

# Inhibition of alanyl-aminopeptidase on CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells enhances expression of FoxP3 and TGF- $\beta$ 1 and ameliorates acute colitis in mice

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**Abstract.** Inhibitors of alanyl-aminopeptidase e.g. phebestin increase the expression of transforming growth factor (TGF)- $\beta$ 1 in mononuclear cells. We investigated whether phebestin also produced this effect in CD4<sup>+</sup>CD25<sup>+</sup> T-cells and whether phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells were capable of ameliorating acute colitis in mice. The suppressive activity of mouse CD4<sup>+</sup>CD25<sup>+</sup> T-cells was assessed *in vitro* by co-culture with splenocytes. mRNA expression associated with the suppressive phenotype was determined *in vitro* and *in vivo*. The *in vivo* role of phebestin-exposed CD4<sup>+</sup>CD25<sup>+</sup> T-cells was studied in sodium dextran sulfate-induced acute colitis in mice. The proliferation of activated effector T-cells or splenocytes *in vitro* was inversely correlated with the number of CD4<sup>+</sup>CD25<sup>+</sup> T-cells. Phebestin pre-treatment substantially enhanced the suppressive activity of these cells and increased expression levels of TGF- $\beta$ 1 and FoxP3. Furthermore, transfer of CD4<sup>+</sup>CD25<sup>+</sup> T-cells exposed to phebestin for a short time *ex vivo* significantly reduced the mouse colitis disease activity index. We conclude that aminopeptidase inhibitors support the suppressive activity as well as TGF- $\beta$ 1 and FoxP3 expression of natural regulatory T-cells.

## Introduction

A unique population of CD4<sup>+</sup> T lymphocytes that constitutively expresses CD25 and comprises 5-10% of circulatory CD4<sup>+</sup> T-cells acts to powerfully suppress responder T-cells *in vitro* and *in vivo*. Sakaguchi *et al* (1) first described these CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells, which are capable of controlling pathogenic T-cell responses in disease models for colitis, autoimmune encephalomyelitis, gastritis, and graft-versus-host disease (2-5).

T-cell receptor (TCR) challenging and, as most authors agree, direct T-cell contact are required for the effective suppression of responder T-cells by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells. In both mice and humans, CD4<sup>+</sup>CD25<sup>+</sup> cells have been shown to constitutively express high levels of transforming growth factor (TGF)- $\beta$ 1, and application of anti-TGF- $\beta$ 1 antibodies leads to a dose-dependent decrease in suppressive activity in humans and mice (6-9). At their cell surface, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells are unique in their expression of both latent and active TGF- $\beta$  (6), a feature that is believed to facilitate contact-dependent inhibition of responder T-cells. The role of TGF- $\beta$ 1 in the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells has been questioned (10). Recent studies, however, clearly established its pivotal role in mediating the suppressive function of CD4<sup>+</sup>CD25<sup>+</sup> cells in a broad variety of autoimmune disease models and experimental settings (11-18).

*In vitro* generation of induced regulatory T-cells is achieved by activation in the presence of the immunosuppressive cytokines, TGF- $\beta$ 1 and interleukin (IL)-10 (7,11). TGF- $\beta$ 1 induced the gene expression of FoxP3-converted naïve CD4<sup>+</sup>CD25<sup>+</sup> T-cells into anergic/suppressor cells upon TCR activation (19), and adenovirally FoxP3-transduced CD4<sup>+</sup>CD25<sup>+</sup> T-cells prevented autoimmune thyroiditis *in vivo* (58). CD4<sup>+</sup>CD25<sup>+</sup> T-cells also play an essential role in the generation *in vivo* and *in vitro* of other regulatory T-cell subsets including Tr1 and Th3 (20-24).

Highly immune-deficient SCID mice develop colitis upon adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> effector T-cells (3,12,25,26).

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In this model, the co-transfer of an equal number of CD4<sup>+</sup>CD25<sup>+</sup> cells prevented colitis, and the disease was cured by the subsequent injection of CD4<sup>+</sup>CD25<sup>+</sup> cells from healthy animals; this curative effect was abolished by anti-IL-10, anti-TGF- $\beta$ 1, and anti-CTLA-4 antibodies (3,25). In a similar experimental setting, both Th1- and Th2-cell-induced colitis was suppressed by transferred CD4<sup>+</sup>CD25<sup>+</sup> T-cells *in vivo* (27). Whereas the role of CD4<sup>+</sup>CD25<sup>+</sup> T-cells in the SCID model of adoptive transfer is well established, little is known about the protective capacity of these cells in colitis models of fully immunocompetent mice.

We previously reported that inhibitors of the enzymatic activity of membrane alanyl-aminopeptidase (mAAP, APN, CD13, EC 3.4.11.2) inhibited proliferation and function of immune cells, including mononuclear cells and T-cells, as well as cell lines derived thereof (28). This immunosuppression resulted, at least partially, from downregulation of the expression and release of pro-inflammatory and inflammatory cytokines such as IL-2, whereas the production/release of the immunosuppressive TGF- $\beta$ 1 was increased (28).

In a previous study we demonstrated the expression of mAAP mRNA in human CD4<sup>+</sup>CD25<sup>+</sup> T-cells of healthy volunteers. Among various leukocyte and T-cell subsets, the CD4<sup>+</sup>CD25<sup>+</sup> T-cells showed by far the highest mAAP mRNA levels (29). These findings, together with the induction of production and release of TGF- $\beta$ 1 observed in response to aminopeptidase inhibition, suggest that CD4<sup>+</sup>CD25<sup>+</sup> T-cells represent essential mediators of the known immunosuppressive effects of these compounds.

In this study, we demonstrated the ability of inhibitors of alanyl-aminopeptidase to i) increase TGF- $\beta$ 1 expression in CD4<sup>+</sup>CD25<sup>+</sup> T-cells, ii) enhance the suppressive activity of these cells, and iii) ameliorate disease activity in a sodium dextran sulfate (DSS)-induced colitis mouse model. Thus, the administration of alanyl-aminopeptidase inhibitors and, in particular, combined cell therapy with immunomodulated CD4<sup>+</sup>CD25<sup>+</sup> T-cells are potentially beneficial for the pharmacological treatment of inflammatory diseases such as inflammatory bowel disease (IBD) and may provide an adjuvant therapeutic concept of functional immunorestitution.

## Materials and methods

**Cells.** Splenocytes were prepared from the spleens of healthy Balb/c mice using density gradient centrifugation over Lymphocyte Separation Medium (PAA Laboratories, Pasching, Austria) (30). CD4<sup>+</sup>CD25<sup>+</sup> T-cells were positively selected from mouse splenocytes using CD25 MicroBeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). For *in vitro* experiments, cells were stimulated as indicated below. Prior to their injection into colitis-afflicted mice, CD4<sup>+</sup>CD25<sup>+</sup> T-cells from healthy mice were exposed to phorbstein (Sigma, Steinheim, Germany) (1  $\mu$ mol/l, 37°C, 45 min) or kept in PBS for the same period of time.

