

# Targeting PKC in Human T Cells Using Sotrastaurin (AEB071) Preserves Regulatory T Cells and Prevents IL-17 Production

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Regulatory T-cells (Treg) are crucial for immune homeostasis and prevention of immune pathology. Yet, Treg may lose Foxp3 and start secreting IL-17, dependent on environmental cues. Our previous data revealed that Treg from severe psoriasis patients are particularly prone to such conversion. The question of how to maintain Treg stability in the context of inflammation awaits immediate resolution. The pan-protein kinase C (PKC) inhibitor sotrastaurin has shown efficacy in clinical trials of psoriasis. Here, we show that sotrastaurin inhibited effector T-cell responses, whereas the regulatory response was enhanced. Sotrastaurin prevented TCR/CD28-induced T-cell activation and pro-inflammatory cytokine production, but preserved a stable Treg phenotype as evidenced by maintenance of suppressive capacity, high Foxp3 and CD25 expression, and lack of IL-17A and IFN $\gamma$  production. Moreover, in both circulating and dermal psoriatic Treg, prone to rapid induction of IL-17, sotrastaurin enhanced Foxp3 expression and prevented IL-17A and IFN $\gamma$  production even when stimulated in the presence of the helper T 17-enhancing cytokines IL-1 $\beta$  or IL-23. Thus, pharmacological inhibition of PKC may serve as a powerful tool to concurrently inhibit effector T cells and to facilitate Treg, thereby showing therapeutic potential for the treatment of psoriasis.

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## INTRODUCTION

Regulatory T cells (Treg) are crucial for maintaining immune homeostasis. Their significance was demonstrated in a variety of preclinical autoimmunity models and their clinical relevance was proven by demonstrating that the suppressive function of Treg was hampered in autoimmunity and allergy (Buckner, 2010). This knowledge is currently exploited for the development of new therapeutic designs in the management of human chronic inflammatory diseases. However, recent insights point to the notion that human Treg may lose the

expression of the Treg-master transcription factor Foxp3 and convert into less suppressive and even IL-17-producing phenotypes under pro-inflammatory environmental stimuli (Koenen *et al.*, 2008; Beriou *et al.*, 2009; Voo *et al.*, 2009). IL-17 is considered an important mediator in psoriatic disease. Recently, we demonstrated that Treg of psoriasis patients revealed a remarkably high propensity to differentiate into IL-17A-secreting cells, with enhanced loss of Foxp3 and a higher ROR $\gamma$ t expression (Bovenschen *et al.*, 2011). The concurrent presence of IL-17 + Foxp3 + CD4+ Treg in human psoriatic skin lesions led us to believe that this relative Treg instability may contribute to perpetuation of skin inflammation. Thus, the question of how to maintain human Treg stability under *in vivo* inflammatory conditions awaits immediate resolution, and the search for pharmaceutical agents that may realize such a feat has only just started. Foxp3 expression is under tight epigenetic control (Huehn *et al.*, 2009; Lal *et al.*, 2009) and pharmaceutical agents such as DNA methyltransferase inhibitors and histone deacetylase inhibitors were shown to maintain Foxp3 expression in human Treg (Koenen *et al.*, 2008; Polansky *et al.*, 2008). In contrast, many of the early T-cell activation inhibitors such as calcineurin inhibitors have disabling effects on Treg function.

In 2008, a proof-of-concept clinical study in psoriasis patients using the pan-protein kinase C (PKC) inhibitor sotrastaurin (AEB071) was reported; sotrastaurin significantly reduced the clinical severity of psoriasis by affecting several

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Abbreviations: PKC, protein kinase C; Tconv, conventional T cells; Th, helper T cells; Treg, regulatory T cells

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immune cells (Skvara *et al.*, 2008). To date, sotrastaurin has shown evidence of efficacy in phase II clinical trials in both psoriasis as well as renal transplantation (Friman *et al.*, 2011; Wagner *et al.*, 2011). Sotrastaurin is a low-molecular-weight immunosuppressant that selectively targets PKC including the PKC $\alpha$ , - $\beta$ , and - $\theta$  isoforms (Evenou *et al.*, 2009). PKC- $\theta$ , a serine/threonine kinase belonging to the calcium-independent PKC subfamily, is the most abundant isoform in T cells. TCR and CD28 signaling trigger PKC $\theta$  catalytic activation and membrane translocation to the immunological synapse, leading to activation of NF $\kappa$ B, activator protein-1, and nuclear factor of activated T-cells in effector T cells. Studies in PKC-knockout mice have shown that PKC $\theta$  is required for productive helper Th2 (Marsland *et al.*, 2004) and Th17 (Tan *et al.*, 2006) responses but is dispensable for Th1 responses. More recently, the function of PKC $\theta$  in Treg has become the focus of attention. Selective inhibition of PKC $\theta$  using a low-molecular-weight inhibitor enhanced Treg function, suggesting that PKC $\theta$  signaling negatively regulates Treg cells via an unknown mechanism (Zanin-Zhorov *et al.*, 2010).

Yet, most knowledge on the differential role of PKC $\theta$  in T-cell biology relies on studies in mice. Little is known about regulation and function of PKC $\theta$  in human T cells. Here, we investigated the *in vitro* effects of sotrastaurin on human effector and Treg-cell subsets. Sotrastaurin significantly inhibited T-cell effector function and the production of inflammatory cytokines. Importantly, sotrastaurin was found to preserve human Treg suppressive function and prevent IL-17A production. In Treg from psoriasis patients with impaired Treg stability, treatment with sotrastaurin restored a *bona fide* Treg phenotype and prevented Treg differentiation into inflammatory effector cells. Thus, pharmacological targeting of PKC in T cells by low-molecular-weight inhibition might be a powerful means to simultaneously control effector T cells and adequate Treg function.

## RESULTS

### AEB071 inhibits effector function of conventional T cells, but preserves the Treg phenotype even under pro-inflammatory conditions

First, we established the effect of the PKC inhibitor sotrastaurin (AEB071) on human conventional CD4 + CD25<sup>-</sup> cells (conventional T; Tconv) from healthy individuals. We observed a clear dose-dependent inhibition of the proliferative capacity of Tconv by AEB071 following anti-CD3/anti-CD28 mAb-coated bead stimulation (Supplementary Figure S1a online). To exclude the possibility that the observed proliferation inhibition was due to induction of cell apoptosis, cells were stained for the active form of Caspase 3, a central mediator of apoptosis. No obvious differences in the percentages of Caspase 3-positive cells between untreated and treated groups were noted (Supplementary Figure S1b online).

