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Rottlerin inhibits human T cell responses

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ABSTRACT

Rottlerin is a pharmacological inhibitor of protein kinase C (PKC) θ, a novel PKC selectively expressed in T lymphocytes. PKC θ is known to regulate T cell receptor (TCR)/CD28 signalling pathways in T lymphocytes, but the impact of PKC θ inhibition on human T cell responses remains undefined. In this work, we describe the effects of rottlerin on the responses of CD4+ and CD8+ human T lymphocytes upon polyclonal activation. We observed a dose-dependent inhibition of CD4+ and CD8+ T cell proliferation in response to anti-CD3/anti-CD28 antibodies stimulation in the presence of rottlerin. This inhibition was associated with impaired CD25 expression and decreased interleukin (IL)-2 production in activated T cells. In contrast, rottlerin did not alter IL-2-induced T cell proliferation. Furthermore, we demonstrated that rottlerin blocked interferon (IFN) γ, IL-10 and IL-13 mRNA expression in TCR/CD28 activated CD4+ T cells. These findings place rottlerin as a potent immunosuppressive agent for the development of novel therapies in T cell mediated immune disorders.

1. Introduction

Protein kinases C (PKC) consist in a family of at least 10 serine threonine kinases classified into three groups based on their structure homology and cofactor regulation. The classical PKC (α, βI, βII and γ) are activated by diacylglycerol (DAG) and negatively charged phospholipids in a calcium (Ca^{2+}) dependent manner. Novel PKC (δ, ε, θ, and η/L) are Ca^{2+} insensitive. Atypical PKC (λ/ι and ζ) are activated in the presence of negatively charged phospholipids but are Ca^{2+} independent and do not response to DAG [1]. PKC are key regulators of signalling cascades in numerous systems. Recent studies using PKC knockout (K.O.) mice have highlighted the major, specific and non-redundant role of several PKC family members in the regulation of innate and adaptative immune response. Indeed, mice deficient for PKC β present impaired humoral immunity associated with alteration of BCR-dependent B cells activation [2] whereas PKC δ K.O. mice exhibit expansion of the B-lymphocyte population and auto-immune diseases [3]. Studies have demonstrated the role of PKC ε in macrophages and monocyte-derived dendritic cells in response to bacterial lipopolysaccharide [4,5]. PKC α deficient mice display a T cell lineage-specific defect in cell cycle progression downstream of IL-2 receptor [6]. PKC θ has the particularity to be selectively expressed in T lymphocytes and in the skeletal muscle [7,8]. Studies in PKC θ K.O. mice [9,10] have identified this enzyme as a central regulator of the signalling pathways induced in response to TCR/CD28

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Abbreviations: AP-1, activator protein-1; APC, antigen-presenting cell; BSA, bovine serum albumin; Ca^{2+}, calcium; DAG, diacylglycerol; DMSO, dimethyl sulphoxide; EAE, experimental autoimmune encephalomyelitis; HePTP, hematopoietic protein tyrosine phosphatase; IFN, interferon; K.O., knockout; IL, interleukin; MFI, median fluorescence intensity; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; PBMC, peripheral blood mononuclear cells; PKC, protein kinase C; PI, propidium iodide; PMA, phorbol myristate acetate; rhIL-2, recombinant human IL-2; SEB, staphylococcal enterotoxin B; TCR, T cell receptor; Th, T helper

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stimulation and leading to interleukin (IL)-2 synthesis, T cell proliferation and differentiation. Inhibition of individual PKC isoenzymes could therefore represent an interesting approach in the development of new immunomodulatory treatments.

To the best of our knowledge, the functional consequences of PKC θ inhibition on the responses of human T lymphocytes have not been established so far. Here, we investigated the effects of rottlerin, a PKC θ pharmacological inhibitor [11–13], on the activation of freshly purified human CD4+ and CD8+ T lymphocytes.

2. Materials and methods

2.1. Culture medium and reagents

Culture medium consisted of RPMI 1640 (Bio Whittaker Europe, Verviers, Belgium) supplemented with 2 mM L-glutamine (Bio Whittaker), gentamicin (20 μg/ml), 1% nonessential amino acids (Bio Whittaker) and 10% fetal calf serum (PAA Laboratories, Pasching, Austria). Rottlerin and rapamycin were purchased from Biomol (Plymouth Meeting, USA). Anti-CD3 and anti-CD28 antibodies were purchased from Analis Immunotech (Namur, Belgique) and recombinant human IL-2 (rhIL-2) from R&D Systems (Abingdon, U.K.).

2.2. Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway) of buffy-coats obtained from healthy donors. PBMC were resuspended in culture medium, and allowed to adhere to 75 ml Falcon flasks for 2 h. CD4+ and CD8+ T lymphocytes were purified from nonadherent cells by negative selection using a cocktail of biotin-conjugated antibodies (against CD8 or CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and Glycophorin A), MACS Anti-Biotin MicroBeads and MACS column following the manufacturer's recommendation (MACS human CD4 and CD8 T cell isolation kit II; Miltenyi Biotec, Gladbach, Germany). This resulted in a purity of more than 95% for CD4+ T cells and 80% for CD8+ T cells, as assessed by flow cytometry analysis. CD4+CD25+ T cells were then depleted from purified total CD4+ T cells using MACS CD25 microbeads (Miltenyi Biotec) with a purity about 99% CD4+CD25− T cells.

2.3. T cell stimulations

Stimulation of T cells was performed in 96-well plates (2×10^5 cells/well) using plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml). For rottlerin treatment, cells were pre-incubated 1 h with the drug before stimulation. Where indicated, rhIL-2 was added. Proliferation was measured after three days of culture and an additional 16 h pulse with [^{3}H]thymidine (1 μCi/well). CD25 and CD69 expression, and IL-2 production were analysed by flow cytometry after, respectively, 24 and 48 h of stimulation. For IL-2 intracytoplasmic staining, brefeldin A (Sigma–Aldrich, Bornem, Belgique) was added (10 μg/ml) for the last 5 h of the culture.

To determine the effects of rottlerin on IL-2 response, CD4+CD25− T cells were stimulated overnight by plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in order to upregulate CD25 expression. Cells were then harvested, washed and rested in fresh medium for 24 h. T cell proliferation was induced with 50 U/ml rhIL-2 in the presence or absence of rottlerin. Finally, to assess the influence of rottlerin in a calcium independent system, purified T cells were stimulated with phorbol myristate acetate (PMA, 10 ng/ml, Sigma–Aldrich) and soluble anti-CD28 (0.1 μg/ml) in the presence or absence of the drug.

