither a pYxxM motif, which is required for PI3-K binding, nor a phosphotyrosine within its cytoplasmic domain. The proline rich regions within the cytoplasmic domain of CD2 were proposed to mediate direct association with PI3-K, however, this association was found insufficient for CD2-mediated activation of PI3-K (Kivens et al., 1998). Another possibility involves the use of an adaptor molecule. Possible adaptor molecules include CD2 binding protein 2 (CD2BP2) (Nishizawa et al., 1998), CD2-associated protein (CD2AP) (Huber et al., 2003), Shc (Umehara et

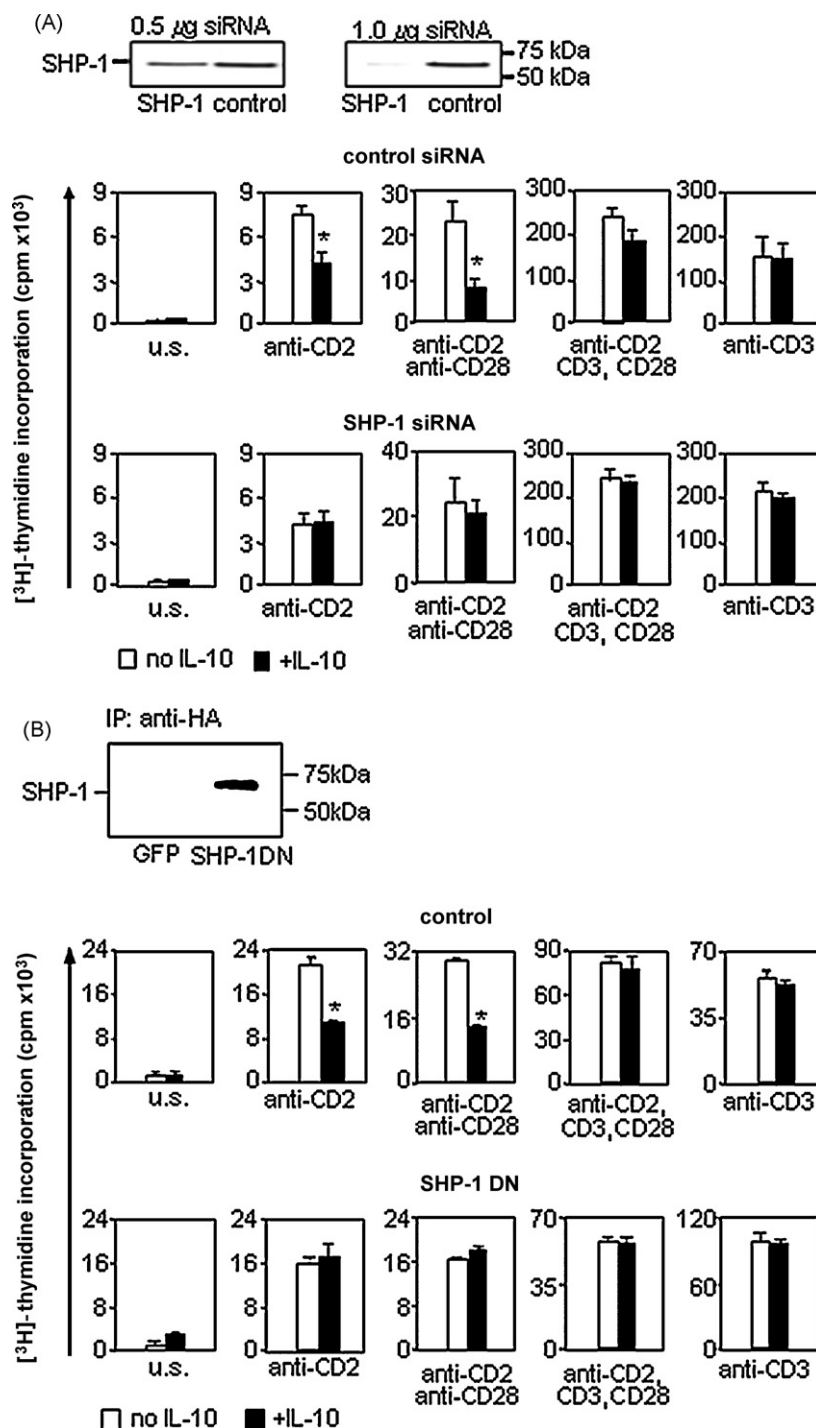


Fig. 4. SHP-1 silencing prevents IL-10-induced T cell suppression. (A) Human CD4⁺ T cells were transfected with SHP-1 siRNA or control siRNA at increasing doses, with 1 μ g efficiently silencing SHP-1 expression. SHP-1 or luciferase (control) siRNA-transfected cells were stimulated with anti-CD2, anti-CD2/CD28, anti-CD2/CD28/CD3 or anti-CD3 mAbs (each at 10 μ g/ml), in the presence or absence of IL-10 (10 ng/ml). Proliferation was measured by [³H] thymidine incorporation after 5 days (B) SHP-1 DN or GFP (control) over-expressing human CD4⁺ T cells were stimulated with the same conditions as specified in (A). Proliferation was measured by [³H] thymidine incorporation after 5 days. (unstimulated: u.s.). One of five-independent experiments with similar results is shown (* $p < 0.001$).