Then, cells were stimulated with anti-CD3/anti-CD28 mAb-coated beads in the presence or absence of AEB071 or rapamycin and phenotypically analyzed by flow cytometry over a 6-day culture period. AEB071 prevented the expression of the T-cell activation markers CD69, CD25, and HLA-DR (Figure 1a). Expression of the negative co-stimulatory

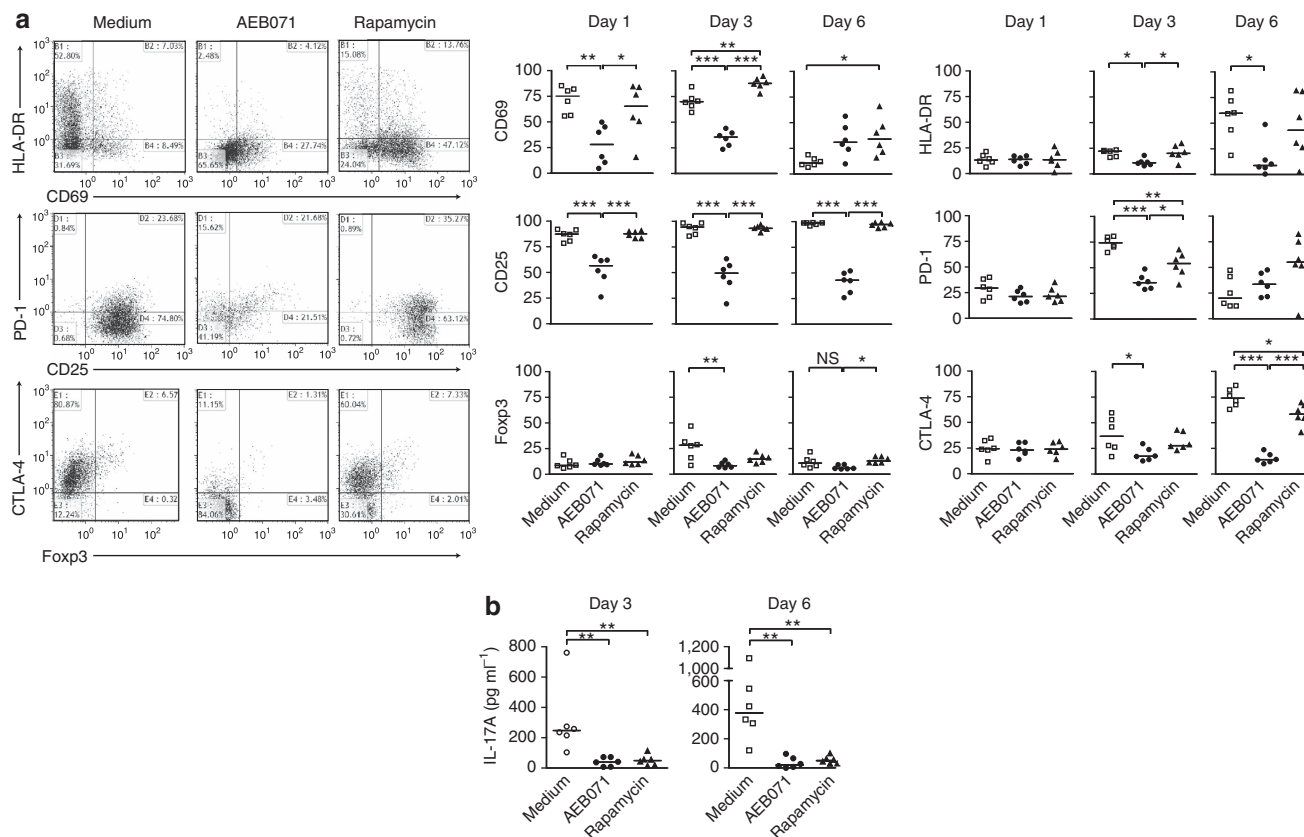
molecules PD1 and CTLA4 during T-cell activation was also prevented by AEB071. Activated human CD4 + CD25<sup>-</sup> cells may transiently express Foxp3, but stable expression of Foxp3 is correlated with suppressor function in Tregs (Tran *et al.*, 2007). In our Tconv cultures, the transient expression of Foxp3 peaked at day 3 of culture. AEB071 treatment significantly prevented not only the increase in the number of Foxp3-positive cells (Figure 1a) but also the expression level of Foxp3 (mean fluorescence intensity, data not shown). Foxp3 expression was low at day 6 of the culture in both the treated and untreated conditions, implying that inhibition of PKC does not favor the generation of induced Treg from Tconv.

Subsequently, we established that addition of AEB071 to cultures of activated Tconv significantly inhibited the production of IL-17A (Figure 1b) and IFN $\gamma$  (Supplementary Figure S1c online) at both day 3 and 6. Addition of rapamycin also completely inhibited IL-17A secretion.

We next assessed the effect of AEB071 on Treg from healthy individuals. As a comparator, we included rapamycin, which is known to harness human Treg function both *in vitro* and *in vivo* (Tresoldi *et al.*, 2011). AEB071 dose dependently inhibited Treg proliferation following stimulation with either anti-CD3/anti-CD28 mAb-coated beads or phorbol 12-myristate 13-acetate (PMA)/soluble anti-CD28 mAb in the presence of exogenously added recombinant human interleukin-2 (rhIL-2, Figure 2a). The IC<sub>50</sub> of AEB071 was 0.09  $\mu$ M under the stimulation with CD3/CD28/rhIL-2 and 0.15  $\mu$ M when PMA/CD28/rhIL-2, a PKC-skewing activation method, was used (Smeets *et al.*, 2012). Rapamycin was less potent in the inhibition of Treg proliferation as compared with Tconv, 30% (Treg, Figure 2a) vs. 70% (Tconv, Supplementary Figure S1a online). Higher concentrations of rapamycin did not result in further inhibition of Treg proliferation. Further, AEB071-treated Treg did not show increased apoptosis (Supplementary Figure S1d online), indicating that the compound does not affect Treg-cell viability by apoptosis induction. Rapamycin treatment also did not affect Treg apoptosis induction.