**Cytofluorimetric analyses.** Cytofluorimetric analyses were performed with an Epics XL-MCL (Coulter, Germany) using monoclonal rat anti-mouse Foxp3, clone FJK-16s (Natutec, Frankfurt, Germany); rat anti-mouse CD13-FITC, clone RS-

242 (BD Pharmingen, Heidelberg, Germany); and rat anti-mouse CD25-PE, clone 7D4 (Miltenyi). PC5-donkey anti-rat IgG (Coulter) was used for secondary staining of unlabelled antibodies.

**DNA synthesis.** Cells were seeded into 96-well plates at a density of 50,000 cells/well/200  $\mu$ l cell culture medium. For stimulation, 1  $\mu$ g/ml biotin hamster anti-mouse CD3e (clone 145-2C11, eBioscience, San Diego, CA), 1  $\mu$ g/ml biotin anti-mouse CD28 (clone 37.51), and 2.5  $\mu$ g/ml streptavidin (Jackson ImmunoResearch Europe, Cambridgeshire, UK) were added. After 72 h, the cultures were pulsed for an additional 6 h with [<sup>3</sup>H]-methylthymidine (0.2  $\mu$ Ci per well; Amersham-Biosciences, Freiburg, Germany), and the incorporated radioactivity was measured by scintillation counting (31).

**RNA preparation.** Total RNA from snap-frozen colonic tissue sections was prepared by using Trizol reagent (Invitrogen, Heidelberg, Germany) (32). Total RNA from splenocytes and T-cells was prepared using the RNeasy mini kit (Qiagen, Hilden, Germany). Contaminating DNA was removed by DNase I digestion.

**Quantitative PCR.** cDNA was generated from 1  $\mu$ g total RNA, and 1/20th of the cDNA mixture was used for quantitative RT-PCR in the iCycler (Bio-Rad, Munich, Germany). A typical 25- $\mu$ l reaction mixture contained 12.5  $\mu$ l HotStarTaq Master mix (Qiagen), 0.3  $\mu$ l of a 1:1000 dilution of SYBR-Green I (Molecular Probes, Eugene, OR), and 0.5  $\mu$ mol of the specific primers: FoxP3-US, 5'-TGTCAGTCAACTTCA CCAAG-3'; FoxP3-DS, 5'-AGCTGGTGCATGAAATGTG G-3'; TGF- $\beta$ 1-US, 5'-CGCTTGCAGAGATTAAAA-3'; TGF- $\beta$ 1-DS, 5'-TGAATCGAAAGCCCTGTA-3'; IL-10-US, 5'-CTGCTATGTTGCCTGCTCTT-3'; and IL-10-DS, 5'-ATCATTCTTCACCTGCTCCAC-3'. An initial denaturation/activation step (15 min at 95°C) was followed by 40 cycles (30 sec at 95°C, 30 sec at 58°C, 45 sec at 72°C). The amounts of mRNA were normalized to mouse ribosomal protein large P0 (P0-US, 5'-GCACTTTCGCTTTCTGGAGGGTGT-3' and P0-DS, 5'-TGACTTGGTTGCTTTGGCGGGATT-3') and analyzed using a one-way analysis of variance.

**Immunoblot analyses.** For immunoblot analyses, 3x10<sup>6</sup> CD4<sup>+</sup>CD25<sup>+</sup> T-cells were washed twice in ice-cold PBS, resuspended in 50  $\mu$ l of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 10% glycerol, 10 mM K<sub>2</sub>HPO<sub>4</sub>, and 0.5% NP-40), containing a protease inhibitor cocktail (Boehringer Mannheim), 1 mM sodium vanadate, 0.5% sodium deoxycholate, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM NaF, and 20 mM glycerol-2-phosphate (all from Sigma, Heidelberg, Germany) and kept on ice for 30 min to complete cell lysis. The homogenates were centrifuged (15,000 x g, 4°C, 30 min) and the resulting supernatant was stored at -20°C until further use. The protein concentration was determined using the micro-Lowry-based protein assay kit (Sigma), following the recommended protocol. Immunoblots were performed as previously described (34) using the following primary antibodies for immunodetection: rat anti-mouse CD13 monoclonal antibody (clone ER-BMDM1, 1:200; Dianova,

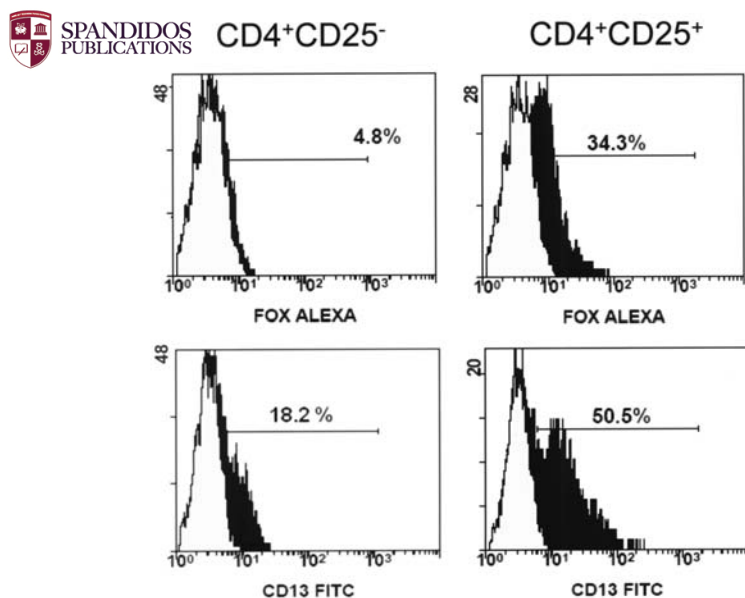


Figure 1. Detection of alanyl-aminopeptidase (mAAP/CD13), the pharmacological target of phebestin, in mouse CD4<sup>+</sup>CD25<sup>+</sup> T-cells. Left panel: FACS analyses demonstrating the expression of FoxP3, CD13, and CD25 in CD4<sup>+</sup>CD25<sup>+</sup>, but not in CD4<sup>+</sup>CD25<sup>-</sup> T-cells. Right panel: The presence of mAAP/CD13 in mouse CD4<sup>+</sup>CD25<sup>+</sup> T-cells was confirmed by immunoblot analysis of total cell lysates using the rat anti-mouse CD13 monoclonal antibody, clone ER-BMDM1. CD4<sup>+</sup>CD25<sup>-</sup> T-cells showed no significant mAAP/CD13 expression. The purified enzyme from mouse kidney (R&D Systems) was used as the positive control.

Hamburg, Germany), goat anti-mouse FoxP3 (polyclonal, 1:500; Acris Antibodies Hiddenhausen, Germany), and monoclonal anti-TGF- $\beta$ 1, - $\beta$ 2, - $\beta$ 3 (clone 1D11, 1:500; R&D Systems, Wiesbaden, Germany). The secondary antibodies were donkey anti-goat IgG HRP (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rat IgG alkaline phosphatase (1:10,000; Sigma), and anti-mouse IgG HRP (1:2000; New England Biolabs, Frankfurt, Germany).

**Animals.** We performed all animal studies in compliance with international and local animal welfare legislations. Female Balb/c mice were purchased from Harlan-Winkelmann (Borchen, Germany) and housed under standard conditions (25°C and 12-h light:12-h dark cycle) for >1 week before starting the experiments. Mice were fed with standard pellets *ad libitum*.

