

Relative impact of CD4+CD25+ regulatory T cells and tacrolimus on inhibition of T-cell proliferation in patients with atopic dermatitis

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Summary

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Accepted for publication

6 January 2005

Key words:

atopic dermatitis, human regulatory T cells, immunosuppressive drugs, Th1/Th2

Conflicts of interest:

M.H.A.R. has acted in a consultancy capacity and has given invited lectures for Fujisawa GmbH. He is also a recipient of a research grant from Fujisawa GmbH.

Background It has been established recently that CD4+CD25+ regulatory T cells (Tregs) play an important role in controlling various immune responses. Immunosuppressive drugs are often used to treat immune dysregulation but are frequently associated with undesirable side-effects.

Objectives We examined the suppressive capacity of circulating Tregs in patients with atopic dermatitis (AD). Combined effects of Tregs and tacrolimus on the inhibition of T-cell proliferation *in vitro* were also assessed.

Methods CD4+CD25+ and CD4+CD25- T cells were isolated from peripheral blood mononuclear cells using immunomagnetic beads. CD4+CD25- T cells were stimulated with purified protein derivative (PPD) or house dust mite allergen (Der p1) for 6 or 7 days, respectively. A dose range of tacrolimus and CD4+CD25+ T cells were added separately, or together. Proliferation was measured by ³H-thymidine incorporation.

Results CD4+CD25+ T cells from normal controls and patients with AD are anergic and inhibit the proliferation of CD4+CD25- T cells in response to PPD and Der p1 *in vitro* in a dose-dependent manner. Addition of tacrolimus and Tregs together showed significantly stronger inhibition of proliferation than either on their own. This was true for both antigens and both in normal controls and in patients with AD.

Conclusions CD4+CD25+ T cells in patients with AD have normal suppressive activity compared with healthy controls. Tregs and tacrolimus have additive effects on the inhibition of proliferation in response to PPD and Der p1.

Atopic dermatitis (AD) is a chronic multifactorial allergic inflammatory disease, characterized by abnormal responses to noninfectious environmental allergens, which manifests itself with eczematous skin lesions, and requires long-term treatment.¹ Its prevalence has increased significantly and it is now the most common chronic disease of children in Europe, the U.S.A. and Japan.¹ For the last four decades topical steroids have been the standard treatment for severe cases of AD, although their clinical utility for long-term treatment is limited by potential side-effects.² More recently, calcineurin inhibitors such as tacrolimus have also been used topically to treat moderate to severe AD without causing the side-effects associated with prolonged use of corticosteroids.²⁻⁴ Despite the huge benefits of calcineurin inhibitors in organ transplantation,

problems have emerged with toxicity.^{5,6} Although the use of topical tacrolimus ointment has so far not been associated with an increased incidence of infections or cutaneous malignancies, this raises the issue of potential side-effects as a consequence of long-term use of this drug.

In the last decade it has become clear that regulatory T cells (Tregs) play a crucial role in immune regulation through the control of T-cell responses. Attention has focused in particular on a subset of naturally occurring CD4+ T cells which can be identified by their constitutive expression of the interleukin-2 receptor α chain, CD25.^{7,8} These CD4+CD25+ T cells have been shown to exist in mice, rats and humans and can be found in the thymus, peripheral blood, lymphoid organs and at sites of inflammation.⁸ Initially, they were thought to play

a critical role in the control of autoimmunity.⁹ However, more recently, they have been shown to play a role in regulation of transplantational tolerance,^{10,11} antitumour immunity and in parasitic and bacterial infections.^{12,13}

The role of Tregs in atopic diseases is not clear. It has been hypothesized that Tregs mediate tolerance to innocuous non-pathogenic antigens (Ags) encountered in respiratory and intestinal tracts, and that the breakdown of this tolerance may lead to atopic disorders.^{14,15} Perhaps the strongest indirect evidence for the role of CD4+CD25+ T cells in atopic disease comes from patients with IPEX syndrome (the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). These patients lack functional Tregs and suffer from severe eczema, elevated IgE levels, eosinophilia and food allergies.¹⁶ Various recent studies that have investigated a potential impairment of Tregs in atopic patients have reached different conclusions. CD4+CD25+ T cells were found to be numerically and functionally impaired in children with cow's milk allergy.¹⁷ Similarly, Ling *et al.* found that Tregs from atopic patients had reduced suppressive capacity in comparison with those from nonatopics.¹⁸ In contrast, in the study by Bellinghausen *et al.*, the majority of atopic patients had Tregs indistinguishable from those of healthy controls, although functionally impaired Tregs were observed in a small subset of patients.¹⁹

It is clear that the balance between Tregs and inflammatory T cells can determine the outcome of an immune response.¹⁴ Therefore enhancement or restraint of Treg activity may be of clinical relevance. Because current therapeutic approaches depend on the use of immunosuppressive drugs, which are often associated with numerous side-effects, the possibility that the body's natural immunoregulatory mechanisms could be utilized to prevent harmful immune responses without the detrimental side-effects is appealing.