To evaluate the impact of PKC inhibition on Treg phenotype, the expression of Foxp3 and CD25 was analyzed upon stimulation with CD3/CD28/rhIL-2 or PMA/CD28/rhIL-2. For comparison and confirmation purposes, we used the selective PKC $\theta$  inhibitor C20. Similar to rapamycin, inhibition of PKC using either AEB071 or C20 largely preserved the expression of Foxp3 and CD25 (both percentage and mean fluorescence intensity) regardless of the stimulation protocol (Figure 2b). Notably, the administration of rapamycin significantly enhanced the mean fluorescence intensity of CD25.

Recent studies provided evidence for functional heterogeneity and lineage plasticity within the Treg compartment. Under certain conditions, Treg may acquire an effector phenotype including the ability to produce IFN $\gamma$  and/or IL-17A. Here, we measured the amount of IFN $\gamma$  and IL-17A produced by Treg after treatment with AEB071, C20, or rapamycin. In the absence of any inhibitors, Treg did produce somewhat IFN $\gamma$  upon stimulation. Interestingly, AEB071 almost completely blocked this IFN $\gamma$  production (Figure 2c).



**Figure 1. AEB071 inhibits conventional T (Tconv)-cell activation and IFN $\gamma$ /IL-17A production.** CD4 + CD25<sup>-</sup> Tconv were stimulated with anti-CD3/anti-CD28 mAb-coated beads with or without AEB071 (10  $\mu$ M) or rapamycin (200 nM) and phenotypically analyzed at day 1, 3, or 6. (a) Representative dot-plots showing CD69, HLA-DR, CD25, PD1, CTLA4, and Foxp3 expression at day 6 of culture (left panel) and cumulative data showing the frequency of each marker analyzed (right panel),  $n = 6$ . (b) Presence of IL-17A in cell culture supernatants at days 3 and 6 as measured by Luminex. Each data point represents a separate experiment performed with CD4 + CD25<sup>-</sup> cells obtained from different healthy blood donors,  $n = 6$ . \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

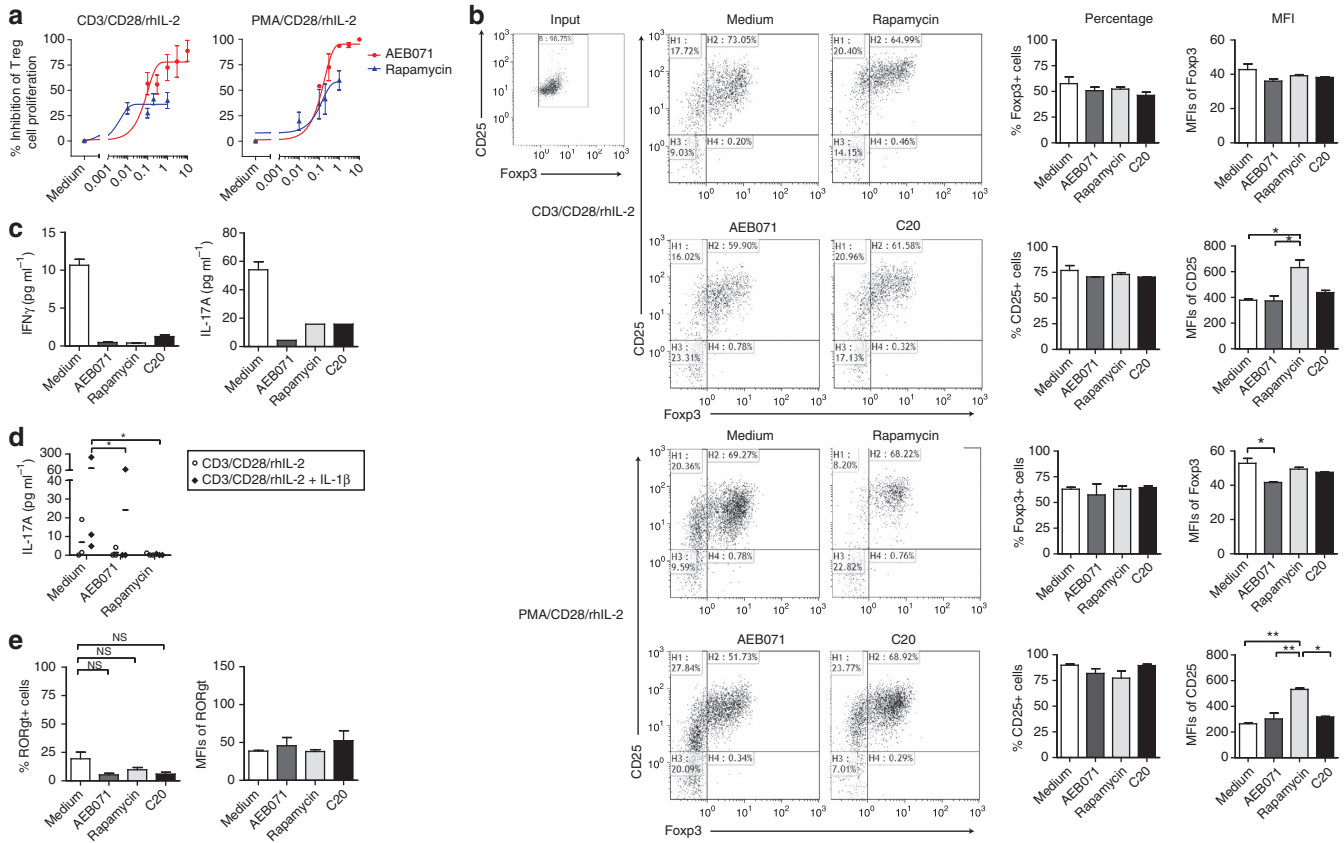
A similar inhibition was seen in the presence of rapamycin or C20. In line with several reports (Koenen *et al.*, 2008; Voo *et al.*, 2009), IL-17A was produced by Treg stimulated with CD3/CD28/rhIL-2, and its production was enhanced by the pro-inflammatory cytokine IL-1 $\beta$  (Figure 2d). In contrast, IL-17A was hardly detected following treatment with AEB071, C20, or rapamycin. Importantly, AEB071, similar to rapamycin, successfully prevented Treg from producing IL-17A, even in the presence of IL- $\beta$  (Figure 2d). It has been suggested that the ratio of Foxp3/ROR $\gamma$ t determines the Treg/Th17 plasticity (Bettelli *et al.*, 2006). However, we were unable to find any significant change of Foxp3 (Figure 2b) and ROR $\gamma$ t (Figure 2e) expression in our set up.