Acute colitis was induced by adding 3% (w/v) dextran sulphate sodium (DSS, MW 36,000-50,000; ICN Biomedicals, Aurora, OH) to the drinking water for 7 days. This concentration of DSS was previously shown to induce severe colitis, but with a low risk of death, within 7 days. The animals had free access to the DSS solutions, which were changed every other day.

The severity and progress of colitis was monitored by daily examination for the general state of health (activity, grooming behaviour, and mean food/water consumption). Weight changes were recorded daily. Feces were visually inspected for signs of diarrhoea and rectal bleeding. The disease activity index (DAI) was determined by summarizing the scores for weight loss, stool consistency, and bleeding (Hemoccult sensa; Beckmann-Coulter, Krefeld, Germany).

Phebestin or CD4<sup>+</sup>CD25<sup>+</sup> T-cells were administered i.v. as indicated in the figure legends. For localization studies, labelling of CD4<sup>+</sup>CD25<sup>+</sup> T-cells was performed by a 15-min incubation of 10<sup>6</sup> cells/100  $\mu$ l PBS in the presence of 2  $\mu$ M

CMFDA (CellTracker Green) at 37°C. Cells were washed twice in PBS and then injected into mice at day 3 of colitis induction.

**Tissue sampling and histological analysis.** Animals were sacrificed by an overdose of carbon dioxide. Blood was collected post mortem by cardiac puncture, and platelet-free plasma was obtained using a two-step centrifugation as described previously (30).

The entire colon plus cecum was dissected, and the length from the colo-cecal junction to the anus was measured as a marker of inflammation. Sections, 1 cm in length, were obtained from distal, transverse and proximal segments of the colon and either fixed in 10% buffered formalin for histological analysis or snap-frozen in liquid nitrogen.

Mucosal lesions and inflammatory cell infiltrates were assessed by light microscopy (x40 objective) on longitudinal sections (4  $\mu$ m) of paraffin-embedded specimens stained with hematoxylin and eosin. Pathological changes were assessed in a 'blinded' fashion for four categories. a) Infiltration: mononuclear cells (presence of large lymph follicles), 1 point; mononuclear cells including high numbers of polymorphonuclear granulocytes, 2 points. b) Histocyte-rich inflammatory sites: focal, 1 point; extensive, 2 points. c) Epithelial erosions: focal, 1 point; in large areas, 2 points. d) Distortion of crypt architecture or crypt loss: focal 1, point; extensive, 2 points. Most severe changes at day 7 were represented by 8 points. Normal morphology without infiltration was assessed with 0 points. Three sections per animal were scored individually and subsequently summarized to obtain the mean score.

**Statistical analysis.** Analysis of any statistical significant differences between the groups was performed with the non-parametric Wilcoxon test. A P value <0.05 was considered significant. Results are expressed as the mean  $\pm$  SEM.



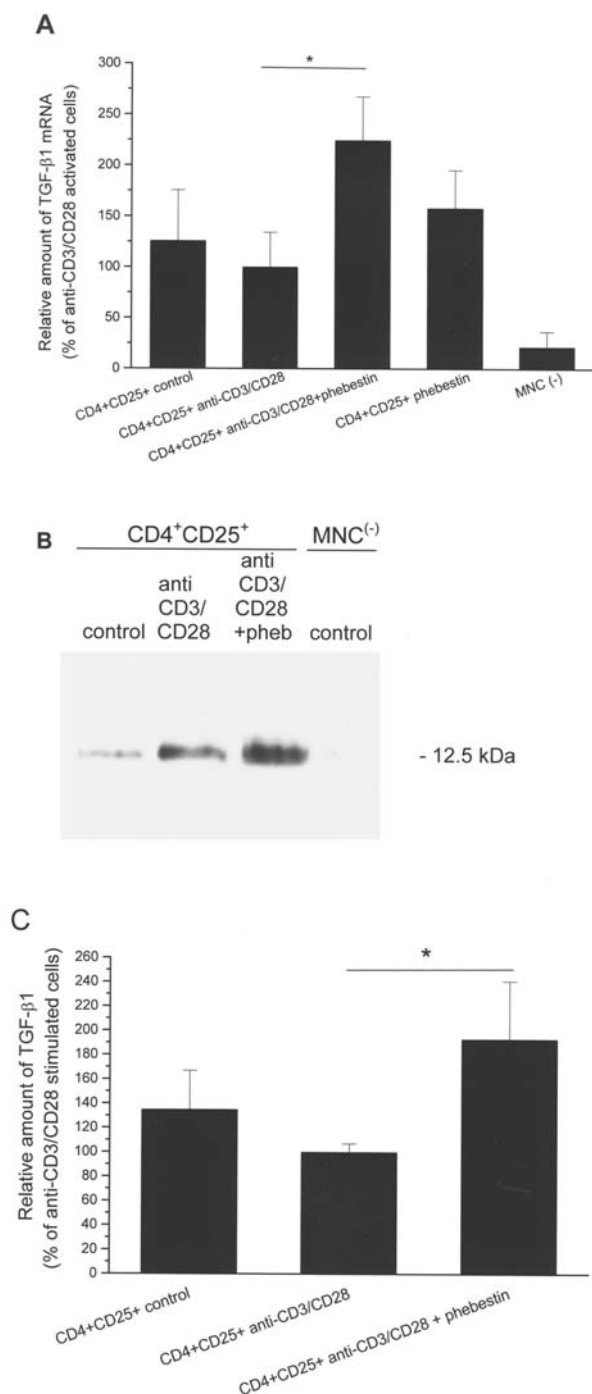


Figure 2. Phebestin induces the expression of TGF-β1 in mouse CD4<sup>+</sup>CD25<sup>+</sup> T-cells. (A) Quantitative RT-PCR revealed the presence of TGF-β1 mRNA in freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> T-cells (~30,000 copies/μg RNA). Amounts of TGF-β1 mRNA were determined in response to 1 μM phebestin and/or activation by anti-CD3/CD28 after 24 h of culture. Mononuclear cells depleted of CD4<sup>+</sup>CD25<sup>+</sup> T-cells (MNC<sup>(-)</sup>) were used for comparison and exhibited lower amounts of TGF-β1 mRNA. (B) Similarly, the effects of phebestin or activation by anti-CD3/CD28, respectively, on TGF-β1 protein were analysed by immunoblot analysis. (C) The amounts of TGF-β1 secreted into the culture medium were determined by ELISA. (A,C: n=6; \*P<0.01; B: shown is one representative immunoblot out of four independent experiments).