al., 1997), CD2 cytoplasmic tail binding protein 3 (CD2BP3), Cbl-interacting protein of 85 kD (CIN85) and Cas ligand with multiple Src homology (SH) 3 domains (CMS) (Tibaldi and Reinherz, 2003). For the moment the target of SHP-1 in the CD2 signaling complex remains to be investigated further, however, the understanding of SHP-1-activating mechanisms could provide insights to how SHP-1 contributes to thresholds for T cell activation.

Several studies have demonstrated the involvement of SHP-1 in the negative regulation of immune responses (Zhang et al., 2000). It is known that SHP-1 responds to an activation signal by binding to membrane bound inhibitory receptors via its SH2 domains to the tyrosine phosphorylated ITIM/ITAM within the cytoplasmic domain of a receptor (Wang et al., 1999). Attempts at identifying the targets of SHP-1 have demonstrated that its suppressive activity is

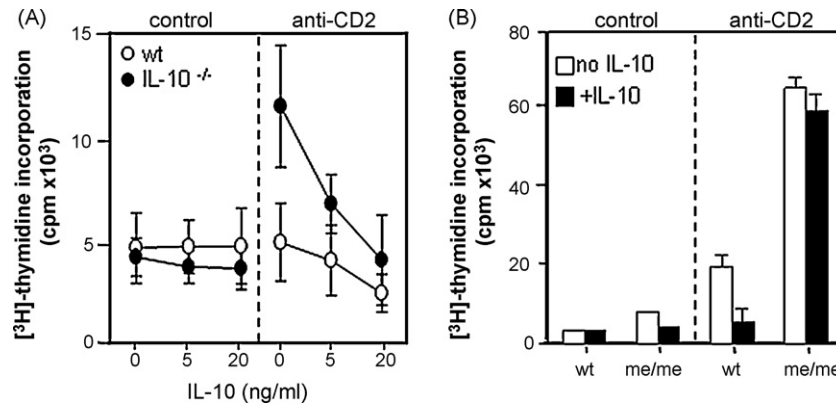


Fig. 5. IL-10 and SHP-1-deficient mouse CD4⁺ T cells demonstrate the suppressor role of IL-10 and SHP-1. (A) Splenic CD4⁺ T cells were isolated from IL-10-deficient and wild type control mice, and stimulated with plate-bound anti-CD2 mAb (10 $\mu\text{g/ml}$) in the presence or absence of IL-10 (5 and 20 ng/ml). Proliferation was measured by [^3H] thymidine incorporation after 3 days. Data representative of duplicate experiments performed from 5 IL-10-deficient mice and 5 wt mice (* $p < 0.05$). (B) Splenic CD4⁺ T cells were isolated from SHP-1-deficient and wild type control mice, and stimulated with plate-bound anti-CD2 mAb (10 $\mu\text{g/ml}$) in the presence or absence of IL-10 (10 ng/ml). Proliferation was measured by [^3H] thymidine incorporation after 3 days. Data representative of duplicate experiments performed from 3 SHP-1-deficient mice and 3 wt mice (* $p < 0.05$).

