

Everolimus and Basiliximab Permit Suppression by Human CD4⁺CD25⁺ Cells *in vitro*

David S. Game, Maria P. Hernandez-Fuentes
and Robert I. Lechler*

Department of Immunology, Faculty of Medicine, Imperial College, Hammersmith Hospital, Du Cane Road, London, UK

*Corresponding author: Professor R.I. Lechler,
r.lechler@imperial.ac.uk

Immunosuppressive drugs are essential for the prevention of acute transplant rejection but some may not promote long-term tolerance. Tolerance is dependent on the presence and regulatory function of CD4⁺CD25⁺ T cells in a number of animal models. The direct effects of immunosuppressive drugs on CD4⁺CD25⁺ cells, particularly those that interfere with IL-2 signaling are uncertain. We studied the effects of the rapamycin derivative everolimus and the anti-CD25 monoclonal antibody basiliximab on the regulatory capacity of human CD4⁺CD25⁺ cells *in vitro*. Both drugs permitted the suppression of proliferation and IFN- γ secretion by CD4⁺CD25⁻ cells responding to allogeneic and other polyclonal stimuli; CTLA-4 expression was abolished on CD4⁺CD25⁺ cells without compromising their suppressive ability. Everolimus reduced IFN- γ secretion by CD4⁺CD25⁻ cells before the anti-proliferative effect: this is a novel finding. Exogenous IL-2 and IL-15 could prevent the suppression of proliferation by CD4⁺CD25⁺ cells and the drugs could not restore suppression. By contrast, suppression of IFN- γ secretion was only slightly impeded with the exogenous cytokines. Finally, CD4⁺CD25⁺ cells were more resistant than CD4⁺CD25⁻ cells to the pro-apoptotic action of the drugs. Together these data suggest that CD4⁺CD25⁺ cells may still exert their effects in transplant patients taking immunosuppression that interferes with IL-2 signaling.

Key words: Immunoregulation, immunosuppression, T cells, tolerance

Received 12 July 2004, revised and accepted for publication 18 August 2004

Introduction

A population of CD4⁺ T cells with high surface expression of the alpha-chain of the IL-2 receptor (CD25) can suppress the activity of effector CD4⁺ or CD8⁺ cells *in*

vitro and *in vivo* (1–5). Murine transplantation models have demonstrated that enrichment of alloantigen-specific CD4⁺CD25⁺ cells can induce transplantation tolerance (6,7). We, and others have demonstrated the existence of CD4⁺CD25⁺ cells with suppressive properties, analogous to those in found in mice, in the peripheral blood of humans; CD4⁺CD25⁺ cells were shown to suppress the responses of CD4⁺CD25⁻ cells to allogeneic as well as other polyclonal stimuli *in vitro* (8–12).

In vitro assays of human alloresponses with cells purified from renal transplant patients suggest that CD4⁺CD25⁺ cells primarily regulate the indirect pathway of allorecognition (13); the principle mechanism of the decreased frequency of direct pathway T cells is not regulation but is likely to be a combination of deletion and anergy (14). Recently, we have described the expansion of CD4⁺CD25⁺ cells *in vitro*, with enhanced alloantigen-specific suppression of CD4⁺CD25⁻ cells to both direct pathway and indirect pathway stimuli, as well as linked suppression (15). This has led to the exciting possibility of manipulating CD4⁺CD25⁺ cells for immunotherapy in order to prevent transplant rejection.

At present, immunosuppressive drugs are essential to prevent allograft rejection in almost all human transplant recipients. Currently, the most widely used immunosuppressive regimes include a calcineurin inhibitor (cyclosporin or tacrolimus), an anti-proliferative agent (azathioprine or mycophenolate mofetil) and steroids. In order to avoid the nephrotoxic effects of calcineurin inhibitors and the undesirable effects of steroid therapy, a number of newer anti-rejection therapies have been explored, with particular targeting of the pro-inflammatory cytokine IL-2. Basiliximab (simulect) is a chimeric monoclonal antibody directed against CD25 and it therefore competes with IL-2 in binding to the high-affinity receptor (16). In the clinical setting, basiliximab used in the induction phase of immunosuppression effectively reduced the number of acute rejection episodes (17). The rapamycin derivative everolimus (RAD) is a macrolide antibiotic related to rapamycin (sirolimus) and inhibits T-cell proliferation by preventing some of the downstream cascades subsequent to ligation of the IL-2 receptor (18,19); clinically, these drugs can be used in various combinations with conventional drugs (20–22). There is accumulating data to suggest that calcineurin inhibitors may prevent the induction of tolerance in some systems, possibly by blocking T-cell anergy and activated induced