## Results

*Characteristics of enriched CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells.* We analysed CD4<sup>+</sup>CD25<sup>+</sup> T-cells enriched from mouse

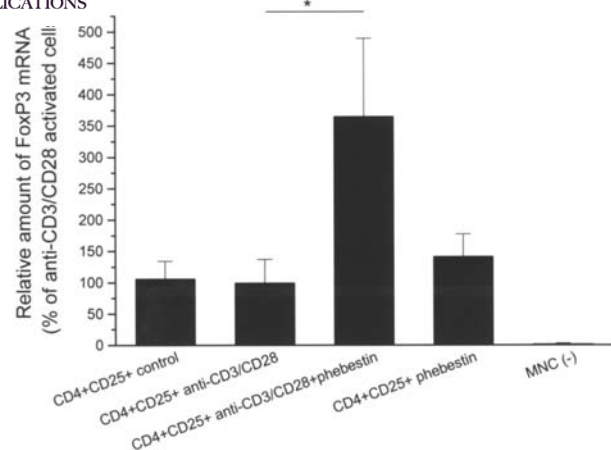
splenocytes using flow cytometry and quantitative RT-PCR. Surface expression of the T-cell antigens CD4 and CD3 was greater by 93% in all experiments, with at least 96.7% being CD4<sup>+</sup>CD25<sup>+</sup> double-positive T-cells. The absence of IL-2 and IFN-γ-mRNA expression indicated the absence of contaminating activated effector T-cells or induced regulatory T-cells (not shown).

*Expression of mAAP/CD13 in CD4<sup>+</sup>CD25<sup>+</sup> T-cells.* CD4<sup>+</sup>CD25<sup>+</sup> T-cells do express mAAP (CD13) and represent a major cellular target of phebestin, a specific inhibitor of alanyl-aminopeptidases (29). Consistent with this finding we previously identified CD13 as a surface marker of the regulatory T-cell line KARPAS-299 (55). Cytofluorimetric analysis of double-stained cells identified 53.2±22.1% CD25<sup>+</sup>CD13<sup>+</sup> (n=3) and 43.9±12.2% FoxP3<sup>+</sup>CD13<sup>+</sup> cells (n=3) among the enriched CD4<sup>+</sup>CD25<sup>+</sup> T-cells (Fig. 1, left panel). By immunoblot analysis using the rat anti-mouse CD13 monoclonal antibody, clone ER BMDM1, we clearly demonstrated the presence of mAAP immunoreactivity in CD4<sup>+</sup>CD25<sup>+</sup> T-cells (Fig. 1, right panel). Furthermore, mouse CD4<sup>+</sup>CD25<sup>+</sup> T-cells exhibited alanine-*p*-nitroanilide-cleaving enzymatic activity, which was almost completely inhibited (14% residual activity) by the addition of phebestin (10<sup>-6</sup> mol/l), but hardly affected by PAQ-22, a selective inhibitor of cytosolic alanyl-aminopeptidase (cAAP, PSA; 95% residual activity), clearly assigning the enzymatic activity to mAAP/CD13.

*The 'suppressive phenotype' of CD4<sup>+</sup>CD25<sup>+</sup> T-cells is lost upon activation in vitro but is preserved by phebestin.* Expression of TGF-β1 has been implicated as an essential phenomenon in the suppression of responder T-cells by CD4<sup>+</sup>CD25<sup>+</sup> T-cells. Activation of CD4<sup>+</sup>CD25<sup>+</sup> T-cells by anti-CD3/anti-CD28 did not cause marked changes in the TGF-β1 mRNA and protein expression, but there was a tendency towards a decrease in the release of TGF-β1 into the culture medium (Fig. 2A-C). Phebestin, when added simultaneously with the activation, led to an increase in TGF-β1 expression at all expression levels investigated, which significantly exceeded those of freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> T-cells (Fig. 2A-C).

Next, we assessed the expression of FoxP3, a transcription factor that is believed to be exclusively expressed in CD4<sup>+</sup>CD25<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> T-cells, and the expression of which correlates with their suppressive phenotype in humans and mice (26,35-40,57). In this study, as expected, we showed that FoxP3 mRNA was abundantly expressed in CD4<sup>+</sup>CD25<sup>+</sup> T-cells, but hardly expressed in CD4<sup>+</sup>CD25<sup>-</sup> T-cells or in MNC<sup>(-)</sup> (Fig. 3A). Upon activation by anti-CD3/anti-CD28, there was no obvious change in FoxP3 mRNA expression. Remarkably, activation in the presence of 1 μM phebestin led to a nearly 4-fold increase in FoxP3 mRNA amounts (Fig. 3A) and a clear induction of FoxP3 protein (Fig. 3B). This finding suggests that phebestin and other inhibitors of alanyl-aminopeptidases exert a stabilizing effect on the suppressive phenotype of CD4<sup>+</sup>CD25<sup>+</sup> T-cells.

*Phebestin increases the immunosuppressive activity of human CD4<sup>+</sup>CD25<sup>+</sup> T-cells in vitro.* As expected, anti-CD3/



B

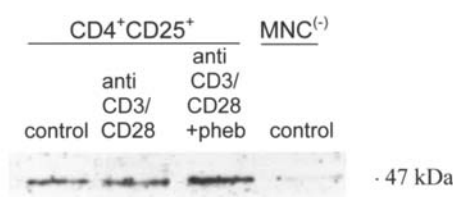


Figure 3. Phebestin induces the expression of FoxP3 in mouse CD4<sup>+</sup>CD25<sup>+</sup> T-cells. Amounts of FoxP3 mRNA were analysed by quantitative RT-PCR. Freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> T-cells contained  $\sim 3.8 \times 10^6$  copies/ $\mu$ g RNA. FoxP3 protein expression was analysed by immunoblot assays. Both at the mRNA (A) and protein level (B) strong expression of FoxP3 was demonstrated which appeared largely unchanged upon activation by anti-CD3/CD28. Phebestin, however, at a concentration of 1  $\mu$ M led to a significant increase in both FoxP3 mRNA and protein after 24 h. MNC<sup>(-)</sup> contained trace amounts of FoxP3 mRNA or protein only. (n=6, \*P<0.01, B: shown is one representative immunoblot out of four independent experiments).

anti-CD28-mediated stimulation of mouse MNC led to an induction of cell proliferation. MNC which had been depleted of CD4<sup>+</sup>CD25<sup>+</sup> prior to activation (MNC<sup>(-)</sup>) showed a markedly elevated proliferative response to anti-CD3/anti-CD28 (Fig. 4). Addition of CD4<sup>+</sup>CD25<sup>+</sup> T-cells to the MNC<sup>(-)</sup> resulted in inhibition of activation-induced proliferation (Fig. 4). CD4<sup>+</sup>CD25<sup>+</sup> T-cells that had been exposed to phebestin (10  $\mu$ M in 20  $\mu$ l PBS, 45 min) prior to their co-culture with MNC<sup>(-)</sup> exhibited a significantly increased suppressive activity when compared with untreated cells (Fig. 4). Activation of purified CD4<sup>+</sup>CD25<sup>+</sup> T-cells alone did not cause a similar proliferative response.

*CD4<sup>+</sup>CD25<sup>+</sup> T-cells are protective in a mouse model of colitis in vivo; enhancement of their suppressive activity by phebestin.* Results of the aforementioned *in vitro* studies prompted us to ascertain whether the administration of i) phebestin, ii) CD4<sup>+</sup>CD25<sup>+</sup> T-cells from healthy mice, or iii) CD4<sup>+</sup>CD25<sup>+</sup> T-cells exposed *ex vivo* to phebestin improve the disease score in a mouse model of colitis.