We have investigated the ability of Tregs to inhibit proliferation in response to purified protein derivative (PPD) and house dust mite allergen (Der p1), in healthy individuals and in patients with AD, as well as their combined effects with tacrolimus. We show that CD4+CD25+ T cells derived from peripheral blood of patients with AD have a similar suppressive capacity *in vitro* to that of Tregs from healthy donors. We also demonstrate that CD4+CD25+ Tregs and tacrolimus have an additive effect on suppression of T-cell proliferation *in vitro*.

Materials and methods

Patient characterization

The study was approved by the Ethics Committee of the Royal Free Hospital NHS trust. Informed, written consent was obtained from all participants. Ten patients with AD were selected according to the U.K. Working Party's modified Hanifin and Rajka criteria.^{20,21} All patients with AD had moderate to severe disease according to the severity grading of Rajka and Langeland²² and had raised serum IgE levels and positive radioallergen sorbent test to house dust Ag. None of

the patients had received phototherapy, systemic corticosteroids or systemic immunosuppressive therapy for 3 months prior to recruitment and no patient had used topical tacrolimus ointment. The control group comprised 20 healthy individuals with no history of AD, allergic rhinitis or asthma.

Purification of T-cell subsets from peripheral blood

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Amersham, U.K.). CD4+ T cells were isolated by negative selection using the CD4 isolation kit (Miltenyi Biotech, Bisley, U.K.). CD4+ T cells were incubated with anti-CD25 Micro Beads (Miltenyi Biotech) and separated into CD4+CD25+ and CD4+CD25- T-cell subsets on a positive selection column. This separation resulted in less than 1% CD25+ cells in the CD4+CD25- fraction and around 80% purity in the CD25+ T-cell subset.^{23,24}

Proliferation and suppression assays

Purified CD4+ T-cell subsets (5×10^4 cells per well) were stimulated in triplicate wells with Ag and irradiated (50 Gy) autologous PBMC as Ag-presenting cells (APCs) (5×10^4 per well) in 96-well round-bottomed plates (Nunc, Roskilde, Denmark). For indirect suppression assays the proliferative responses of CD4+ and CD4+CD25- T cells were compared. For direct suppression assays 5×10^4 CD4+CD25- T cells were stimulated in the absence or presence of CD4+CD25+ T cells (5×10^4 , 2.5×10^4 or 1.25×10^4 cells per well), resulting in responder/suppressor ratios of 1 : 0, 1 : 1, 1 : 0.5 and 1 : 0.25. Ags used were PPD (Statens Seruminstitut, Copenhagen, Denmark) at $1 \mu\text{g mL}^{-1}$ and Der p1 (kind gift from Leti Laboratories, Madrid, Spain) at $20 \mu\text{g mL}^{-1}$. Cells were cultured in RPMI 1640 medium (Gibco, Life Technologies, Paisley, U.K.) supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% pooled human AB serum (all Sigma, Poole, U.K.). Cells were cultured for 6 days with PPD and for 7 days with Der p1. During the last 18 h of culture ^3H -thymidine was added at $1 \mu\text{Ci}$ per well. Indirect and direct suppression were then expressed as percentage of control proliferation by comparing the proliferation of CD4+ and CD4+CD25- T cells or CD4+CD25- T cells alone and in the presence of CD25+ T cells. Alternatively, a wide dose range of tacrolimus (0.05 – 2 ng mL^{-1}) was added to CD4+CD25- T cells stimulated as above. In some experiments, CD25+ T cells and tacrolimus were added together at various doses.

Suppression of an ongoing response

Purified CD4+CD25- T cells (5×10^4 cells per well) were stimulated in triplicate wells with Ag and APCs (5×10^4 cells per well) as before. Freshly purified CD4+CD25+ (1 : 1) cells or tacrolimus (1 ng mL^{-1}) were added on day 0 (onset of cultures), day 2 or day 4 following the initiation of proliferation.

All cultures were harvested at day 6 after stimulation. To confirm that CD4+CD25+ T cells purified on different days had equal suppressive capacity, basic direct suppression experiments (as described in the previous paragraph) were set up on day 2 and day 4.