cell death (AICD); this was not the case for basiliximab or RAD, which has led to the idea that the latter agents could be 'tolerance promoting' (23–28).

Several lines of evidence suggest that the generation and peripheral homeostasis of CD4⁺CD25⁺ cells are intimately entwined with the action of IL-2. Of note, CD4⁺CD25⁺ cells produce negligible IL-2 of their own (2,10): IL-2 must be provided by other T-cell subsets. *In vitro* susceptibility of CD4⁺CD25⁺ cells to IL-2 deprivation has been described for some time: from the first descriptions of these cells, exogenous IL-2 was found to drive their proliferation (2,10,11). Later, *in vivo*, functional regulatory cells could not be identified in IL-2^{-/-} or CD25^{-/-} mice (29). In another study, CD4⁺ cells from IL-2^{-/-} mice could protect EAE-prone IL-2-sufficient mice from disease whereas CD4⁺ cells from CD25^{-/-} mice could not (30). This implies that IL-2 signaling in the regulatory cell is required for the development of suppressive function.

Therefore, the tolerance-promoting effects of basiliximab and RAD could potentially be offset by the prevention of regulatory cell function.

The aim of this study was to determine the effect of basiliximab and RAD on the suppressive capabilities of human CD4⁺CD25⁺ cells *in vitro*. To our knowledge this is the first attempt to address this clinically important question in the human system. In addition, the dissection of the suppression of proliferation versus the suppression of effector function revealed a novel mechanism of action for RAD.

Materials and Methods

T-cell purification

Peripheral blood mononuclear cells (PBMC) from healthy volunteers were obtained from buffy coats by density gradient centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Adherent cells were then removed by incubation for 1 h at 37°C in 250-mL flasks containing RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 100 µg/mL penicillin or 100 µg/mL streptomycin (Life Technologies), L-Glutamine 2 mM (Life Technologies) and 10% fetal calf serum (SeraQ, Sussex, UK). From the non-adherent fraction, CD4⁺ T cells were obtained by incubation with a cocktail of MoAbs: CD14 (Sigma, Gillingham, Dorset), CD33 (Caltag, Burlingame, CA), CD19, CD16, CD56 and CD8 (Diaclone, Besançon, France); TCR γ/δ (Becton Dickinson, 'BD', San Jose, CA), followed by negative selection on goat-anti-mouse-bound magnetic beads (Biomag, Qiagen, Hilden). CD4⁺ cells were then subjected to further selection by 30 min incubation with CD25-linked magnetic beads (Dynal). Those cells not selected by the beads on magnetic column were designated CD4⁺CD25⁻. The beads were then washed four times and any cells present in the washings were discarded. The CD4⁺CD25⁺ cells were released from the beads by mixing with CD4/CD8 Detachabead (Dynal) for 1 h and the beads removed by magnetic column. Isolated subsets were incubated with CD4-TC (Caltag) and CD25-PE (BD) or the appropriate isotype-matched controls to study efficacy of purification, which resulted in 90–95% for CD4⁺CD25⁻ and 85–95% for CD4⁺CD25⁺.

Preparation of dendritic cells and adherent cells

Adherent cells were isolated as above and either used immediately or used to generate dendritic cells. For the latter, adherent cells were cultured in X-vivo medium (Biowhittaker, UK) supplemented with 2% human AB serum (Biowest, Ringmer, East Sussex, UK) and penicillin/streptomycin/glutamine as above. IL-4 and GM-CSF (First Link) were added at days 0, 2 and 4 at 50 ng/mL. In both cases, cells were detached with a cell scraper.