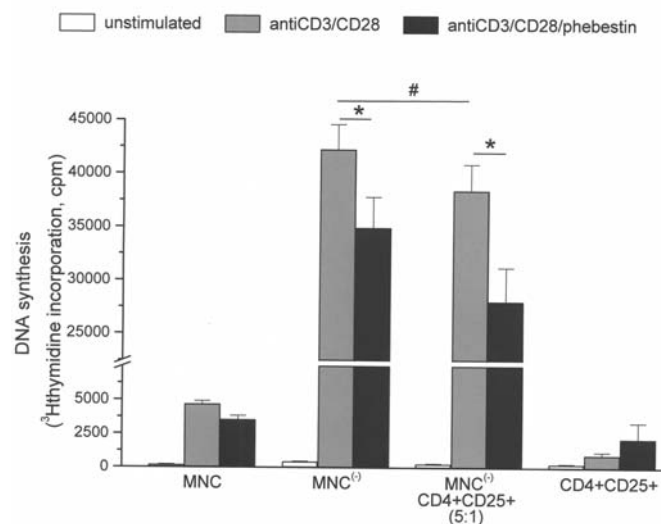


Figure 4. Phebestin increases *in vitro* suppression of mouse MNC by CD4<sup>+</sup>CD25<sup>+</sup> T-cells. Anti-CD3/CD28 leads to a proliferative response (increased DNA synthesis) in mouse MNC. In MNC which have been depleted of CD4<sup>+</sup>CD25<sup>+</sup> T-cells (MNC<sup>(-)</sup>) prior to activation, this proliferative response is significantly aggravated, indicating a lack of suppressive cells. On the contrary, reconstitution of MNC<sup>(-)</sup> by adding CD4<sup>+</sup>CD25<sup>+</sup> T-cells to the co-culture decreased DNA synthesis. This suppressive activity was further increased by the addition of 1  $\mu$ M phebestin at the different cellular ratios (n=4, \*P<0.01, #P<0.05).

First, after i.v. administration of CD4<sup>+</sup>CD25<sup>+</sup> T-cells which had been labelled *ex vivo* with CellTracker Green CMFDA (Molecular Probes), we demonstrated by fluorescence microscopy on colon tissue sections that at least a fraction of these cells was targeted to the local site of inflammation *in vivo* (Fig. 5).

Under the applied conditions, the typical time course for the development of colitis in response to DSS comprised an onset of detectable disease scores at day 3, followed by a daily increase resulting in maximum severity at day 7 (Fig. 6A, PBS-treated control). Single injections of phebestin on day 3 markedly reduced the disease activity index (DAI) on days 5 and 7, whereas the score was only marginally affected by the administration of untreated CD4<sup>+</sup>CD25<sup>+</sup> T-cells. This finding was in contrast with results from studies performed in lymphopenic and immunodeficient mice in which the disease was prevented by transferring relatively small numbers of CD4<sup>+</sup>CD25<sup>+</sup> T-cells (3,12,41,42). This discrepancy might reflect the fact that, in these mice, colitis was induced by a severe impairment of the delicate natural balance of immune homeostasis, either by an adoptive transfer of 'disease-specific' CD4<sup>+</sup>CD25<sup>+</sup> T-cells, or by depletion of CD4<sup>+</sup>CD25<sup>+</sup> T-cells. Correcting this imbalance improves symptoms of the disease (12). In our study, we induced acute colitis in fully immunocompetent mice that bore considerably higher numbers of immune cells, including the different T-cell subsets. In this setting, higher numbers of CD4<sup>+</sup>CD25<sup>+</sup> T-cells are required to effectively modulate the immune response. More remarkably, we found that a profound reduction in the colitis disease activity resulted from a single administration of CD4<sup>+</sup>CD25<sup>+</sup> T-cells that had previously been exposed to phebestin (Fig. 6a). Under these conditions, we observed

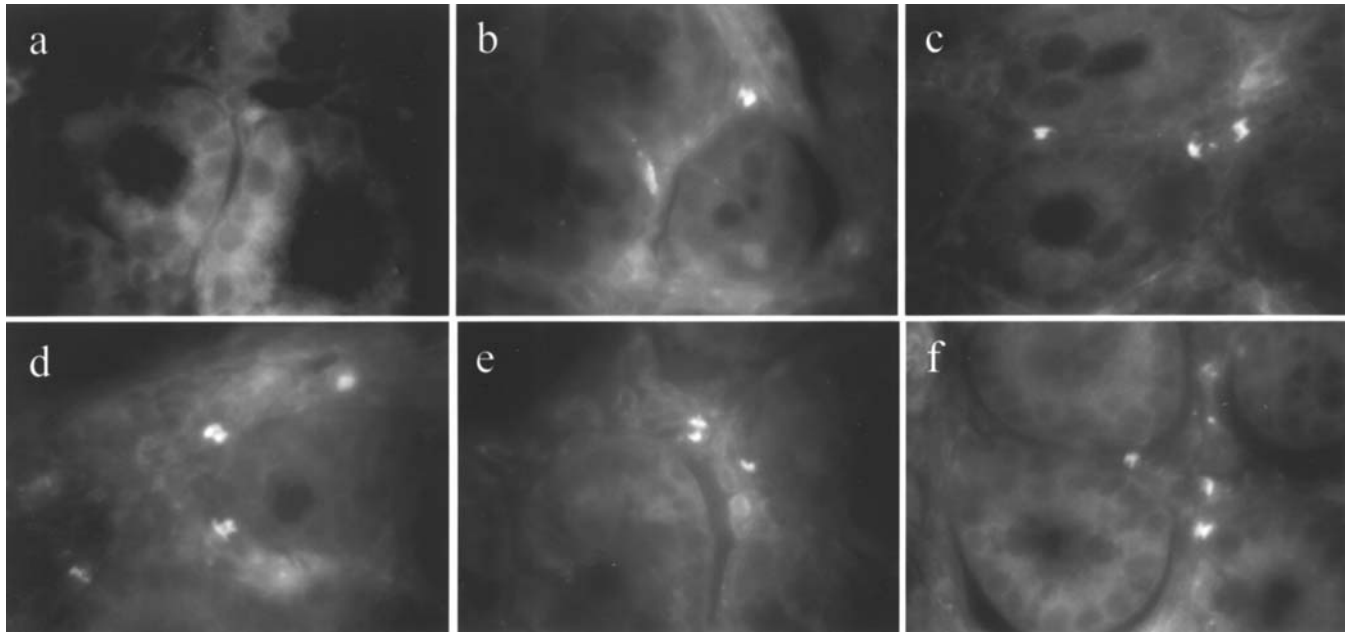


Figure 5. CD4<sup>+</sup>CD25<sup>+</sup> T-cells locate to the local inflammatory site in colitis-induced mice. CD4<sup>+</sup>CD25<sup>+</sup> T-cells derived from the spleen of healthy mice were labelled *ex vivo* with CellTracker Green (CMFDA) and administered ( $10^6$  cells, i.v. tail vein) into colitis-induced mice at day 3 of disease induction. After 24 h, colons were excised and prepared for examination by fluorescence microscopy (Axiovert TV135 Zeiss, filter set 10, x1000). (a) No cells administered, (b-f) CMFDA-labelled cells administered.

strong protective effects (up to -3 score points), which were obvious 1 day after application (day 4) and persisted until day 7, albeit at less pronounced levels. The beneficial clinical effects of phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells included a profound reduction in the incidence of intestinal bleeding, loss of body weight, and shortening of the intestine (Fig. 6b). It should be emphasized that these effects were obtained even though during the 7 day period of treatment the disease-inducing exposure to DSS was fully maintained.