To explore the effect of PKC inhibition on Treg suppressor function, Treg were stimulated with anti-CD3/anti-CD28/rhIL-2 for a week in the presence/absence of AEB071, rapamycin, or C20. Thereafter, viable expanded Treg were co-cultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4 + CD25<sup>-</sup> responder T cells (Tresp) at different ratio's of Treg:Tresp, and the dilution of CFSE was determined after 3 days of culture. According to expectation (Coenen *et al.*, 2006; Tresoldi *et al.*, 2011), Treg expanded in the presence of rapamycin demonstrated preserved suppressor

function when compared with the untreated condition (Figure 3a and b). Interestingly, treatment with AEB071 proved just as successful in maintaining suppressor function (Figure 3a and b), similar to C20 treatment (Figure 3c). At a ratio of 1:1 (Treg:Tresp), the proliferation of Tresp was inhibited >80% under all conditions tested, including Treg treated with AEB071. At a ratio of 1:2 (Treg:Tresp), still over 50% inhibition of Tresp proliferation was observed in the AEB071-treated condition.

#### AEB071 preserves phenotype and function of circulating Treg from psoriasis patients

Previously, we showed that, as compared with healthy controls, Treg from psoriasis patients upon stimulation exhibit enhanced loss of Foxp3, the master transcription factor associated with suppressor function, and increased production of IL-17A (Bovenschen *et al.*, 2011). This was particularly so when stimulated in the presence of IL-23 or IL-1 $\beta$ . Therefore, having established that in healthy Treg AEB071 preserves Treg function and phenotype, we explored whether AEB071 may improve Treg function in psoriasis patients. We isolated either CD4 + lymphocytes or CD4<sup>+</sup>CD25<sup>high</sup> Treg from patient peripheral blood mononuclear cells, and stimulated the cells

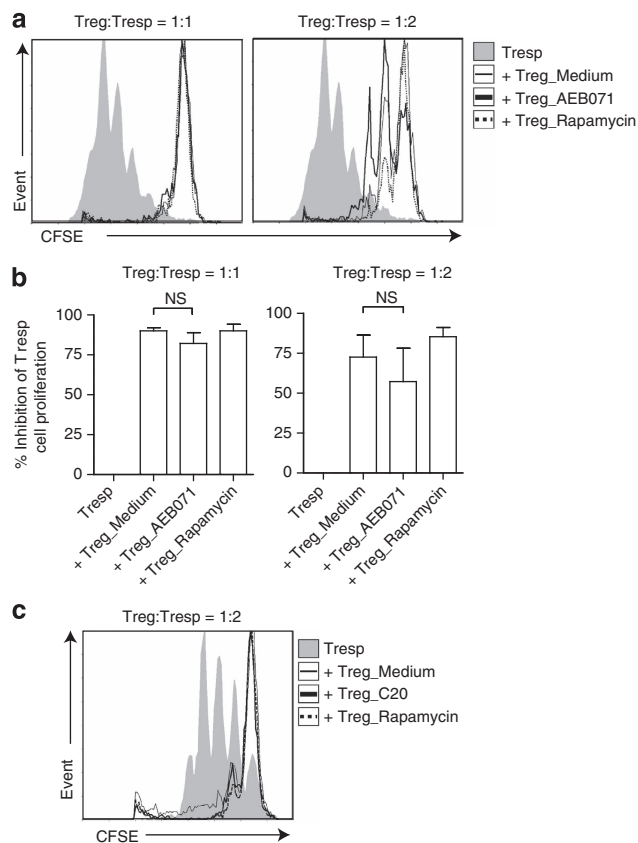


**Figure 2. AEB071 preserves regulatory T cell (Treg) phenotype and prevents Treg from producing IFN $\gamma$ /IL-17A.** FACS-sorted CD4 + CD25<sup>high</sup> Treg were stimulated with anti-CD3/anti-CD28 mAb-coated beads or phorbol 12-myristate 13-acetate (PMA)/anti-CD28 mAb plus recombinant human interleukin-2 (rhIL-2) in the presence of a graded dose of AEB071 (red line) or rapamycin (blue line). (a) Proliferation of Treg was measured by [<sup>3</sup>H]-thymidine incorporation during the last 6 hours of a 7-day culture, n = 5. (b) Flow cytometry of Treg cultured for 7 days as described above in the presence of AEB071 (1  $\mu$ M), rapamycin (200 nM), or C20 (1  $\mu$ M). Representative dot-plot showing CD25 + Fopx3 + expression (left panel) and cumulative data showing the frequency and mean fluorescence intensity (MFI) of CD25 + and Fopx3 + cells (right panel), n = 3. (c, d) Presence of IFN $\gamma$  and/or IL-17A in cell culture supernatant at day 7 as measured by Luminex. IL-1 $\beta$  was added to the culture where indicated. (e) Cumulative data showing the frequency of ROR $\gamma$ t + cells. All experiments were performed with Treg obtained from different healthy blood donors. Mean + SEM are shown, n = 3. NS, not significant; \*P < 0.05; \*\*P < 0.01.

with anti-CD3/anti-CD28 mAb-coated beads plus rhIL-2 in the presence/absence of AEB071 or rapamycin for a week. Th17 polarizing cytokines IL-1 $\beta$  or IL-23 were also administered to the cell culture. In the total psoriatic CD4 + T-cell pool, the addition of AEB071 or rapamycin resulted in enhanced Fopx3 + cell numbers as compared with the standard stimulation, especially in the presence of IL-23 (Figure 4a). More importantly, under these conditions, AEB071 significantly inhibited both IL-17A and IFN $\gamma$  production (Figure 4b). Then, we analyzed FACS-sorted Treg from patient peripheral blood. Confirming our previous results (Bovenschen *et al.*, 2011), these cells showed a markedly diminished expression of Fopx3 following stimulation, which was enhanced by the addition of IL-1 $\beta$  or IL-23 (Figure 4c, upper part left panel). Whereas in healthy Treg, AEB071 treatment maintained a similar level of Fopx3 + cells (Figure 2) as compared with the untreated condition, in patient Treg, treatment with AEB071 or rapamycin not only prevented the loss of Fopx3, but slightly enhanced it, even in the presence of IL-1 $\beta$  or IL-23 (Figure 4c). Moreover, this feature was accompanied by a reduced production of IL-17A (Figure 4d).