Results

Regulatory T cells control both Th1 and Th2 responses in patients with atopic dermatitis and normal controls

We have shown previously that human CD4+CD25+ T cells can suppress *ex vivo* T-cell responses to mitogens and various Ags²⁴ and we wanted to determine the capacity of these cells to suppress Th1 and Th2 Ag-specific T-cell responses. Furthermore, we wanted to investigate a potential impairment of CD4+CD25+ T-cell function in patients with AD. In this study

we chose PPD as a classical Th1 Ag and Der p1 as a Th2 Ag²⁵ because most donors have been vaccinated against tuberculosis and have good PPD responses, while Der p1 is one of the ubiquitous aeroallergens that has been implicated in the pathogenesis of AD.²⁶

We compared the numbers and phenotype of Tregs in peripheral blood of patients with AD and normal controls. The percentage of CD4+CD25+ T cells in peripheral blood of normal controls and patients with AD was similar (mean $\pm$ SEM 11.9 $\pm$ 2.5% and 10.8 $\pm$ 3.21%, respectively). The intensity of CD25 staining and the phenotype of Tregs in patients with AD were also not different from those in normal controls (data not shown). We observed good proliferative responses to both Ags when unfractionated CD4+ T cells from normal donors were stimulated, and proliferation was significantly increased when CD4+CD25+ cells were depleted (mean $\pm$ SEM increase in proliferation 158 $\pm$ 63% for PPD, and