2.4. Immunoblotting

At indicated time intervals, T lymphocytes (1×10^6) were collected and directly lysed in Laemmli buffer. Equal volumes of whole cell extract were resolved by 8% SDS-polyacrylamide gel electrophoresis, and analysed by Western blotting. Immunoblots were probed with phospho-PKC θ Thr^{538} specific antibody obtained from Cell Signalling Technology (Leudsen, The Netherlands). Equal loading was verified by re-probing with total PKC θ specific antibody (Santa Cruz Biotechnology, Boechout, Belgium). The immunoreactive bands were revealed using the ECL Advanced detection kit (Amersham Biotechnologies, Roosendaal, The Netherlands). The images were captured using a ChemiDoc XRS camera W/MZL (Biorad, Nazareth, Belgium) and acquired with Quantity One® SW 1D analysis software (Biorad).

2.5. Flow cytometry analysis

For detection of apoptosis, double staining with FITC-conjugated annexin V and propidium iodide (PI) was performed on T cells washed with PBS and resuspended in 100 μl of annexin V-binding buffer (Becton Dickinson, Erembodegem, Belgium) containing 5 μl FITC annexin V (Pharmingen, San Diego, CA). After 15 min of incubation in the dark at room temperature, 1 μg/ml PI (Sigma Chemicals) was added and flow cytometric analysis was performed.

For CD25 and CD69 expression analysis, cells were washed with PBS supplemented with 0.5% bovine serum albumin (BSA; Sigma Aldrich) and incubated for 20 min at 4 °C with PE-conjugated anti-CD25 and FITC- conjugated anti-CD69 (Becton Dickinson). For IL-2 intracytoplasmic staining, cells were washed in PBS supplemented with 0.5% BSA and incubated for 20 min at room temperature in Cytofix/Cytoperm buffer (Becton Dickinson). They were then washed in Perm/Wash buffer (Becton Dickinson) and incubated for 20 min at room temperature with PE-conjugated anti-IL-2 antibody (Pharmingen, Erembodegem, Belgium). Cells were washed in PBS/BSA and fixed with cell fix buffer (Becton Dickinson). Analysis was done using a DAKO flow cytometer and data were analysed using the Summit software (Dakocytomation, Heverlee, Belgium).

2.6. Real time RT-PCR

mRNA levels were analysed as previously described [14]. Briefly, mRNA content of 5×10^5 viable T cells was isolated using the MagNA Pure LC mRNA isolation kit I (Roche Applied Science, Brussels, Belgium) on the MagNA Pure instrument (Roche Applied Science) following manufacturer's instructions

("mRNA I cells" Roche's protocol, final elution volume 50 μl). CD25, IL-2, Interferon γ (IFNγ), IL-13, and IL-10 mRNA levels were then quantified by real-time RT-PCR using on a Lightcycler instrument (Roche Applied Science). The following oligonucleotide sequences were used as sense primers, antisense primers and probes, respectively: 5′-CTCACCAGGATgCTCACATTTA-3′, 5′-TCCAGAGGTTTGAGTTCTTCTTCT-3′ and 5′-(6Fam)-TGCCCAAgAAGGCCACAGAACTG-(BHQ-1)-3′ for IL-2; 5′-GAAATGCAAAGTCCAATGCA-3′, 5′-CCCACCACGAAATGATAAATT-3′, and 5′-(6Fam)-CCAAGCGAGCCTTCCAGGTCA-(BHQ-1)-3′ for CD25; 5′-CTAATTATTCGGTAACTGACTTGA-3′, 5′-ACAGTTCAGCCATCACTTGGA-3′and 5′-(6Fam)-TCCAACGCAAAGCAATACATGAAC-(BHQ-1)-3′ for IFNγ; 5′-TGAGGAGCTGGTCAACATCA-3′, 5′-CAGGTTGATGCTCAATACCAT-3′and 5′-(6Fam)-AGGCTCCGCTCTGCAATGGC-(BHQ-1)-3′ for IL-13 and 5′-CATCGATTTCTTCCCTGTGAA-3′, 5′-TCTTGGAGCTTATTAAAGGCATTC-3′, and 5′-(6Fam)-ACAAGAGCAAGGCCGTGGAGCA-(BHQ-1)-3′ for IL-10.

The real time RT-PCR for CD3 was performed using CD3 kit containing primers and probe (Applied Biosystems; PE, Norwalk, CT). mRNA levels were expressed as the absolute number of copies normalized against CD3 mRNA.

2.7. Calcium mobilization

Freshly purified CD4+CD25− T cells were washed two times in calcium- and magnesium-free Hank's medium (BioWhittaker). At the concentration of 10×10^6 cells/ml, T lymphocytes were incubated in Hank's medium containing 250 μM sulfinpyrazone (Sigma–Aldrich), 100 μg/ml pluronic acid (Molecular Probes, Leiden, The Netherlands), and 3 μM Fluo-4 AM (Molecular Probes) for 30 min at 37 °C in the dark. Cells were then washed two times with complete medium supplemented with 250 μM sulfinpyrazone and resuspended in this medium at a final concentration of 5×10^5/ml. Loaded T lymphocytes were then treated with 1.25 μM rottlerin or left untreated at 37 °C for 1 h. T cells were stimulated with 10 μg/ml OKT3, 5 μg/ml anti-CD28 and 40 μg/ml rabbit antibody against mouse immunoglobulins (Dakocytomation), and instantly analysed for intracellular Ca^{2+} influx by flow cytometry.

2.8. Cell conjugation and confocal microscopy

The MEC.B7 SigOVA engineered mice fibroblasts [15] were used as antigen-presenting cells (APC). They were seeded at 1×10^5 cells per chamber slide and cultured overnight. The next day, the slides were washed twice with medium to remove nonadherent cells or cell debris. 2×10^5 OT-I CD8+ T cells were added to the monolayer of APC in 1 ml of medium and the chamber slides were centrifuged at $500 \times g$ for 30 s to allow the two cell populations to make contact. After 1 h at 37 °C, the cells were carefully washed twice with warm phosphate-buffered saline and fixed in ice-cold acetone. After