enhanced by using phosphorylated tyrosine-containing sequences of ITIM motifs to occupy its SH2 domains and thereby activating SHP-1 (Brockdorff et al., 1999). This has been further shown in studies using a recently discovered member of the B7 superfamily, B and T lymphocyte attenuator (BTLA). Phosphorylation of the cytoplasmic domain of BTLA was required for SHP-1 association (Watanabe et al., 2003). BTLA contains two ITIM motifs and the mutation of one of these was sufficient to abrogate SHP-1 association, thus indicating a requirement for a dual motif. However, in other receptors such as gp49B, which only have one motif (Wang et al., 1999), this appeared to be sufficient for phosphatase activation. The sequence of the ITIM motif remains under debate. For example, the sequence (I/V)xYxxL has been suggested for association with the inhibitory receptor CD22 (Burshtyn et al., 1996). The inhibitory motif GxYxxM in CTLA-4 has also been termed an ITIM (Cambier, 1997). Overall, further analysis of SHP-1, its activation and the motifs that it recognizes, could prove an invaluable tool for the treatment of T cell-mediated diseases.

The present study reveals a novel mechanism by which IL-10 tunes the threshold of T cell activation by suppressing CD2 co-stimulation. Anti-CD2 stimulation efficiently induces T cell proliferation on its own, in the absence of IL-10, and enhances T cell responsiveness as a co-stimulator, when combined with low amounts of anti-CD3 mAb.

We demonstrate here that proliferation of the T cell fraction, in response to CD2 stimulation, is inhibited by IL-10. By blocking the function of SHP-1, this suppressive effect of IL-10 on CD2-induced proliferation no longer occurs. The expanse of this direct regulation of T cell proliferation by IL-10 and its mediator SHP-1 is becoming more and more profuse. The co-stimulatory molecule CD28 and the inducible co-stimulator (ICOS), another member of the B7 superfamily, also induce T cell proliferation and this proliferation has been shown to be suppressed by IL-10 (Akdis et al., 2000; Taylor et al., 2007). Moreover, we have shown that the suppression in these cases was also mediated by SHP-1. Silencing of SHP-1 mRNA by siRNA transfection and the generation of SHP-1 DN over-expressing T cells abrogated the suppressive effect of IL-10 on proliferation induced by anti-CD2 or a combination of anti-CD2 and -CD28 mAbs. Furthermore, we demonstrated that SHP-1-deficient murine T cells proliferated in response to anti-CD2 mAb stimulation, but the suppressive effect of IL-10 was no longer apparent. When SHP-1 is inactive, the inhibition pathway triggered by IL-10 no longer functions, which leads to increased and prolonged

T cell activation (Cuevas et al., 1999). This may provide a possible explanation for the autoimmune phenotype seen in motheaten mice characterized by multi-organ infiltration with inflammatory cells (Shultz et al., 1993) and the altered immune tolerance against bacterial flora in IL-10-deficient mice that develop colitis (Kuhn et al., 1993).

These data provide substantial evidence for the model we envisage starting from the ligation of IL-10 with its receptor. This then activates Tyk2 and the associated SHP-1 (Yetter et al., 1995), allowing SHP-1 to dephosphorylate pYxxM motifs with the cytoplasmic tail of co-stimulatory molecules, such as CD28, ICOS (Taylor et al., 2007) and in this case, CD2 combined with its adaptor molecule. This prevents initialization of the signaling cascade and proliferation is suppressed.

In conclusion, SHP-1 appears to play an essential role in IL-10-mediated suppression of T cell activation by suppressing many co-stimulatory molecules, such as CD2, CD28, ICOS, BTLA and possibly several others. Therefore, SHP-1 may be considered as a potential therapeutic target in diseases due to dysregulated immune tolerance against allergens, autoantigens, infectious agents, tumor antigens and transplantation antigens.

Acknowledgements

We thank S. H. Shen, National Research Council of Canada, Montreal, Canada, for mutant SHP-1 plasmid and Marco Colonna, St Louis, USA for the motheaten mice. The authors' labs are supported by Swiss National Science Foundation 32-112306 (MA), 32-105865 (CAA) and German Science foundation (EF) grants and Global Allergy and Asthma European Network (GA²LEN).

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