In the placebo-treated group, morphological analysis of colon tissue samples by light microscopy showed infiltration of mononuclear cells and polymorphonuclear granulocytes in 85% of the tissue samples evaluated as well as the presence of histiocyte-rich inflammation in 40% of the samples at day 4. Up to day 7, a progression toward extensive inflammation including strong alterations in crypt architecture were detectable. The mean histological score in this group reached  $1.8 \text{ points} \pm 0.34$  (SEM) at day 4 and  $5.83 \pm 0.5$  at day 7. These alterations appeared to be ameliorated under the treatment regimens (Fig. 6b and c-f). However, significantly lower histological score values were detected for the group receiving phebestin-exposed CD4<sup>+</sup>CD25<sup>+</sup> T-cells only ( $0.66 \pm 0.23$  at day 4,  $P=0.0261$ ;  $3.77 \pm 0.61$  at day 7,  $P=0.0105$  in comparison to the control group, respectively). One day after treatment, 66% of the evaluated tissue samples from animals of this group showed normal morphology, and 33% of the samples had signs of moderate inflammation.

Since the administration of *ex vivo*-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells does not appear to be an easy option for the treatment of patients afflicted with colitis, we next attempted to ascertain whether daily administration of phebestin induced a similar improvement in the DAI in the mouse model of acute colitis. As shown in Fig. 6g, phebestin administered

twice a day ( $50 \mu\text{g}$ , i.p.) induced a highly significant decrease in the DAI at days 4 ( $P=0.0038$ ), 5 ( $P=0.035$ ), 6 ( $P=1.31 \times 10^{-6}$ ), and 7 ( $P=1.24 \times 10^{-4}$ ). A prominent feature of this improved DAI was the capability of phebestin to prevent the disease-associated loss of body weight (Fig. 6h). These effects appeared to be dose-dependent, as the daily administration of either  $1 \times 50 \mu\text{g}$  ( $P<0.05$  at days 4-7) or  $1 \times 10 \mu\text{g}$  ( $P$ , not significant) phebestin reduced the DAI with gradually decreasing efficacy (not shown).

*Reduced colitis disease scores are associated with increased local expression of TGF- $\beta$ 1 and FoxP3 mRNA in the intestine.* To determine whether phebestin exerts its beneficial clinical effects *in vivo* via mechanisms similar to those thought to preserve the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> T-cells *in vitro*, we assessed the local expression of TGF- $\beta$ 1 and FoxP3 mRNA in the intestine of the four groups of colitis-afflicted mice.

The onset of clinical symptoms was accompanied by a significant loss of local (intestinal) expression levels of FoxP3 and TGF- $\beta$ 1. Intestinal expression of TGF- $\beta$ 1 mRNA appeared to be elevated 24 and 48 h (days 4 and 5) after application of phebestin, CD4<sup>+</sup>CD25<sup>+</sup> T-cells, or phebestin-exposed CD4<sup>+</sup>CD25<sup>+</sup> T-cells compared with the PBS control (Fig. 7A). The highest amounts of TGF- $\beta$ 1 mRNA were detected in response to the administration of phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells. Expression levels were highest on day 4, but markedly decreased on day 5. The changes in TGF- $\beta$ 1 mRNA expression observed locally were mirrored at the systemic level where we demonstrated elevated concentrations of TGF- $\beta$ 1 in platelet-free plasma under the three treatment regimens (Fig. 7B;  $P$ , not significant). In accordance with the expression changes in the intestine, the administration of