**AEB071 prevents IL-17A and IFN $\gamma$  production by skin-resident T cells**

Recruitment of T cells into the skin and their effector responses are considered to be key features in the pathogenesis of psoriasis. We therefore investigated the effect of AEB071 on skin-resident T cells. Single dermal cell populations were prepared from skin biopsies from lesional skin of psoriasis patients (n = 5) or skin from healthy individuals (n = 3). Figure 5a shows the percentage of cutaneous lymphocyte antigen and Fopx3-positive cells in the CD3 + population thus obtained (Figure 5a). Culture strategies are outlined in Figure 5b. First, we stimulated freshly isolated primary dermal T cells with anti-CD3/anti-CD28 mAb-coated beads plus rhIL-2 and IL-1 $\beta$  for 12 days, in the presence or absence of AEB071. Similar to its effect on blood-derived circulating T cells (Supplementary Figure S1a online), and as compared with the nontreated condition, AEB071 strongly inhibited proliferation of primary dermal T cells in both samples from psoriasis patients and those from healthy controls (data not shown). As very few dermal T cells proliferated in the presence of AEB071, hampering more extensive analysis, we



**Figure 3. Inhibition of protein kinase C (PKC) preserves regulatory T cell (Treg) suppressive function.** Carboxyfluorescein diacetate succinimidyl ester (CFSE)-based co-culture suppression assay of CD4 + CD25<sup>high</sup> Treg that were stimulated with anti-CD3/anti-CD28/rhIL-2 in the absence or presence of AEB071 (1  $\mu$ M), C20 (1  $\mu$ M), or rapamycin (200 nM). (a, c) A representative overlay histogram of CFSE-based suppression assay is shown on day 3 of co-culture. (b) Cumulative data showing %inhibition of Tresp proliferation induced by expanded Treg that were cultured according to the conditions mentioned. All experiments were performed with CD4 + CD25<sup>high</sup> and CD4 + CD25<sup>-</sup> cells obtained from different healthy blood donors,  $n = 5$  (a, b) or  $n = 3$  (c). Mean + SEM are shown. NS, not significant; Tresp, responder T cells.

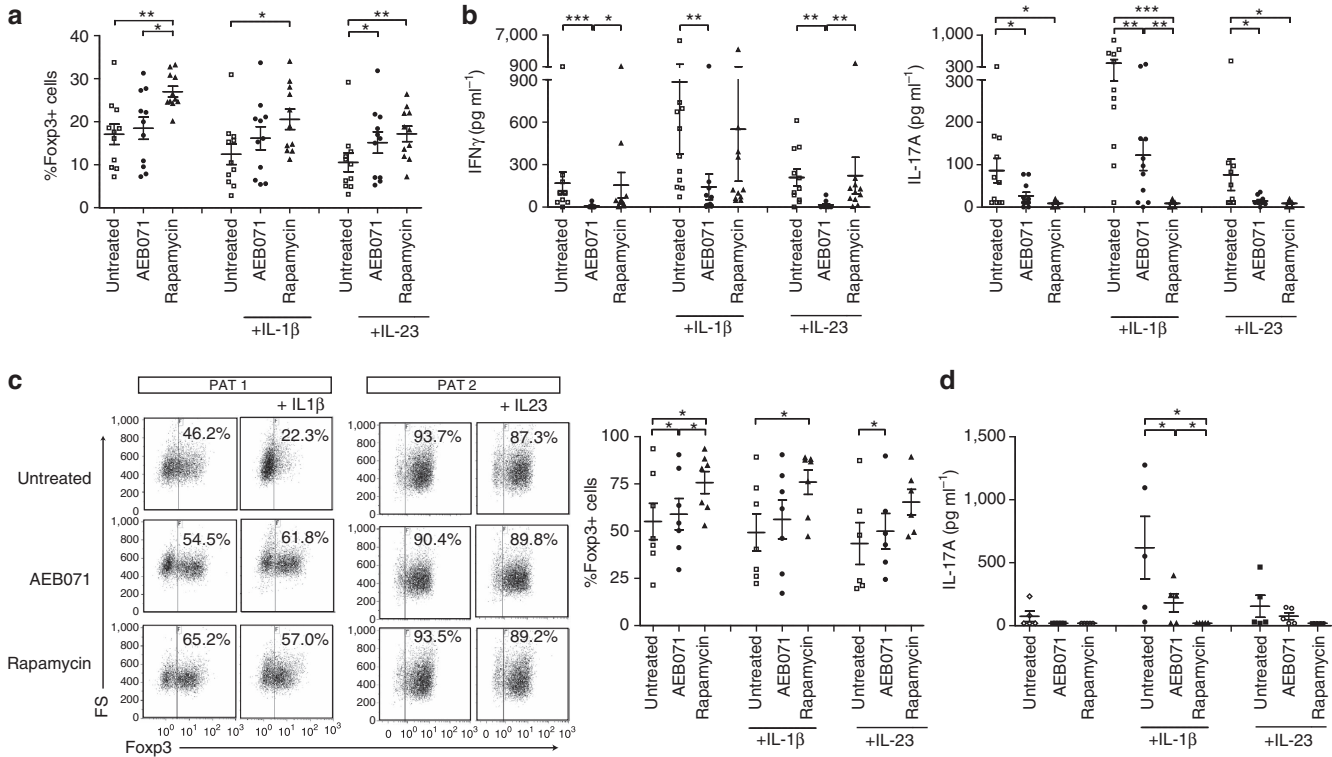
employed an alternative strategy to investigate the effect of AEB071 on dermal T cells. First, we expanded primary dermal T cells with anti-CD3/anti-CD28/rhIL-2 for 12 days. Thereafter, expanded dermal T cells were collected and rechallenged in the presence/absence of AEB071 for another 7 days before performing the phenotypic analysis. Consistent with our observation in circulating Treg, AEB071 enhanced Foxp3 expression in dermal T cells derived from both healthy individuals and patients (Figure 5c). In addition, patient-derived dermal T cells (primary and expanded) showed enhanced capacity to lose Foxp3 expression upon stimulation, which was largely prevented by treatment with AEB071 (Figure 5c). Again, similar to circulating Treg, upon stimulation, psoriasis patient-derived dermal T cells produced more IL-17A than those from healthy individuals. Also, AEB071 clearly diminished the percentage of IL-17A- as well as IFN $\gamma$ -producing cells in patients (Figure 5d).