Proliferation studies

All assays were performed in RPMI 1640 supplemented as above, but with 10% human AB serum instead of fetal calf serum. Cells were incubated at 37°C with 5% CO₂. 5 × 10⁴ CD4⁺CD25⁻ cells, 5 × 10⁴ CD4⁺CD25⁺ cells or a coculture of the same number of both subsets from the same donor were cultured (total cell number 1 × 10⁵ in coculture). Unless otherwise stated, cultures were stimulated with 0.1 µL/mL of anti-CD3/anti-CD28-coated beads (Dynal) or with 30 Gy irradiated 1 × 10⁵ dendritic or 2 × 10⁵ adherent cells. Where specified RAD was added at 5–25 nM or basiliximab at 10–25 µg/mL (both Novartis Pharma AG, Basel, Switzerland). Where specified exogenous recombinant human IL-2, rh-IL-2 at 10 U/mL (Roche, UK) or rh-IL-15 (R&D Systems, MN) at 25 ng/mL were added. After 4 days for beads stimulation or 5 days for allogeneic stimulations 1 µCi of Thymidine-H³ was added to each well. Plates were harvested after further 16 h. Incorporation of Thymidine-H³ was measured in a scintillation counter (Wallac, Turku, Finland).

To calculate the ratio of suppression of the CD4⁺CD25⁺ cells the following formula was applied: [(cpm of CD4⁺CD25⁻ – cpm of coculture)/cpm of CD4⁺CD25⁻].

ELISA

After 36 h of culture, supernatants from the above cultures were harvested and frozen (–20°C) until further use. Paired antibodies for IFN-γ (Immunokontakt, Frankfurt, Germany) were used. ELISA assays were performed using a standard protocol. Plates were coated overnight with purified anti-IFN-γ Ab at 1 µg/mL, followed by a blocking step with PBS/1% Bovine Albumin (Sigma). Standards and samples were incubated for 2 h at room temperature in the presence of biotinylated anti-IFN-γ Ab at 0.45 µg/mL, washed, followed by a 45-min incubation with Streptavidin-horseradish peroxidase conjugate (Biosource, Camarillo, CA) at 68 ng/mL. Samples were revealed by a short incubation with TMB-single solution (Zymed, San Francisco, CA), stopped with 100-mM solution of H₂SO₄. IFN-γ levels were obtained comparing the optical density (OD) values of the standards and the samples when read in a spectrophotometer at 450 nm. The limit of detection varied between 5 and 10 pg/mL of IFN-γ.

CTLL bioassay

CTLL is a murine cell line that proliferates in a dose-dependent manner to human IL-2. Cells were maintained in supplemented RPMI, 10% fetal calf serum and rh-IL-2 at 10 U/mL (Roche, UK). Prior to assay the CTLL cells had no supplemental rh-IL-2 for 2 days and were thoroughly washed to ensure that no exogenous IL-2 could confound the assay. To each 50 µL of supernatant, 5 × 10³ CTLL in 100 µL of medium were added. The amount of IL-2 in the supernatant was assessed by comparison with a standard curve of CTLL proliferation as measured by Thymidine-H³ incorporation.

CFSE labeling

CD4⁺CD25⁻ and CD4⁺CD25⁺ subsets were labeled independently with 5-µM CFSE (Molecular Probes, Leiden, The Netherlands) for 3 min at room temperature and washed extensively. Cells are then counted and set in culture as follows: CD4⁺CD25⁻CFSE⁺ or CD4⁺CD25⁺CFSE⁺ or a mixture of CD4⁺CD25⁻CFSE⁺ with unlabeled CD4⁺CD25⁺ and activated as for

proliferation studies. When mixed, the CFSE-negative cells were gated out of the assays of proliferation (Figure 1E).