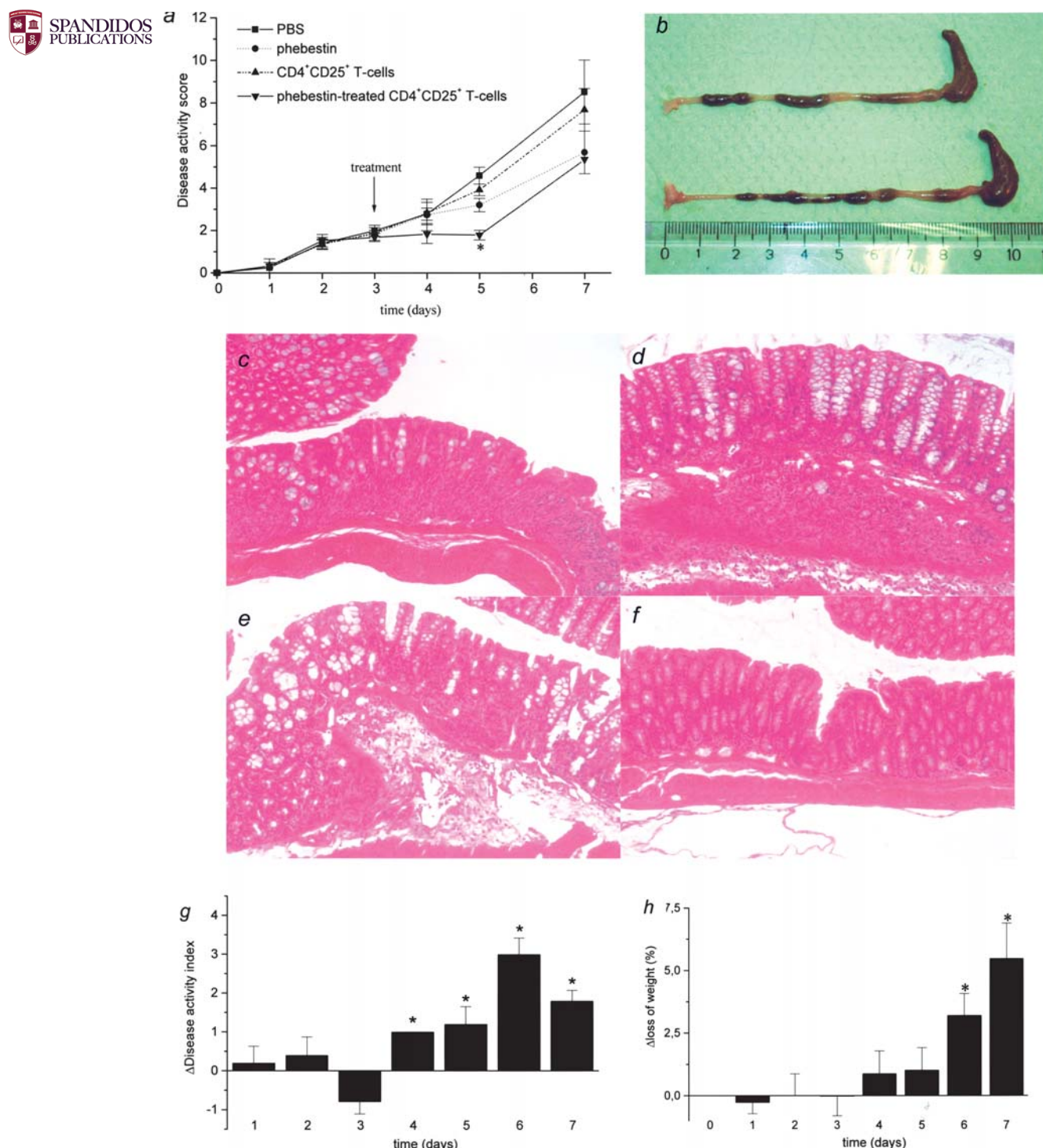


Figure 6. Phebestin and phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells are protective in a mouse model of acute colitis *in vivo*. (a) The mice developed acute colitis with a disease activity index (DAI) of ~8 during a 7-day period (control). A single administration of phebestin (10  $\mu$ g in 20  $\mu$ l PBS) at day 3 improved the disease score from day 5 onwards. Similarly, the administration of 10<sup>6</sup> CD4<sup>+</sup>CD25<sup>+</sup> T-cells provoked a slight decrease in the DAI. Remarkably, CD4<sup>+</sup>CD25<sup>+</sup> T-cells that had been exposed to phebestin (10  $\mu$ g in 20  $\mu$ l PBS) for 45 min prior to administration showed a complete and immediate prevention of a further increase in the disease activity during the next 2 days. This treatment regimen remained most protective until day 7 (n=15, \*P<0.05). The DAI was determined by summarizing the scores for weight loss, stool consistency and bleeding. Weight loss: <5% = 0, 5-10% = 1, 10-15% = 2, 15-20% = 3, and >20% = 4. Consistency of feces: well-formed pellets = 0, pasty, semi-formed stool = 2, and liquid stool = 4. Rectal bleeding: none = 0, hemocult-positive = 2, and visible gross bleeding = 4. \*\*Determination of daily weight changes in healthy animals revealed variations of up to 4% on consecutive days; therefore, weight loss scoring started at 5% weight loss. (b) Shortening of the colon, typically observed in acute colitis, was partially prevented by phebestin. One day after treatment (day 4) the colon of mice treated with phebestin-exposed CD4<sup>+</sup>CD25<sup>+</sup> T-cells (bottom) was significantly longer than that of PBS-treated mice (top). (c-f) Histological examination of colon sections revealed leukocyte infiltration, with or without impairment of the surface epithelium, and focal or diffuse distribution of granulation tissue. Some of the active inflammatory infiltrates were situated within the submucosal layer in PBS-treated colitis-afflicted mice (c). These typical signs of acute colitis were partially prevented by a single administration of phebestin on day 3 (d) or CD4<sup>+</sup>CD25<sup>+</sup> T-cells from healthy mice (e). The administration of CD4<sup>+</sup>CD25<sup>+</sup> T-cells that had been exposed to phebestin *ex vivo* largely preserved normal morphology and reduced local inflammation significantly (f). Repeated administration of phebestin improved the DAI of acute colitis. (g) The single administration of phebestin twice a day (50  $\mu$ g each time, i.p.), resulted in a significant decrease in the DAI compared to the PBS-treated control ( $\Delta$ DAI) and the effect was equal to that observed when using phebestin-exposed CD4<sup>+</sup>CD25<sup>+</sup> T-cells. (h) As part of the DAI, mice showed significantly lower weight loss in response to the repeated administration of phebestin ( $\Delta$ loss of weight of the PBS control). (n=6, \*P<0.01).

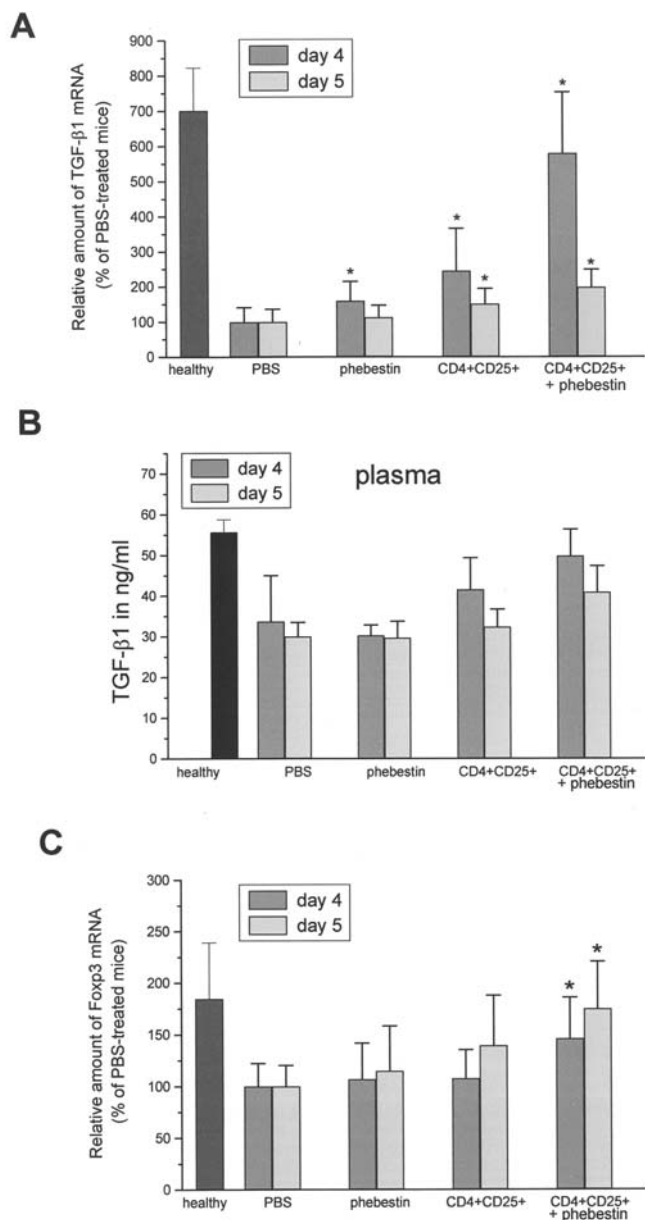


Figure 7. CD4<sup>+</sup>CD25<sup>+</sup> T-cells, phebestin, and phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells modulated local expression levels of TGF-β1 and FoxP3 as well as systemic TGF-β1 amounts in colitis-induced mice *in vivo*, indicating an induction/preservation of the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> T-cells. After a single administration of phebestin or CD4<sup>+</sup>CD25<sup>+</sup> T-cells on day 3, the amounts of TGF-β1 (A) and FoxP3 mRNA (C) were determined by quantitative RT-PCR at the site of inflammation in the colon on the following two days. Phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells (CD4<sup>+</sup>CD25<sup>+</sup> + phebestin) provoked by far a stronger induction of TGF-β1 mRNA expression in the colon and led to significantly elevated colonic expression of FoxP3. (B) The systemic plasma levels of TGF-β1 were assessed by ELISA and were found slightly altered by phebestin alone, but were slightly elevated in response to untreated and, in particular, to phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells. (n=7, \*P<0.05 vs. PBS).

phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells provoked the most prominent increase in plasma TGF-β1 (Fig. 7B).