## DISCUSSION

IL-17 is an important mediator in the pathogenesis of psoriasis. Previously, we reported that Treg of psoriasis patients have an enhanced propensity to convert into an inflammatory phenotype and start producing IL-17 (Bovenschen *et al.*, 2011). We also found evidence for these IL-17-producing Treg in psoriatic plaques. Recently, the importance of defining agents that can prevent this type of conversion was highlighted in view of improved therapeutic regimens for the management of (auto)inflammatory processes (Soler and McCormick, 2011). We here hypothesized that the pan-PKC inhibitor sotrastaurin (AEB071), now used in clinical trials to diminish clinical symptoms in psoriasis patients (Skvara *et al.*, 2008), might be such an agent. Indeed, our study provides evidence that sotrastaurin preserves the stability of human Treg; *in vitro* treatment with sotrastaurin prevented circulating Treg of both psoriasis patients and healthy controls from producing IFN $\gamma$  and IL-17A, even in the presence of the IL-17-driving cytokine IL-1 $\beta$ . In addition, Treg that were cultured in the presence of sotrastaurin, similar to rapamycin, efficiently suppressed the proliferation of responder T cells, indicating that both compounds favor the maintenance of suppressor function. Moreover, also in dermal T cells from lesional skin sotrastaurin preserved Foxp3 expression and inhibited IL-17A and IFN $\gamma$  production upon stimulation, features that may contribute to the clinical effects observed upon treatment.

On the basis of data obtained in PKC $\theta$ -knockout mice, contrasting roles for PKC $\theta$  in the development and function of thymus-derived Treg were reported: while Treg numbers were reduced, Treg suppressor function proved intact (Gupta *et al.*, 2008). The recent finding that the PKC $\theta$ -specific inhibitor C20 increases the suppressor activity of human Treg (Zanin-Zhorov *et al.*, 2010) is in line with our findings, and suggests that therapeutic strategies designed to inhibit PKC in T cells may hold promise for the treatment of autoimmune conditions. Sotrastaurin is a low-molecular-weight immunosuppressant selectively targeting PKC (Evenou *et al.*, 2009). In patients with moderate-to-severe psoriasis, sotrastaurin resulted in a mean reduction up to 69% for the psoriasis area and severity index score compared with baseline after 2 weeks of treatment (Skvara *et al.*, 2008), suggesting the efficacy of sotrastaurin in T cell-mediated diseases. Indeed, sotrastaurin markedly prolonged graft survival times in experimental heart and kidney allotransplantation animal models (Weckbecker *et al.*, 2010; Bigaud *et al.*, 2012). Although a phase II clinical trial using sotrastaurin plus MPA in renal transplant recipients was associated with increased rejection from week 4 onward, this calcineurin inhibitor-free regimen showed an acceptable safety profile and improved renal function (Friman *et al.*, 2011), which support further study of sotrastaurin as a component of multidrug immunosuppressive regimens.

Several studies revealed that under pro-inflammatory environmental stimuli, human Treg may lose Foxp3 and convert into less suppressive and even inflammatory phenotypes (Koenen *et al.*, 2008; Beriou *et al.*, 2009; Voo *et al.*, 2009). Here, we showed that sotrastaurin maintained the regulatory phenotype of Treg as characterized by the expression of Foxp3 and CD25 and preserved suppressor function. More



**Figure 4. AEB071 prevents the extensive loss of Foxp3 and inhibits IFN $\gamma$ /IL-17A production by peripheral regulatory T cells (Treg) from psoriasis patients.**

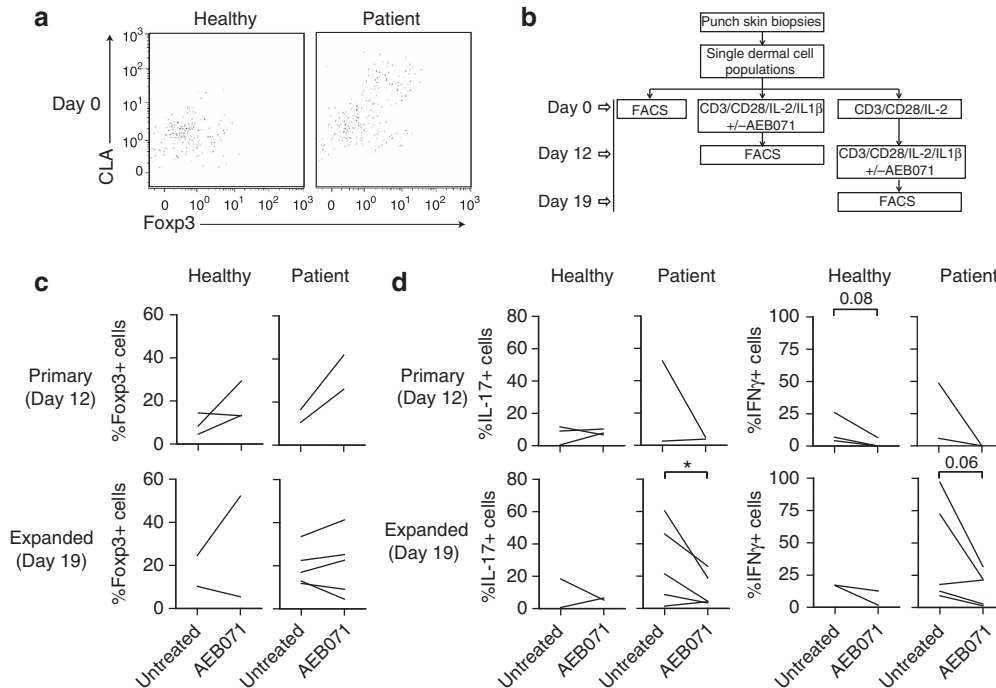
CD4+ T cells or CD4+CD25<sup>high</sup> Treg isolated from psoriasis peripheral blood were stimulated with anti-CD3/anti-CD28 mAb-coated beads in the absence/presence of AEB071 (1  $\mu$ M) or rapamycin (200 nM) for 7 days. IL-1 $\beta$  or IL-23 was included where indicated. In case of Treg, rhIL-2 was also administered to the cultures. (a) Cumulative data showing the frequency of Fxp3+ cells as measured by flow cytometry,  $n = 11$ . (b) Cumulative data showing the amount of IFN $\gamma$  and IL-17A in the culture supernatants of patient CD4+ cells as measured by Luminex,  $n = 11$ . (c) Representative dot-plots showing Fxp3 expression on Treg from two different psoriatic patients following stimulation as described above for 7 days. Cumulative data showing the frequency of Fxp3+ cells (right panel),  $n = 7$ . (d) Cumulative data showing the amount of IL-17A in psoriatic patient Treg-cell culture supernatants at day 7 of culture. Each data point represents a separate experiment, performed with cells isolated from a different psoriasis patient,  $n = 7$ . \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . PAT, patient.

importantly, upon stimulation of Treg, sotrastaurin, similar to rapamycin (Yurchenko *et al.*, 2012), not only preserved expression of Fxp3 and CD25 but also prevented Treg from producing IL-17A. The stabilizing effect of sotrastaurin was found in Treg from healthy individuals as well as from those of psoriasis patients, even in the presence of the Th17-enhancing cytokines IL-1 $\beta$  or IL-23. As psoriatic Treg are far more prone to convert into IL-17 producers and being in an inflammatory environment, this stabilizing feature is of particular relevance in the treatment of psoriasis patients.