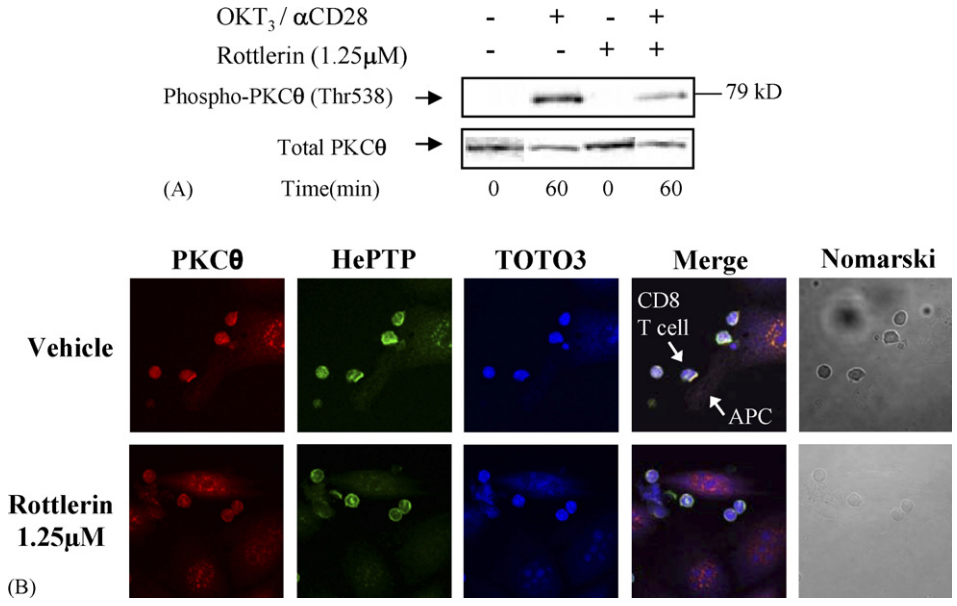

Fig. 1 – (A) TCR/CD28 stimulation activates PKC θ in T lymphocytes: inhibition by rottlerin. CD4+CD25− T lymphocytes were purified from healthy blood donors and stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in 96-well plate in the presence or absence of 1.25 μM rottlerin. At 60 min, cells were harvested, lysed and the protein extracts were analysed by Western blotting using phospho-PKC θ specific antibody. Protein loading was controlled by reprobing with total PKC θ antibody. One representative out of three independent experiments is shown. (B) Active PKC θ is required for hematopietic protein tyrosine phosphatase (HePTP) translocation to the immune synapse. Confocal microscopy of CD8+ antigen-specific, TCR-transgenic OT-I T cells (CD8) in contact with APC for 60 min and stained for PKC θ (red) and HePTP (green), the nuclei are stained with TOTO-3 iodide (blue). The fourth panel is an overlay of the first three and the last panel is a Nomarski differential phase contrast image. The synaptic area is indicated with arrows. Upper panels: the CD8+T cells were treated with DMSO for 1 h. Lower panels: the CD8+T cells were treated with 1.25 μM of rottlerin for 1 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

permeabilization in 0.1% saponin/0.02% NaN3 in phosphate buffered saline for 10 min, the cells were incubated with the primary antibodies for 1 h. After three washes in 0.01% saponin in phosphate-buffered saline, the primary antibodies were revealed using Alexa 546 goat-anti-rabbit and Alexa 488 goat-anti-mouse (Molecular Probes) then with TOTO-3 iodide (Molecular Probes) to stain nuclei. The stained cells were mounted with ProLong antifade kit (Molecular Probes) and then viewed under a confocal laser scanning microscopy TCS SP2 (Leica TCS SP2, Van Hopplynus) using TCS software.

2.9. *Statistical analysis*

Data were compared using the nonparametric Wilcoxon's paired test.

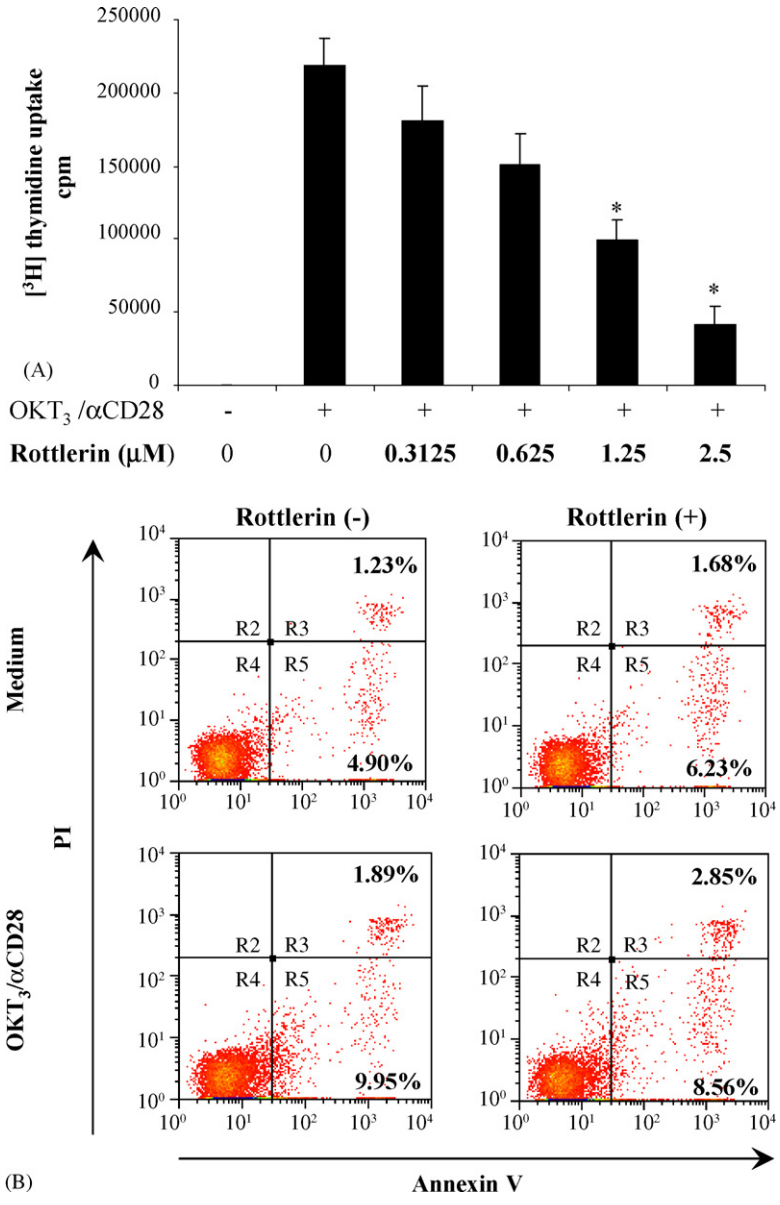

Fig. 2 – Rottlerin inhibits CD4+ T lymphocyte proliferation induced by TCR/CD28 stimulation. (A) CD4+CD25− T lymphocytes were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in 96-well plate for 72 h (2×10^5 cells/well) in the presence of graded doses of rottlerin. T cells were then pulsed for an additional 16 h with 1 μCi [^{3}H]thymidine per well, harvested and analysed using standard procedures. Results represent the means ± S.E.M. [^{3}H]thymidine incorporation of five independent experiments.$^*p < 0.05$. Statistical significance is calculated in comparison with stimulated untreated cells. (B) CD4+CD25− T lymphocytes were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in 96-well plate for 72 h (2×10^5 cells/well) in the presence of rottlerin (1.25 μM). After 24 h, the percentages of apoptotic cells were assessed with use of flow cytometry after staining with FITC-conjugated annexin V and propidium iodide (PI). One representative experiment out of four is shown.