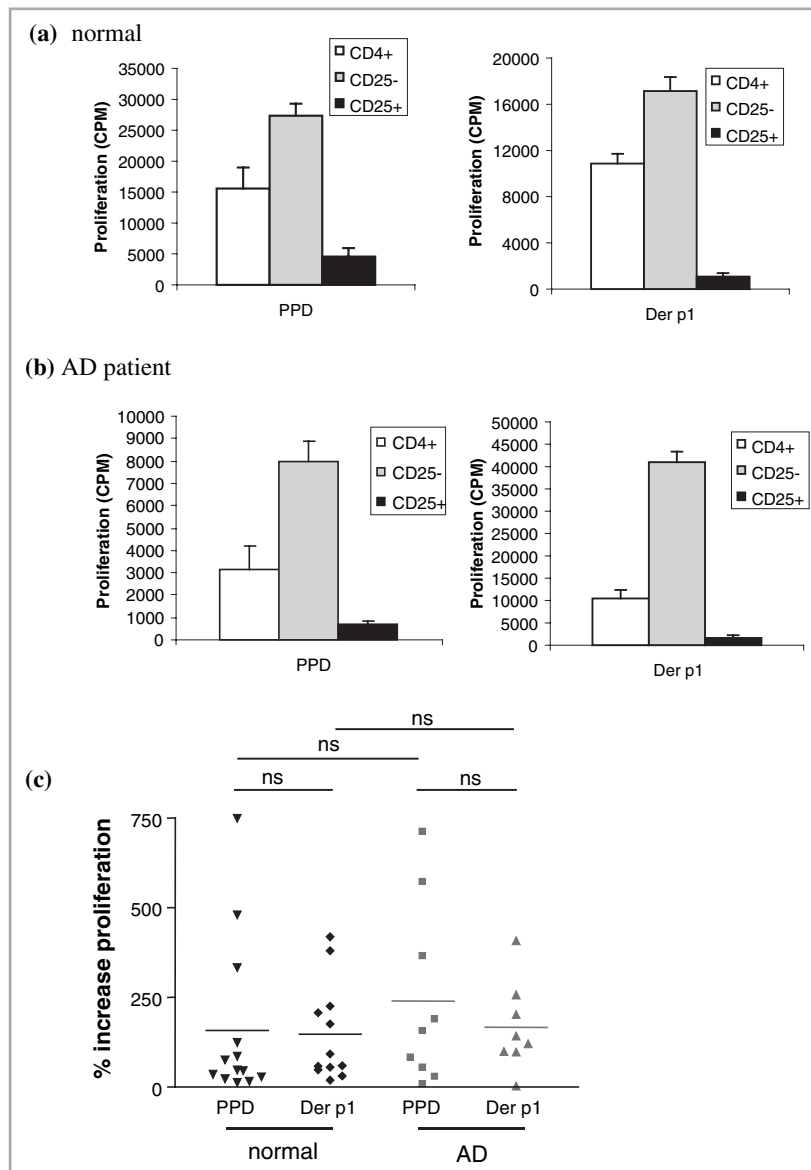


Fig 1. CD4+CD25+ T cells are anergic and suppressive upon stimulation with Th1 and Th2 antigens. CD4+, CD4+CD25- and CD4+CD25+ T cells were isolated from peripheral blood mononuclear cells (PBMC) from (a) healthy controls and (b) patients with atopic dermatitis (AD). T cells were incubated with purified protein derivative (PPD) or house dust mite allergen (Der p1) with irradiated autologous PBMC as antigen-presenting cells. Proliferative responses were assessed by ³H-thymidine incorporation. Panels (a) and (b) show the results from one of at least eight independent experiments for each antigen, presented as mean $\pm$ SEM of triplicate cultures. Panel (c) shows cumulative data expressed as increase in proliferation upon removal of CD4+CD25+ cells by comparison between proliferation of CD4+ T cells (100%) and CD4+CD25- T cells. No significant difference was observed between PPD ($n = 13$) and Der p1 ($n = 12$) in normal controls or patients with AD ($n = 9$ and $n = 8$, respectively). No difference was observed between the two groups of subjects (t -test, $P > 0.05$).

147 ± 40% for Der p1; Fig. 1a,c). CD4+CD25+ cells themselves did not proliferate significantly, in agreement with their anergic phenotype *in vitro*. In patients with AD removal of CD4+CD25+ T cells led to similar increases in proliferation in response to both Ags (mean ± SEM 239 ± 84% for PPD and 163 ± 43% for Der p1) and this was not significantly different from the levels seen in normal donors (Fig. 1b,c). CD4+CD25+ T cells from patients with AD were also anergic (Fig. 1b). In both groups of patients, proliferation was negligible when no Ag was added (spontaneous proliferation) and the removal of CD25+ T cells did not enhance proliferation to a significant level.

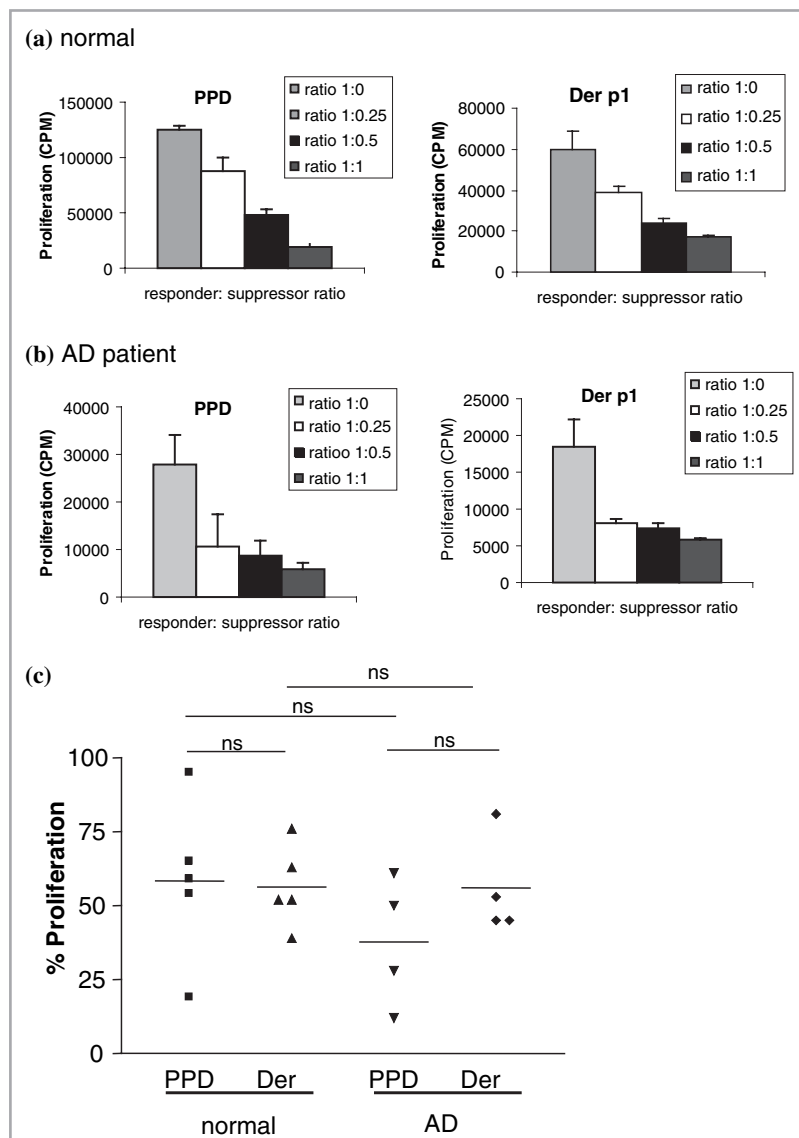
To confirm their suppressive capacity, increasing numbers of CD4+CD25+ T cells were added to the responding CD4+CD25- T cells stimulated with Ag and APCs. In normal donors, the addition of Tregs led to a dose-dependent suppression of proliferation (mean ± SEM direct suppression at 1 : 0.5 responder/suppressor ratio was 58 ± 12% for PPD and