The mechanism by which PKC affects Treg fate and function is largely unclear. In effector T cells, PKC $\theta$  is recruited to the immune synapse with the specific purpose of destabilizing the connection and possibly allowing for subsequent antigen encounter, thereby favoring Th2 and Th17 development (Yokosuka *et al.*, 2008). In contrast, mouse studies revealed that in Treg, the PKC/CARMA/NF $\kappa$ B complex was found localized outside the immune synapse (Zanin-Zhorov *et al.*, 2011). This implies that inhibition of PKC-mediated activation of NF $\kappa$ B/Api is beneficial during Treg activation, whereas inhibition of the NF $\kappa$ B/Api-mediated inflammatory pathway is detrimental for (Th2 and Th17) effector function. The fact that rapamycin is able to stabilize Treg suggests that multiple

pathways are likely involved. Treg stability through rapamycin-mediated inhibition of mammalian target of rapamycin may be achieved in a direct way through inhibition of protein synthesis or indirectly by modulating cellular metabolism via protein kinase B (PKB/Akt). This is underlined by the phenomenon that effector T cells and Treg require distinct metabolic programs to support their functions; Th1, Th2, and Th17 cells are highly glycolytic, whereas Treg have high lipid oxidation rates *in vitro* (Michalek *et al.*, 2011). Importantly, hypoxia-inducible factor-1, a key metabolic sensor, was shown to control the balance between the reciprocally regulated Th17 and Fxp3+ Treg cell lineage, which appeared to be dependent on mammalian target of rapamycin (Dang *et al.*, 2011; Shi *et al.*, 2011). Thus, the data imply that pharmacological inhibition targeting either PKC or mammalian target of rapamycin (by sotrastaurin and rapamycin, respectively) may be used to control Treg- and Tconv-specific functions in inflammatory disease.

Further, we found that different IC<sub>50</sub>s of sotrastaurin were obtained for Tconv and Treg subsets, and under different stimulation conditions (e.g., CD3/CD28 and PMA/CD28). Some of this can be explained by our previous finding that PMA/CD28-induced T-cell stimulation is highly dependent on



**Figure 5. AEB071 prevents IL-17A and IFN $\gamma$  production by skin-resident T cells.** Punch skin biopsies obtained from healthy individuals and psoriasis patients were used for preparation of the single dermal cell populations as described in Material and Methods. Thereafter, cells were either analyzed by FACS analysis for CD3, cutaneous lymphocyte antigen (CLA), and Foxp3 expression or cultured as schematically indicated (b) with or without AEB071 (10  $\mu$ M). (a) Dot-plots showing the expression of CLA and Foxp3 based on the CD3 + -gated derma cell population at day 0. Dot-plots show one representative healthy donor and one merged dot-plot of three patients. (c) Cumulative data showing the percentage of Foxp3 + cells after the first round of 12-day stimulation (primary (D12)) or after the second round of 7-day stimulation (expanded (D19)). (d) Cumulative data showing the percentage of IL-17A and IFN $\gamma$ -positive cells at days 12 and 19 of culture. In total, three healthy and five patient skin biopsies were used.

the PKC $\theta$  pathway, whereas CD3-mediated pathways are dependent on both Lck and PKC $\theta$  signal transduction (Smeets *et al.*, 2012). Although sotrastaurin dose dependently inhibited both Tconv- and Treg-cell proliferation, the IC<sub>50</sub> for Treg was always lower than that for Tconv regardless of the stimulus provided. This may be explained by the following: (1) differences in expression of environmental sensors and ensuing signal transduction pathways involved in Treg and Tconv subsets; (2) differences in PKC $\theta$  cellular concentrations and cellular localization as reported recently for Treg and Tconv. Higher sensitivity of Treg to PKC inhibition may be favorable for preventing Treg reprogramming *in vivo*. More studies are needed to establish optimal dosing of PKC inhibitors for stabilizing Treg function while maintaining T-cell homeostasis.

In conclusion, here, we showed that sotrastaurin inhibited effector T-cell responses, whereas the regulatory response was enhanced, a feature observed even in the presence of IL-1 $\beta$ . In addition, we provide evidence for the role of PKC in the balance of Treg and Th17 differentiation and show that this mechanism also holds true for both circulating and skin-derived T cells from psoriasis patients. Inhibition of PKC thus appears to control Treg fate by preventing Treg differentiation into inflammatory effector T cells. Pharmacological inhibition of PKC may thus add to strategies for Treg facilitation in the treatment of autoimmune-related disorders, such as psoriasis.

## MATERIALS AND METHODS

### Cell isolation and culture

Buffy coats were obtained from healthy blood donors (Sanquin Blood Bank, Region South-East, The Netherlands) upon written informed consent, according to the Declaration of Helsinki Principles. In total, 16 psoriasis patients were recruited to this study and peripheral blood was collected by vena puncture. The average psoriasis area and severity index score was 10.3  $\pm$  2.5 (mean  $\pm$  SEM). Patients were either untreated ( $n$  = 5) or treated with methotrexate ( $n$  = 7), fumaric acid ( $n$  = 3), or UVB ( $n$  = 1). This study was carried out in Radboud University Medical Centre in accordance with the applicable rules concerning the review of research ethics committees and upon written informed consent. Peripheral blood mononuclear cells and CD4+ were isolated and cultured as described previously (He *et al.*, 2011). Briefly, the resultant CD4+ fraction was labeled with anti-CD25-PE (M-A251; BD Biosciences, Erembodegem, Belgium) and anti-CD4-PC5 (13B8.2, Beckman-Coulter, Mijdrecht, The Netherlands), conjugated mAbs; thereafter, Tconv (CD4 + CD25<sup>-</sup>) and Treg (CD4 + CD25<sup>high</sup>) were sorted using an Altra or Aria cell-sorter (Beckman-Coulter). Cell purity was over 98%.