3. Results

3.1. Rottlerin inhibits PKC θ activity in TCR/CD28 stimulated human CD4+CD25− T lymphocytes

We first tested the effect of the novel PKC inhibitor rottlerin on the phosphorylation-dependent [16] activation of PKC θ in human T lymphocytes. CD4+ T lymphocytes were purified from healthy volunteers and CD25 positive T cells were then depleted to prevent any potential interference with regulatory CD4+CD25+ T cells. CD4+CD25− T lymphocytes were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) antibodies in the presence (1.25 μM) or absence of rottlerin. As shown in Fig. 1A, phosphorylated forms of PKC θ appeared in T lymphocytes within 60 min following polyclonal stimulation and the addition of rottlerin markedly inhibited PKC θ activation.

To verify the efficacy of the drug in a physiological model, we evaluated the effect of rottlerin in naïve CD8+ antigen-specific OT-I TCR- transgenic T cell activation with antigen-presenting cells (APC). Freshly isolated OVA257–264/Kb-specific CD8+ T cells from OT-I TCR transgenic mice [17] were overlaid on adherent antigen-expressing SigOVA257–264MEC/B7.1 cells (SAMBOK) [15] at 37 °C for 1 h, fixed and stained for endogenous PKC θ and its target hematopoietic protein tyrosine phosphatase (HePTP) [18]. With the vehicle treatment, both PKC θ and HePTP accumulate in the immune synapse (Fig. 1B, upper panels). The rottlerin treatment as low as 1.25 μM completely inhibited the recruitment of the HePTP to the immune synapse (Fig. 1B, lower panels). Indeed, HePTP accumulation in the immune synapse depends on its phosphorylation on Ser^{225} by PKC θ upon antigen recognition by T cells [18].

3.2. Rottlerin inhibits CD4+CD25− T lymphocyte proliferation in response to polyclonal stimulation

To investigate the functional outcomes of PKC θ inhibition in human T lymphocytes, we first analysed the effects of rottlerin on T cell proliferation induced by polyclonal activation. CD4+CD25− T lymphocytes were incubated in the presence of graded doses of rottlerin and stimulated as described with anti-CD3 and anti-CD28 antibodies. After 4 days of culture, proliferation was measured by [^{3}H]thymidine incorporation. As shown in Fig. 2A, we observed a dose-dependent inhibition of T cell proliferation in the presence

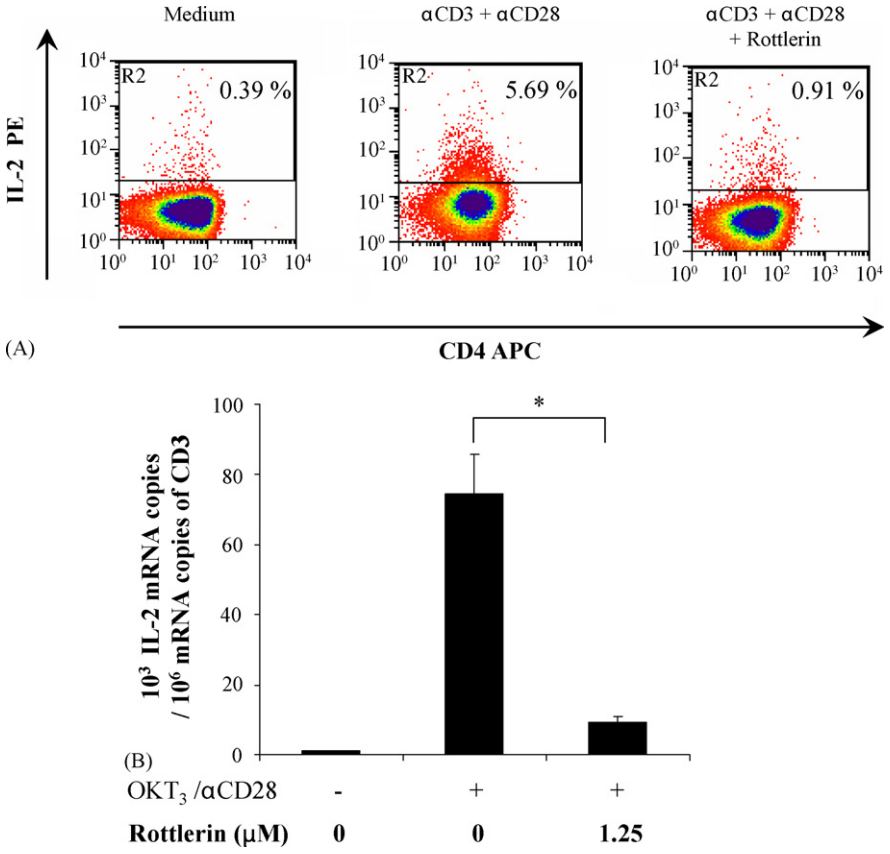

Fig. 3 – Rottlerin decreases IL-2 production by polyclonally activated CD4+ T cells. (A) CD4+CD25− T lymphocytes were stimulated for 48 h with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in the presence or absence of 1.25 μM of rottlerin. Brefeldin A (10 μg/ml) was added in the culture for the last 5 h of stimulation and IL-2 production was assessed by flow cytometry. Results show one representative donor out of five. (B) After 24 h of stimulation, CD4+CD25− T cells were harvested and IL-2 and CD3 mRNA accumulation were quantified by quantitative RT-PCR. IL-2 mRNA levels were normalized against CD3 mRNA levels. Results show the means ± S.E.M. of five independent experiments. *$p < 0.05$.

of rottlerin. This inhibition was statistically significant at the dose of 1.25 μM and was not due to a toxic effect of the drug as assessed by annexin V/propidium iodide staining (Fig. 2B).