56 ± 6% for Der p1; Fig. 2a,c), and no difference was observed between the capacity of CD4+CD25+ T cells to suppress PPD and Der p1 responses (Fig. 2a,c). Similarly, in patients with AD, addition of CD4+CD25+ T cells led to a dose-dependent suppression of proliferation by CD4+CD25- T cells (mean ± SEM suppression 63 ± 11% for PPD and 56 ± 8.5% for Der p1 at 1 : 0.5 ratio), and the level of suppression for either Ag was not significantly different compared with normal donors (Fig. 2b,c).

Tacrolimus and CD4+CD25+ cells become less effective after T-cell activation has occurred

It is well established that immunosuppressive agents such as tacrolimus and naturally occurring Tregs prevent activation and initial expansion of T lymphocytes. To establish the efficacy of tacrolimus and CD4+CD25+ T cells in suppressing proliferative responses that have already been initiated, we

Fig 2. CD4+CD25+ T cells suppress proliferation to purified protein derivative (PPD) and house dust mite allergen (Der p1) in patients with atopic dermatitis (AD) and normal controls. CD4+ T-cell subsets were isolated from (a) healthy controls and (b) patients with AD. CD4+CD25- T cells (5×10^4 per well) were stimulated with PPD or Der p1 in the absence or presence of CD4+CD25+ T cells (5×10^4 , 2.5×10^4 or 1.25×10^4 cells per well), resulting in responder/suppressor ratios of 1 : 0, 1 : 1, 1 : 0.5 and 1 : 0.25. Panels (a) and (b) show the results from one of at least five independent experiments for each antigen, presented as mean ± SEM of triplicate cultures. Panel (c) shows cumulative data for five normal controls and four patients with AD expressed as percentage of control proliferation [(proliferation of cocultures of responder to suppressor at 1 : 0.5 ratio/proliferation of CD4+CD25- cells alone) × 100]. No significant difference was observed in the potency of CD4+CD25+ cells between the antigens or between the two groups of donors (t-test, $P > 0.5$).