As observed with TGF-β1 mRNA, a clear increase in FoxP3 mRNA levels in both the intestine and splenocytes was demonstrated under the three treatment regimens in comparison with the PBS control (Fig. 7C). Again, phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells were the most effective, and the expression changes appeared more pronounced on day 5. Enhancement

of FoxP3 expression very likely represents a specific response of CD4<sup>+</sup>CD25<sup>+</sup> T-cells to alanyl-aminopeptidase inhibition. This view is supported by the lack of a similar response *in vivo* to the daily administration of the p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580 (not shown), which has been recently shown capable of suppressing the DSS-induced colitis in mice (43).

Generally, the effects obtained with phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells appeared to be additive to those obtained with either phebestin or freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> T-cells alone.


## Discussion

The data presented here demonstrated the capability of phebestin, an inhibitor of alanyl-aminopeptidase, to increase TGF-β1 and FoxP3 expression in CD4<sup>+</sup>CD25<sup>+</sup> T-cells and to enhance the suppressive activity of these cells. Furthermore, phebestin-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells reduced the disease activity in the DSS-induced colitis in mice. This protective activity of phebestin *in vivo* was correlated with increased levels of TGF-β1 and FoxP3 mRNA at the local site of inflammation.

It is clear from previous studies that FoxP3, a member of the forkhead winged-helix protein family of transcription factors, is a highly specific marker for CD4<sup>+</sup>CD25<sup>+</sup> T-cells, and its expression is crucial for cell development and function (36). The function of the highly conserved FoxP3 is identical in humans and mice, as mutations result in fatal multi-organ autoimmune diseases in both species (44,45). The same phenotype has been observed with animals that are null for TGF-β1 (46), pointing to a direct link between FoxP3 and TGF-β1. Indeed, transgenic expression of FoxP3 converts naïve CD4<sup>+</sup> T-cells into functional CD4<sup>+</sup>CD25<sup>+</sup> T-cells (35,37), supporting the role of FoxP3 as a master regulator of CD4<sup>+</sup>CD25<sup>+</sup> T-cell differentiation. Based on the above facts, it is not surprising that we observed the simultaneous induction of both FoxP3 and TGF-β1 expression when exposing CD4<sup>+</sup>CD25<sup>+</sup> T-cells to phebestin. We also observed a parallel increase in FoxP3 and TGF-β1 mRNA expression within the colon of colitis-afflicted mice treated with phebestin-exposed CD4<sup>+</sup>CD25<sup>+</sup> T-cells. It could be concluded, therefore, that indeed the number and/or suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> T-cells at the site of inflammation increased upon the administration of this therapy. This view is supported by the observed 'homing' of *ex vivo*-labelled CD4<sup>+</sup>CD25<sup>+</sup> T-cells to the inflamed colon.

The precise mode of suppression by CD4<sup>+</sup>CD25<sup>+</sup> T-cells is not fully understood, but includes direct cell-cell contact and the production of immunosuppressive cytokines, namely TGF-β1 and IL-10 (22,47,48). A key to understanding the contact-dependent suppression of effector T-cells was the finding of a surface-expression of both latent and active TGF-β1 (6). Nakamura *et al* (6) showed that the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> T-cells was abrogated if the surface-bound TGF-β1 was blocked. Although another study reported opposite results (10), recent studies have provided compelling evidence for a crucial role of TGF-β1 in the suppressive properties of CD4<sup>+</sup>CD25<sup>+</sup> T-cells in different settings, including colitis (11-13,15), autoimmune pneumonitis (14),



 SPANDIDOS (16), oral tolerance (17), and natural killer cell (18). A recent revision of the role of TGF- $\beta$ 1

revealed the basis of this apparent discrepancy by demonstrating that CD4<sup>+</sup>CD25<sup>+</sup> T-cell function is indeed dependent on TGF- $\beta$ 1-signaling which, however, could be at least partly provided by TGF- $\beta$ 1 derived from cells other than CD4<sup>+</sup>CD25<sup>+</sup> T-cells (49). TGF- $\beta$ 1 is definitely required to induce and/or maintain FoxP3 expression via TGF- $\beta$  receptor type II (TBR2) signaling (50). In addition to this effect on the CD4<sup>+</sup>CD25<sup>+</sup> T-cells themselves, activation-dependent induction of TBR2 expression on responder T-cells is a central mechanism that renders them susceptible for surface-bound TGF- $\beta$ 1 of suppressive CD4<sup>+</sup>CD25<sup>+</sup> T-cells (47).

For different autoimmune diseases, functional deficits of the CD4<sup>+</sup>CD25<sup>+</sup> T-cell population were demonstrated (16,50,51) and, notably, these were paralleled by decreased expression levels of FoxP3 (16,51). Another study (52) demonstrated the presence of higher numbers of TGF- $\beta$ 1-positive CD4<sup>+</sup>CD25<sup>+</sup> T-cells in patients with active ulcerative colitis (UC) compared to inactive UC, emphasizing the role of TGF- $\beta$ 1 in the remission of UC. Taken together, these findings clearly indicate that FoxP3 and TGF- $\beta$ 1 are tightly linked functional markers of CD4<sup>+</sup>CD25<sup>+</sup> T-cells.

In this study, we showed that the onset of acute colitis was associated with a loss of both FoxP3 and TGF- $\beta$ 1 expression in the colon. This loss was prevented to a significant extent by the administration of phebstein-treated CD4<sup>+</sup>CD25<sup>+</sup> T-cells, an observation that is in line with the observed induction of TGF- $\beta$ 1 and FoxP3 expression in response to phebstein in CD4<sup>+</sup>CD25<sup>+</sup> T-cells *in vitro*. In both mice and humans, a loss of CD4<sup>+</sup>CD25<sup>+</sup> T-cell suppressive activity is likely to coincide with the onset of autoimmune or inflammatory disease. Two studies have demonstrated the ability of *in vitro* expanded CD4<sup>+</sup>CD25<sup>+</sup> T-cells to suppress autoimmune diabetes in lymphocyte-sufficient NOD mice, paving the way for cellular immunotherapy of autoimmunity (53,54). Extending these findings, we provide a tool to further strengthen the suppressive activity of natural regulatory T-cells via inhibition of membrane alanyl-aminopeptidase (mAAP/ CD13). The molecular mechanisms and signaling pathways that lead to TGF- $\beta$ 1 and FoxP3 expression and that consecutively lead to the observed strengthening of the suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> T-cells remain to be identified. An equal induction of TGF- $\beta$ 1 expression in response to mAAP inhibitors has been observed in the KARPAS-299 T-cell line, which has been identified as a regulatory T-cell line (55), but also in other cell types including human monocytes, HUVECs or the glioblastoma cell line U87 (U.L., C.W. unpublished data). It is tempting to speculate, therefore, that the induction of TGF- $\beta$ 1 expression is due to a signaling pathway which is not restricted to CD4<sup>+</sup>CD25<sup>+</sup> T-cells, but here serves to maintain/enhance their FoxP3 expression and, thus, their suppressive phenotype. Microarray analyses aimed at the identification of the signaling pathways involved pointed to an induction of the wnt pathway (56). This raises the interesting possibility that the wnt/BMP pathway contributes to the differentiation and preservation of functional CD4<sup>+</sup>CD25<sup>+</sup> T-cells, a hypothesis that clearly warrants further investigation.

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