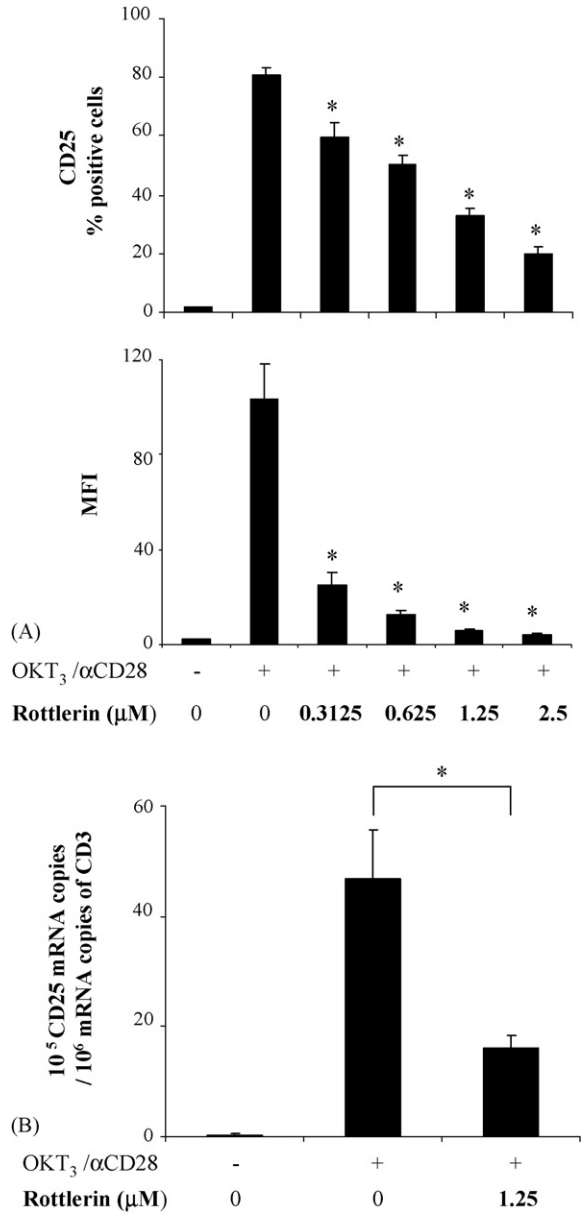

Fig. 4 – Rottlerin prevents CD25 upregulation on activated CD4+ T cell surface. (A) CD4+CD25− T lymphocytes were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in the presence of graded doses of rottlerin. Flow cytometry analysis of CD25 expression was performed after 24 h of stimulation. Data are expressed as percentage of CD25 positive T cells and as median fluorescence intensity (MFI). Results show the means ± S.E.M. of five independent experiments. *$p < 0.05$. (B) After 24 h of stimulation, CD25 and CD3 mRNA accumulation were quantified by quantitative RT-PCR. CD25 mRNA levels were normalized against CD3 mRNA levels. Results show the means ± S.E.M. of five independent experiments. *$p < 0.05$.

3.3. *Inhibition of proliferation is associated with impaired IL-2 production and decreased CD25 expression in activated CD4+CD25− T cells*

T cell proliferation in response to TCR/CD28 stimulation requires IL-2 production by activated T cells and upregulation of IL-2 receptor alpha chain (CD25) on their surface. Therefore, we analysed the consequence of rottlerin treatment on IL-2 production by CD4+CD25− upon 48 h of stimulation. Using flow cytometry analysis, we observed a marked decrease in IL-2 synthesis in the presence of 1.25 μM of rottlerin (Fig. 3A). This effect was statistically significant as percentages (means ± S.E.M.) of IL-2 positive CD4+ T cells among activated T cells in the absence of rottlerin was 7.05 ± 2.30% versus 2.17 ± 0.62% in activated cells in the presence of rottlerin ($n = 5$; $p < 0.05$). In parallel, real time RT-PCR analysis at 24 h of stimulation revealed that rottlerin inhibited the upregulation of IL-2 mRNA levels (Fig. 3B). We then evaluated CD25 expression on activated CD4+ T cells in the same experimental settings. As shown in Fig. 4, rottlerin prevented CD25 upregulation in a dose-dependent manner and CD25 mRNA levels were significantly decreased in the presence of 1.25 μM of the drug. Moreover, upregulation of the T cell activation marker CD69 was also inhibited by the addition of rottlerin (Fig. 5).

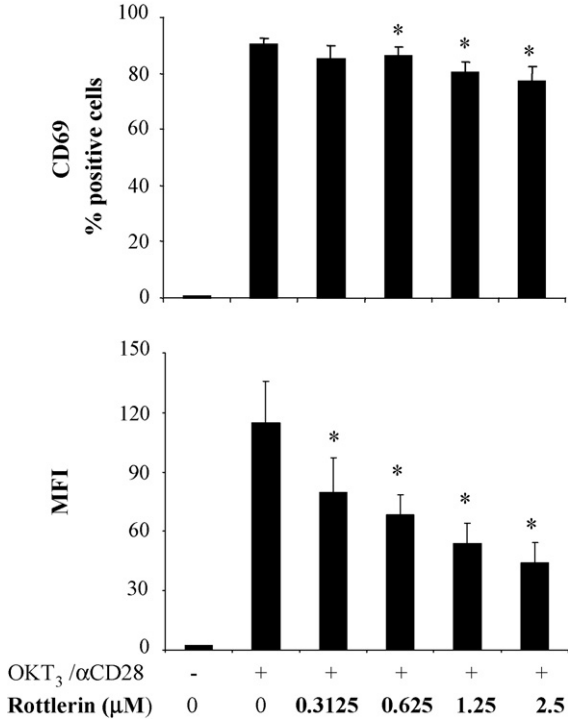

Fig. 5 – CD69 expression is downregulated by rottlerin. CD4+CD25− T lymphocytes were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in the presence of graded doses of rottlerin. CD69 expression on activated T cell was assessed by flow cytometry after 24 h of stimulation. Results are expressed as percentage of CD69 positive T cells and as median fluorescence intensity (MFI). Data represent the means ± S.E.M. of five independent experiments. *$p < 0.05$.