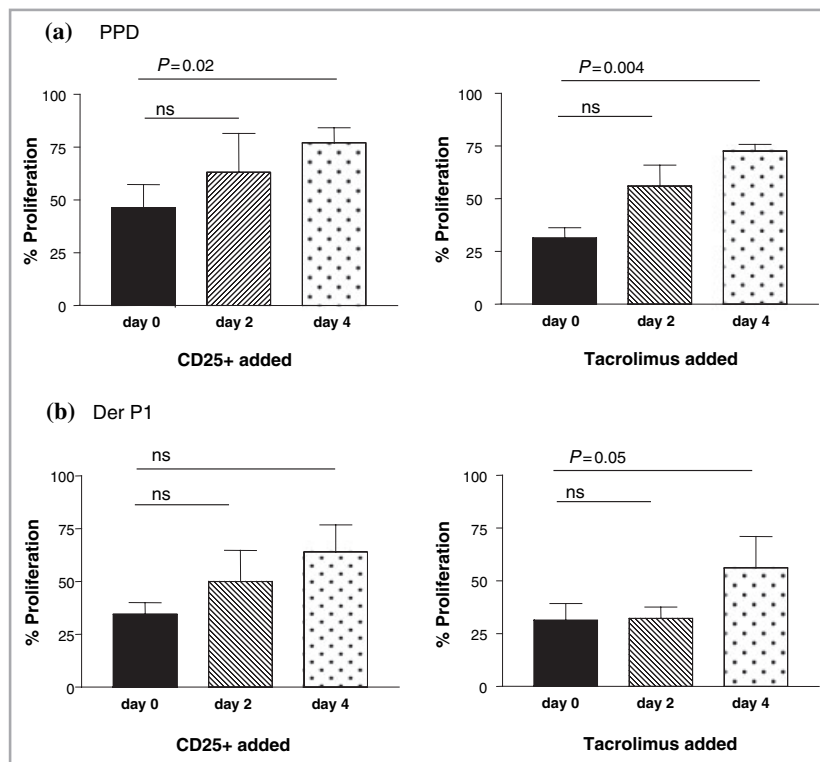


Fig 3. Tacrolimus and CD4+CD25+ T cells can inhibit ongoing proliferation but become less effective with time. CD4+CD25+ T cells were stimulated with (a) purified protein derivative (PPD) or (b) house dust mite antigen (Der p1) and antigen-presenting cells as before. Tacrolimus or CD4+CD25+ T cells were added at the onset of culture (day 0) and also on day 2 and day 4 following stimulation. Suppression is expressed as percentage proliferation compared with control (CD4+CD25+ cells stimulated alone), and cumulative data for five normal donors are expressed as mean $\pm$ SEM (t-test, P-values as indicated).

stimulated CD4+CD25+ T cells from normal individuals, with Ag and APCs as before. Tacrolimus or Tregs were added either at the onset of culture (day 0) or on day 2 or day 4 following stimulation. Both tacrolimus and CD4+CD25+ cells were most effective when added on day 0, leading to a mean $\pm$ SEM reduction in proliferation to $47 \pm 11\%$ of control for CD4+CD25+ cells and $31 \pm 5\%$ for tacrolimus. If the addition of tacrolimus or Tregs was delayed until day 2 or day 4, less suppression was observed (Fig. 3).

Tacrolimus and naturally occurring regulatory T cells have an additive effect on proliferation

We next examined if the addition of CD4+CD25+ T cells and tacrolimus together can increase the level of suppression obtained *in vitro*. CD4+CD25+ T cells were stimulated with Ag and APCs as before. CD4+CD25+ T cells were added at a 1 : 0.5 ratio, tacrolimus was added at a suboptimal dose (0.1 ng mL^{-1}) or both were added together. The addition of tacrolimus alone reduced proliferative responses to PPD and Der p1 in normal donors to $46 \pm 9\%$ mean $\pm$ SEM (range 15–60%) and $61 \pm 9\%$ (range 30–84%) of control proliferation (Fig. 4a). Addition of CD4+CD25+ cells alone at a 1 : 0.5 ratio inhibited proliferation to $58 \pm 14\%$ (range 19–95) and $56 \pm 6\%$ (range 39–76) of control for PPD and Der p1, respectively (Fig. 4a). Significantly more inhibition was observed when both suppressive agents were added. Proliferation was significantly reduced for both PPD ($P = 0.01$, $P = 0.008$ compared with tacrolimus or Tregs alone) and Der p1

stimulation ($P = 0.01$ compared with either tacrolimus or Tregs alone). Similar results were observed in patients with AD (Fig. 4b). Tacrolimus alone lowered the proliferation in response to PPD to $70 \pm 9\%$ (range 55–96) and in response to Der p1 to $45 \pm 7\%$ of control (range 30–68). When added on their own, CD4+CD25+ cells also inhibited proliferation: to $43 \pm 12\%$ of control (range 12–81) in response to PPD and to $41 \pm 6\%$ of control (range 22–51) in response to Der p1. When tacrolimus and Tregs were added together, suppression was significantly enhanced (Fig. 4b, $P < 0.05$ in all cases). Proliferation was reduced to $24 \pm 8\%$ of control (range 6–50) for PPD stimulation and to $32 \pm 4\%$ of control (range 20–45) for Der p1 (Fig. 4b). Taken together, these data suggest that tacrolimus and CD4+CD25+ T cells can have additive effects in suppressing proliferation.

To confirm this, we cultured CD4+CD25+ T cells with increasing doses of tacrolimus alone or in the presence of CD4+CD25+ T cells (cocultured at 1 : 0.25 and 1 : 0.75 responder/suppressor ratio). At the whole range of concentrations the added contribution by CD4+CD25+ T cells to the inhibition of proliferation was clearly demonstrable. When CD4+CD25+ T cells were stimulated on their own, tacrolimus at 2 ng mL^{-1} inhibited proliferation by 40%. The same dose of tacrolimus inhibited proliferation by 66% when a low number of CD25+CD4+ T cells (1 : 0.25 responder/suppressor ratio) was added (Fig. 5). At a higher number of Tregs (ratio 1 : 0.75) the same dose of tacrolimus reduced proliferation by 86%. Thus, while 2 ng mL^{-1} of tacrolimus was necessary to achieve 40% suppression in the absence of Tregs,

Fig 4. Tacrolimus and CD4+CD25+ regulatory T cells have an additive effect in inhibiting proliferation. CD4+CD25- T cells isolated from (a) healthy controls or (b) patients with atopic dermatitis (AD) were stimulated with purified protein derivative (PPD) or house dust mite antigen (Der p1). A suboptimal dose of tacrolimus (0.1 ng mL^{-1}), CD4+CD25+ T cells (1 : 0.5 ratio) or both were added at the onset of culture. Suppression is expressed as percentage proliferation compared with control (CD4+CD25- cells stimulated alone), and cumulative data for five normal donors and four patients with AD are expressed as mean $\pm$ SEM (t-test, P-values as indicated).