CD4 + CD25<sup>-</sup> Tconv were stimulated with anti-CD3/anti-CD28 mAb-coated beads (T Cell Expanders, Dynal Biotech, Oslo, Norway) in a bead:cell ratio of 1:5, in the absence/presence of the indicated concentrations of inhibitors for 4 days. CD4 + CD25<sup>high</sup> Treg were stimulated with either anti-CD3/anti-CD28 mAb-coated beads, in a bead:cell ratio of 1:2, and recombinant human cytokines IL-2 (rhIL-2,

25 U ml<sup>-1</sup>, Proleukine, Amsterdam, The Netherlands), or PMA (12.5 ng ml<sup>-1</sup>), soluble CD28 mAb (1 µg ml<sup>-1</sup>) and rhIL-2 (25 U ml<sup>-1</sup>) in the absence/presence of the indicated concentrations of inhibitors for a week. IL-1β (50 ng ml<sup>-1</sup>) or IL-23 (50 ng ml<sup>-1</sup>) was added into cell cultures where described.

### Cell isolation from skin

Skin punch biopsies from healthy individuals were obtained from abdominal skin leftover of healthy individuals undergoing elective plastic surgery at the Radboud University Medical Centre Department of Plastic Surgery after oral or written informed consent for scientific use. Skin punch biopsies from psoriasis patients (psoriasis area and severity index score, 15.8 ± 1.7, *n* = 5) were obtained from lesional skin at the University of Essen Department of Dermatology after oral or written informed consent for scientific use. The use of human skin was approved and in accordance with the regulations set by the Medical Ethical Committees for human research of the Radboud University Medical Centre.

Biopsies (4 mm) were incubated in a 24-well plate in which each well contains 32 mg ml<sup>-1</sup> Dispase II (Roche, Woerden, The Netherlands) at 4 °C overnight for peeling-off of the epidermis. Thereafter, biopsies were mechanically dissociated using gentaMACS tubes (Miltenyi Biotec, Leiden, The Netherlands) followed by the incubation with collagenase I (10,000 U ml<sup>-1</sup>, Sigma-Aldrich, Zwijndrecht, The Netherlands) for 1 h. After the addition of DNase I (5 MU ml<sup>-1</sup>, Calbiochem, Amsterdam, The Netherlands) and one more cycle of mechanical dissociation, samples were filtered through a cell strainer to obtain the single dermal cell population, referred to as primary dermal T cells in this manuscript.

Primary dermal T cells were cultured with anti-CD3/anti-CD28 mAb-coated beads together with rhIL-2 for 12 days before either directly performing the phenotypic analysis or used as expanded dermal T cells for further analysis. At day 12, the expanded dermal T cells were collected and restimulated for another 7 days before phenotypic analysis. IL-1β (50 ng ml<sup>-1</sup>) and AEB071 (10 µM) were added where indicated.

### Inhibitory compounds

AEB071 and C20 were kindly provided by Organon NV (now Merck Research Laboratories, MSD, Oss, The Netherlands) and Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT), respectively. Rapamycin was purchased from Sigma. Cells were pretreated with indicated concentrations of the inhibitors or vehicle control for 30 min at 37 °C. Thereafter, stimulators as indicated were added to the culture mixture.

### Cell proliferation assay

Typically, 5 × 10<sup>4</sup> Tconv or 2 × 10<sup>4</sup> Treg per well were stimulated in the presence of graded doses of inhibitors. Cell proliferation was monitored by [3H]-thymidine incorporation using a Gas Scintillation Counter (Canberra Packard, Matrix 96 Beta-counter, Meriden, CT) at day 4 (Tconv) or 7 (Treg). Percentage of cell proliferation inhibition was normalized to the control. The sigmoid curve was generated using GraphPad Prism5.0 (San Diego, CA).

### Antibodies and CFSE labeling

Cells were phenotypically analyzed by multi-colors flow cytometry (Beckman-Coulter), using the following: anti-CD3-PeCy5 (UCHT1),

anti-CD4-PeCy5 (T4), anti-CD8-ECD (SFC121Thy2D3), anti-CD69-ECD (TP1.55.3), and anti-CD62L-ECD (DREG56; all from Beckman-Coulter), anti-CD25-PeCy7 (BC96; eBioscience, Uithoorn, The Netherlands), anti-HLA-DR-FITC (L243), and anti-PD1-FITC (MIH4; both from BD Bioscience) mAbs. Anti-Foxp3-FITC or -eFluo450 (PCH101), anti-RORγt-PE or -APC (AFKJS-9; both from eBioscience), and anti-CTLA4-APC (BNI3, BD Bioscience) were used after Fix-Perm-treatment (eBioscience). Appropriate isotype mAbs were used to define marker settings. Fixable viability dye (eBioscience) was used in some experiments. Data were analyzed using the Kaluza software (Beckman-Coulter).

Cells (5 × 10<sup>6</sup> cells ml<sup>-1</sup>) were labeled with 0.5 µM CFSE (Molecular Probes, Leiden, The Netherlands) to monitor cell division (Koenen *et al.*, 2005).

### Co-culture suppression assays

The suppressor capacity of expanded Treg was studied in co-culture assays. Tregs were expanded in the absence/presence of inhibitors for a week. Thereafter, viable Tregs were collected, washed, and added at different ratios to CFSE-labeled CD4 + CD25 – responder T cells (Tresp) together with anti-CD3/anti-CD28 mAb-coated beads for 3 days. Proliferation of Tresp was determined by analyzing CFSE dilution as described previously (Koenen *et al.*, 2005).

### Cytokine production

IL-17A and IFNγ were determined in the culture supernatants using Luminex cytokine assays (Invitrogen, Veenendaal, The Netherlands), according to the manufacturer's instructions. The lower level of detection of IFNγ was <5 pg ml<sup>-1</sup> and that of IL-17A was <10 pg ml<sup>-1</sup>.

### Statistics

Results are presented as mean ± SEM. *P*-values were determined by paired *t*-test or one-way analysis of variance, using the GraphPad Prism5.0 software. *P*-values < 0.05 were considered statistically significant.

### CONFLICT OF INTEREST

RLS and AMHB were previously employed by Organon NV, Oss, The Netherlands.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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