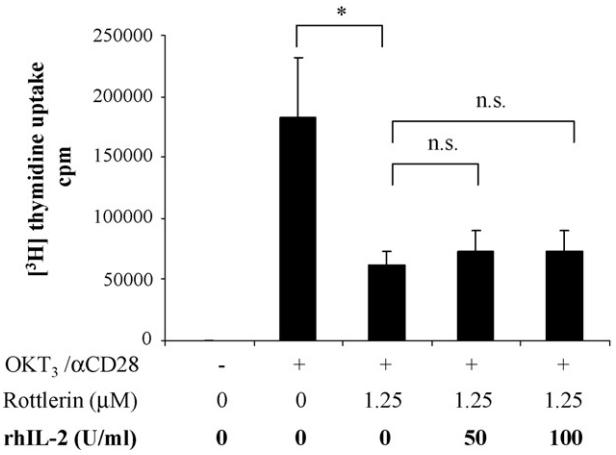

Fig. 6 – Exogenous human recombinant IL-2 does not restore T cell proliferation. CD4+CD25− T cells were stimulated for 72 h with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) with or without rottlerin and rhIL-2 where indicated. T cells were then pulsed for an additional 16 h with 1 μCi [^{3}H]thymidine and harvested for proliferation assessment. Results represent the means ± S.E.M. [^{3}H]thymidine incorporation of five independent experiments (n.s. = non-significant; $^*p < 0.05$.).

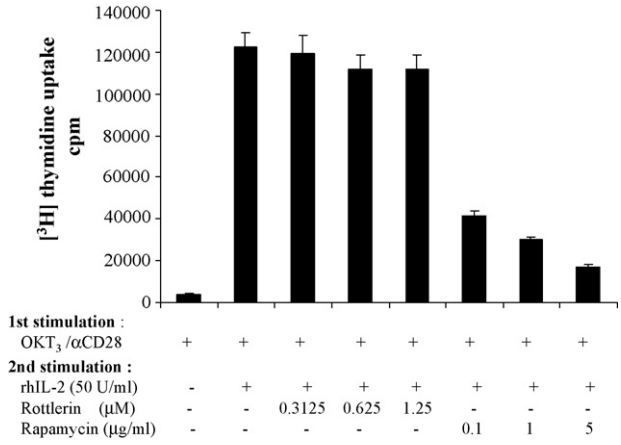

Fig. 7 – IL-2-induced T cell proliferation is not affected by rottlerin. CD4+CD25− T cells were stimulated overnight by plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in order to upregulate CD25 expression. After extensive washing and 24 h rest, proliferation was induced with rhIL-2 in the presence or absence of rottlerin or rapamycin where indicated. After 72 h of stimulation, cells were pulsed for 16 h with 1 μCi [^{3}H]thymidine and proliferation analysed. Results represent the means ± S.E.M. [^{3}H]thymidine incorporation of five independent experiments.

3.4. *IL-2-induced T cell proliferation is not affected by rottlerin*

We next evaluated if the impaired T cell proliferation following treatment with rottlerin could be restored by exogenous rhIL-2. As shown in Fig. 6, the addition of rhIL-2 up to 100 U/ml did not significantly restore the proliferation.

To determine the influence of rottlerin on IL-2 signalling pathway, a two steps stimulation was performed in a second set of experiments. CD4+CD25− T cells were stimulated overnight by anti-CD3 and anti-CD28 antibodies in order to upregulate CD25 expression. After extensive washing and 24 h rest, T cells were cultured for 4 days with 50 U/ml rhIL-2 in the presence or absence of rottlerin. Rapamycin was used as a control drug known to inhibit IL-2 signalling pathway [19]. As shown in Fig. 7, rottlerin did not affect IL-2-induced T cell proliferation whereas rapamycin was efficient in the same conditions.

3.5. *Rottlerin inhibits T helper 1 (Th1) and T helper 2 (Th2) cytokine mRNA expression*

To determine whether rottlerin could also influence the production of Th1 or Th2 cytokines, we measured by real time RT-PCR the mRNA levels in CD4+ T cells stimulated with anti-CD3 and anti-CD28 in the absence or presence of 1.25 μM of the drug. A time course experiment previously established that stimulation was optimal after 24 h of culture. As shown in Fig. 8, IFNγ, IL-10 and IL-13 mRNA levels were significantly upregulated upon 24 h stimulation and rottlerin was able to inhibit the increase of IFNγ, IL-10 and IL-13 mRNA levels.

3.6. *CD8+ T lymphocyte proliferation is inhibited by rottlerin*

We next tested the effects of rottlerin on the response of CD8+ T cells. Purified CD8+ T cells were stimulated with anti-CD3/anti-CD28 antibodies in the absence or presence of graded doses of rottlerin. As observed with CD4+ T lymphocytes, the drug induced a dose-dependent inhibition of CD8+ T cell proliferation (Fig. 9A) and a significant decrease in IL-2 production and CD25 expression (Fig. 9B).

3.7. *Rottlerin does not impair TCR/CD28-induced calcium mobilization*

As rottlerin was previously shown to inhibit calcium mobilization in different systems [20,21], we evaluated the effect of the drug on calcium influx in activated human T cells. Rottlerin treated (1.25 μM) and control CD4+CD25− T lymphocytes were stimulated with anti-CD3 (10 μg/ml), anti-CD28 (5 μg/ml) and rabbit antibody against mouse immunoglobulins (40 μg/ml) and calcium mobilization was directly assessed by flow cytometry. As shown in Fig. 10A and B, rottlerin did not inhibit calcium influx in these conditions. Next, we analysed the influence of rottlerin in a calcium-independent setting. For that purpose, CD4+CD25− T lymphocytes were stimulated with PMA (10 ng/ml) and anti-CD28 antibodies (0.1 μg/ml) in the presence of graded doses of rottlerin. As shown in Fig. 11A, we observed a dose-dependent inhibition of T cell proliferation in the presence of rottlerin. In addition, CD25 upregulation was also prevented in a dose-dependent manner (Fig. 11B).