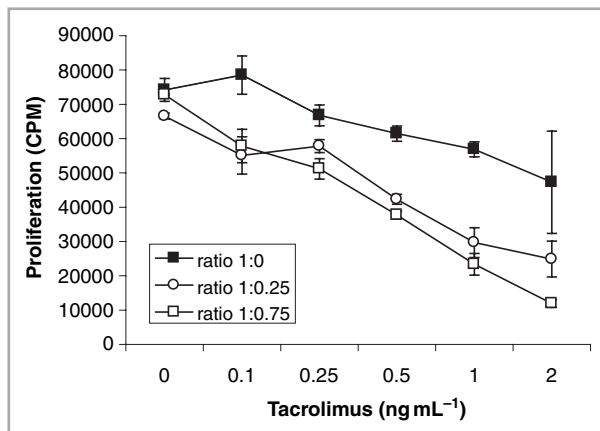
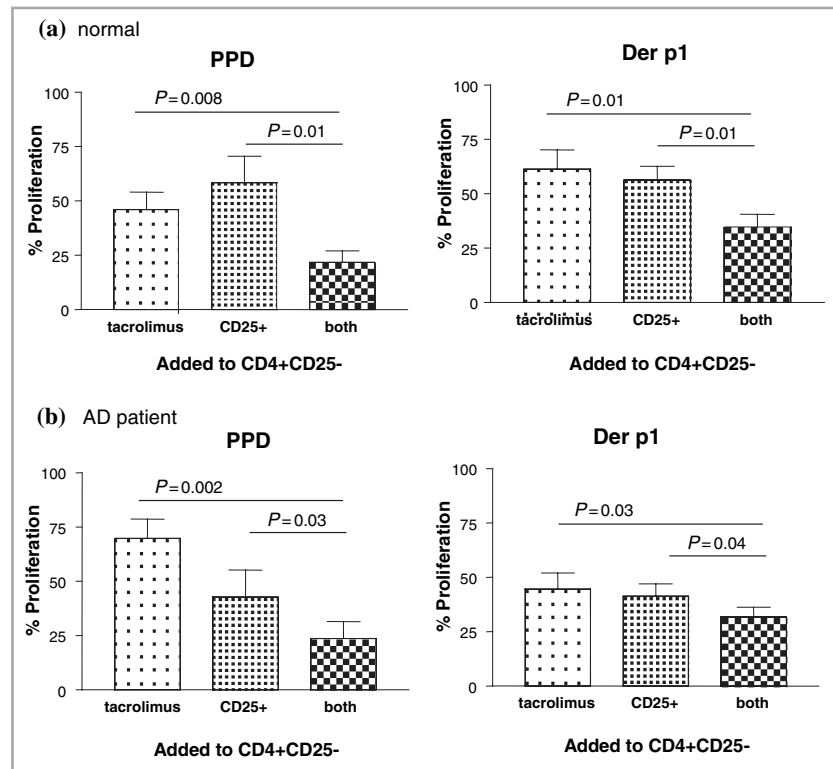


Fig 5. CD4+CD25+ regulatory T cells augment the inhibition of proliferation induced by tacrolimus over a wide range of doses. CD4+CD25- T cells were stimulated with purified protein derivative and increasing doses of tacrolimus. CD4+CD25+ T cells were added to the culture at the responder/suppressor ratio of 1 : 0.25 or 1 : 0.75. Both tacrolimus and regulatory T cells were added at the onset of culture and a representative experiment is shown. Points represent mean $\pm$ SEM of triplicate cultures.

similar levels of suppression were seen with doses of 0.25 – 0.5 ng mL^{-1} tacrolimus in the presence of Tregs. This suggests that in the presence of Tregs the dose of tacrolimus that can be used to induce equivalent suppression can be reduced significantly (four- to eightfold).

Discussion

The current approach to treatment of immune dysregulation, as seen in autoimmune diseases, allergic disorders and transplantation, is often the use of immunosuppressive agents.^{27,28} T cells play a major role in immunological disorders and they are the main targets for these immunosuppressive agents; however, generalized immunosuppression is often associated with detrimental side-effects.^{27,28} As our understanding of the critical role Tregs play in these diseases increases, the challenge is to understand the potential of manipulating these cells with the aim of enhancing their activity. This may allow for integration of intrinsic immunoregulation (mediated by Tregs) and therapeutic immunosuppression, which could be a major advance in the treatment of immunopathologies.

In this study we set out to answer two main questions: (i) Is there a difference in Treg capacity to suppress proliferation to Th1- and Th2-type Ags between normal donors and patients with AD? (ii) Could Tregs work together with immunosuppressants such as tacrolimus and have an additive effect? We found no significant difference in the number of CD4+CD25+ T cells present in peripheral blood of patients with AD compared with normal controls. This is in contrast to the study of Ou *et al.*,²⁹ who observed the number of CD4+CD25+ cells to be higher in patients with AD. An important caveat for many of the human studies is the limitation of investigating T-cell function *in vitro* and limited accessibility of cells from the sites of allergic disease, such as the

airway or skin. Therefore, we cannot exclude the possibility that Treg numbers may be decreased or their function impaired in the affected skin of patients with AD.

It has been suggested that Tregs can control both Th1 and Th2 responses.^{30–32} To confirm that this was the case in both normal donors and patients with AD, we examined proliferative responses to two classical Th1 and Th2 Ags, PPD and Der p1.²⁵ CD4+CD25+ T cells in normal controls suppress proliferation of responder T cells to the same level to both PPD and Der p1. The suppressive capacity of CD4+CD25+ T cells from patients with AD was equivalent to that from normal donors. This is in agreement with studies from Ou *et al.* and Bellinghausen *et al.*, who have also observed functional Tregs in allergic donors and patients with AD, respectively.^{19,29} In contrast, Ling *et al.* have found that CD4+CD25+ T cells from atopic patients have reduced capacity to suppress allergen-driven T-cell proliferation, which was even further reduced upon allergen exposure.¹⁸ This suggests that CD4+CD25+ T cell-mediated immune regulation is impaired during the pollen season. However, in this study Tregs from atopic patients were able to suppress mitogen-induced responses, even during the hayfever season, suggesting that impaired Treg function is limited to the activating Ag. AD is a multifactorial disease and although inhalant and food allergens have been implicated, the exact pathogenesis remains unclear.²⁶ Therefore we were not able to examine Treg function in terms of activating Ags, and it is possible that this could be impaired.

The ability of CD4+CD25+ T cells to prevent immune pathology *in vivo*, and proliferative responses *in vitro*, is well established. In this study we also demonstrate that Tregs have the capacity to suppress proliferative responses that have already been initiated *in vitro*, although they become less effective with time. This observation is in agreement with the *in vivo* model of intestinal inflammation, where CD4+CD25+ T cells have been shown to ameliorate an established pathological response.³³ This is important, as it suggests that manipulation of Tregs may be beneficial in the treatment of chronic inflammation.

The capacity of Tregs as intrinsic immune regulators to affect the balance between immune responses and immune pathology has led to speculations that these cells may be manipulated to enhance or reduce the immune response.^{14,34,35} We hypothesized that an increase in Treg activity could allow lower doses of immunosuppressive drugs to achieve clinical effectiveness. In view of numerous side-effects associated with the use of immunosuppressive drugs, this could potentially ensure clinical effectiveness while limiting side-effects. To address this hypothesis, we investigated the potential additive effects of Tregs and tacrolimus on proliferation of effector cells *in vitro*. Our data indicate that addition of CD4+CD25+ T cells together with tacrolimus leads to significant reduction in Ag-specific proliferative responses *in vitro*, in comparison with addition of the same number of CD4+CD25+ T cells alone, or the same dose of tacrolimus alone. Furthermore, when tacrolimus was titrated in the presence of increasing numbers of CD4+CD25+ T cells, Tregs

lowered the dose required for 50% inhibition of proliferation by four- to eightfold. Therefore in principle, lower doses of immunosuppressants might be therapeutically effective if used together with a strategy that would enhance Treg activity.

In conclusion, our results indicate that, at least in the periphery, patients with AD have a CD4+CD25+ regulatory population which is neither numerically nor functionally different from that of normal donors. Furthermore, our data suggest the possibility of Tregs augmenting the inhibition of T-cell activation by tacrolimus. Given the undesirable side-effects associated with immunosuppressive drugs, this observation has potential clinical implications.

Acknowledgments

This work was funded by Fujisawa GmbH, BBSRC ERA initiative, The Sir Jules Thorne Charitable Trust and Dermatrust.

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