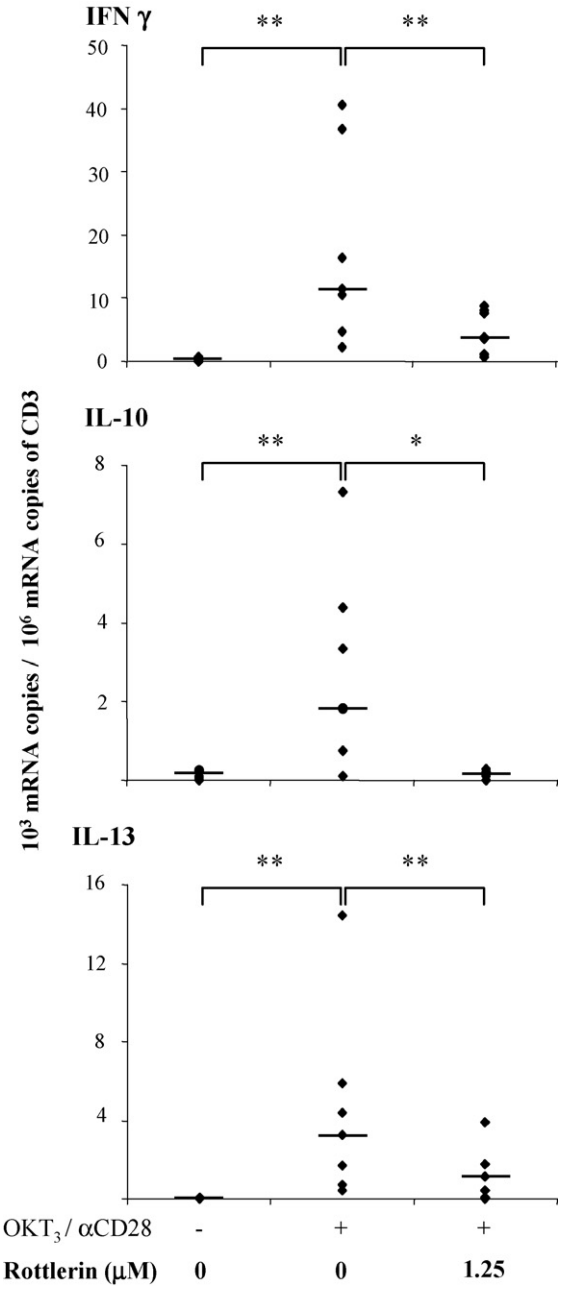

Fig. 8 – Rottlerin inhibits IFNγ, IL-10 and IL-13 gene expression. CD4+CD25− T lymphocytes were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml) in the presence or absence of rottlerin. After 24 h of stimulation, cells were harvested, mRNA levels of IFNγ, IL-10, IL-13 were quantified by real time RT-PCR and normalized against CD3 mRNA. Data represent the median of seven independent experiments. *$p < 0.05$; **$p < 0.01$.

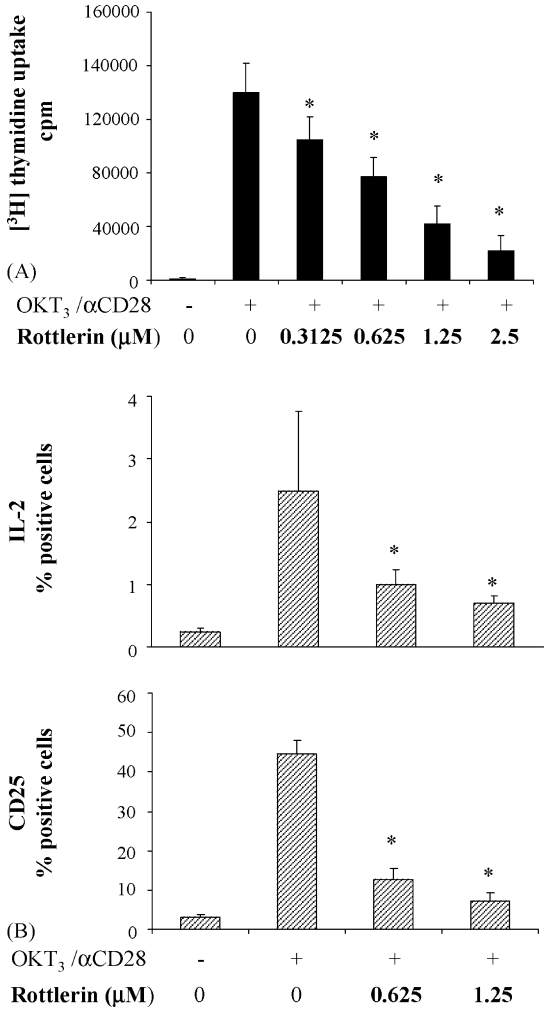

Fig. 9 – Rottlerin inhibits CD8+ T lymphocyte proliferation induced by TCR/CD28 stimulation. CD8+ T lymphocytes purified from healthy blood donors were stimulated in the presence of graded doses of rottlerin with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (0.1 μg/ml). (A) Proliferation was evaluated after 72 h of stimulation and an additional 16 h pulse with [3H]thymidine. (B) IL-2 production and CD25 expression were analysed by flow cytometry after 48 and 24 h of stimulation, respectively. Results represent the means ± S.E.M. of five independent experiments. *$p < 0.05$. Statistical significance is calculated in comparison with stimulated untreated cells.

4. Discussion

In the present study, we showed the suppressive effects of rottlerin, a pharmacological inhibitor of PKC θ [12,13], on polyclonal activation of human CD4+ and CD8+ T cells. We observed that rottlerin inhibits the proliferation of CD4+ and CD8+ T cells upon activation with anti-CD3 and anti-CD28 monoclonal antibodies, which mimic the activation signals provided by professional antigen-presenting cells. Inhibition of T cell proliferation is associated with decreased IL-2 production and impaired CD25 and CD69 expression. In contrast, rottlerin did not affect IL-2 signalling pathway. Our results suggest that rottlerin selectively targets TCR/CD28 signalling pathway while not IL-2-induced cell cycle division. Our data are in agreement with observations made in PKC θ K.O. mice which display a marked deficit in TCR/CD28 induced T cell proliferation and in IL-2 production [9,10] as well as in CD25 expression [9]. Despite an impaired calcium influx in PKC

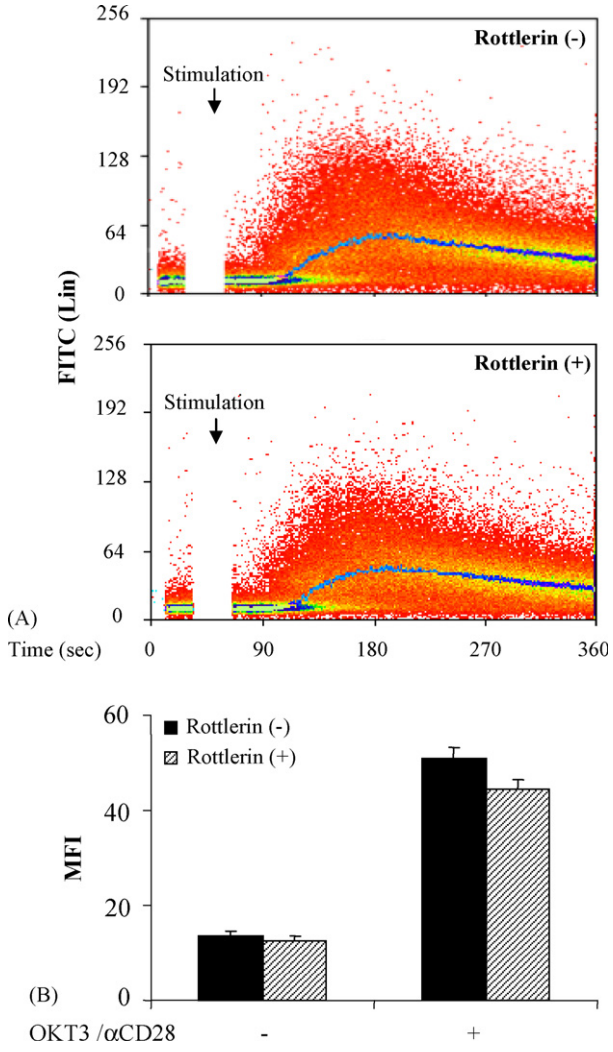

Fig. 10 – Rottlerin does not modify calcium mobilization in response to TCR/CD28 stimulation. Calcium mobilization was evaluated by flow cytometry in rottlerin (1.25 μM) treated and control CD4+CD25− T cells after stimulation with anti-CD3 (10 μg/ml), anti-CD28 (5 μg/ml) and rabbit antibodies against mouse immunoglobulins (40 μg/ml). (A) One representative donor out of six is shown. Curve represents median fluorescence intensity (MFI). (B) Data show means ± S.E.M. of basal vs. peak MFI, respectively, before and after stimulation in the presence or absence of rottlerin (n = 6).

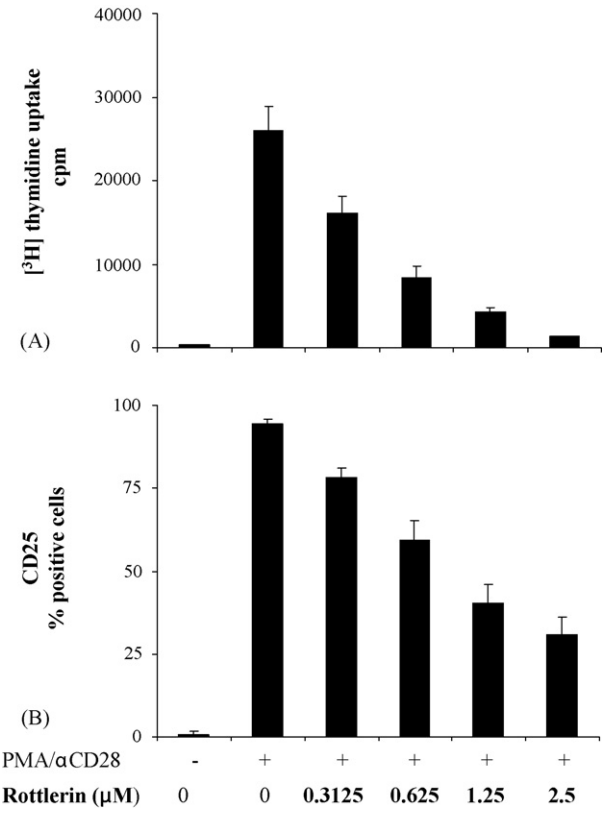

Fig. 11 – Rottlerin inhibits CD4+ T lymphocyte proliferation induced by PMA/CD28 stimulation. CD4+CD25− T lymphocytes were stimulated with PMA (10 ng/ml) and anti-CD28 antibody (0.1 μg/ml) in the presence of graded doses of rottlerin. (A) Proliferation was evaluated after 48 h of stimulation and an additional 16 h pulse with [^{3}H]thymidine. (B) CD25 expression was analysed by flow cytometry after 24 h of stimulation. Results show means ± S.E.M. of three independent experiments.

θ K.O. T cells [22], rottlerin did not modify calcium mobilization in our system. Moreover, we observed that rottlerin exerted inhibitory effects on PMA/CD28 activated T cell proliferation, demonstrating that the action of rottlerin is independent on its effect on calcium mobilization.

At the molecular level, activation of nuclear factor κB (NF-κB) [9,10], activator protein-1 (AP-1) [9,10] and nuclear factor of activated T cells (NFAT) [10] were found to be compromised in PKC $\theta^{-/-}$ T cells. In parallel, studies using reporter gene assays have demonstrated the specific implication of PKC θ on IL-2 promoter activity [23,24] as well as on AP-1 [23–25] and NF-κB [26–28] activation, both transcription factors required for IL-2 gene transcription. Interestingly, rottlerin was previously shown to inhibit TCR/CD28-induced NF-κB activation [26,27].

CD25 promoter activity is regulated by at least five positive regulatory regions including NFAT, NF-κB and AP-1 [29]. CD25 expression is also regulated in an IL-2 dependent manner [30]. However, addition of exogenous IL-2 was not sufficient to reverse the inhibitory effect of rottlerin on T cell proliferation. Furthermore, we have demonstrated that rottlerin inhibits the upregulation of IFNγ, IL-13 and IL-10 mRNA levels in polyclonally activated CD4+ T cells showing that differentiation of both Th1 and Th2 are blocked by rottlerin. Similar inhibition of INFγ and IL-10 production was observed in an antigen presenting cell dependent system where PBMC were stimulated with the superantigen SEB (staphylococcal enterotoxin B) in the presence of rottlerin (Fig. 12; supplementary data). Interestingly, PKC θ K.O. mice failed to develop Th2 cell immune response during helminth infection [31]. In a model of atopic asthma, they did not develop features of Th2 allergic airway inflammation [31]. Moreover, albeit a conserved Th1 response during in vivo infection to Leishmania major [31], PKC θ K.O. mice were protected from experimental autoimmune encephalomyelitis (EAE) [32,33], an antigen-induced

Th1 dependent model of multiple sclerosis. These last observations could be set in close relation with the beneficial effects of the pan-PKC inhibitor bisindolymaleimide VIII in a rat model of EAE [34]. Remarkably, a novel drug NVP-AEB071, inhibitor of classical and novel PKC isoforms, was recently described to inhibit T cell activation and to prolong rat and monkey organ allografts survival times [35,36].

Altogether, our results highlight the potential therapeutic value of rottlerin in T cell-mediated immune disorders such as allograft rejection, autoimmune diseases and allergic disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2006.10.034.

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