Annexin V staining

CFSE-labeled cells cultured as above were washed and resuspended in binding buffer (0.1-M HEPES/NaOH (pH 7.4) 1.4-M NaCl, 25-mM CaCl₂) in the presence of 5 μ L of Annexin V-PE per 10⁶ cells (BD-Pharmingen) for 20 min in the dark at 4°C. Cells were analyzed by flow cytometry (FacsCalibur and CellQuest 3.3 software, BD).

Total CTLA-4 staining

Recently, isolated or cultured cells were fixed with a 4% paraformaldehyde (Sigma)/PBS solution for 10 min at 4°C. Cells were then washed in PBS and incubated for 30 min in the dark at 4°C in a solution containing 0.5% Saponin (Sigma), 2% FCS (SeraQ, Sussex, UK) and PBS in the presence of 2 μ L of anti-CD152-PE or mouse IgG2a-PE (BD-Pharmingen). After washing twice with 0.1% saponin/2% FCS/PBS cells were analyzed by flow cytometry (FacsCalibur and CellQuest 3.3 software, BD).

Statistical analyses were performed with Prism 3.02 (GraphPad Software, San Diego, CA, USA). Statistical analysis between groups has been carried out using a Wilcoxon signed-rank test.

Results

RAD and basiliximab permit suppression of proliferation to anti-CD3/anti-CD28-coated beads

In a coculture of 1:1 regulatory cell:effector cell, CD4⁺CD25⁺ cells suppressed the proliferation of CD4⁺CD25⁻ cells stimulated by beads coated with anti-CD3 and anti-CD28 antibodies as measured by the uptake of tritiated thymidine. Data from a representative experiment are shown by the black bars in Figure 1A. The ratio of suppression can be calculated by [cpm of CD25⁻ cells alone – cpm of CD25⁻ with CD25⁺]/cpm of CD25⁻ alone, as shown by the black bars in Figure 1B. If basiliximab or RAD was added to the coculture, the overall proliferation was decreased, as expected, but the remaining proliferation was still amenable to suppression by the CD4⁺CD25⁺ cells (Figure 1A,B, spotted and lined bars). Clearly, for the calculation of suppression ratio with drugs, the cpm of CD25⁻ cells in the presence of drug is used. Cumulative data from 12 different experiments are depicted in Figure 1C: There was no significant difference in the median values for the ratios of suppression, which were 0.58 drug free, 0.62 with RAD and 0.53 with basiliximab. As can be seen from the figure, there was considerable variability in the suppressive ability of the CD4⁺CD25⁺ cells between experiments. This may relate to a genuine variability in the suppressive ability in CD4⁺CD25⁺ cells from humans or may be a reflection of the purification method employed, where a proportion of non-regulatory CD4⁺CD25⁺ cells were isolated (data not shown).

In a similar experiment, if the cell cultures were stimulated with serial dilutions of beads (1–0.001 μ L/mL), plotting the suppression ratio against the cpm of the assay showed

that, consistent with the literature, suppression was superior at lower bead concentration (31,32). Representative data from three independent experiments are shown. At very high cpm, the suppression ratio in the presence of basiliximab was less than that for the drug free and RAD conditions, but this was not significant.

Suppression of proliferation can also be depicted with CFSE staining of the cells. Representative data of five independent experiments are shown in Figure 1E. Here, it can be seen that given sufficient stimulation, CD25⁺ cells can proliferate vigorously, although to a lesser degree than CD25⁻ cells and yet suppression of proliferation can occur: In the drug-free analysis, 8.7% of CD25⁻ cells are within three cycles of division compared with 35.7% of purified CD25⁺ cells and 14.9% of CD25⁻ cells in coculture with unstained CD25⁺ cells (Figure 1E, top row). Addition of drugs to this assay confirmed their permissive nature (Figure 1E, lower 2 rows). Again, there was some variability in the suppressive potency of the CD4⁺CD25⁺ cells between experiments but the permissive nature of the drugs was robust.

The doses of RAD and Basiliximab were chosen according to the manufacturer's instruction and in accordance with the literature: preliminary dose-response experiments confirmed the suitability of these doses (data not shown). RAD and basiliximab exert maximal suppressive effects at the order of 5 nM and 10 μ g/mL, respectively; at this dose neither drug completely inhibits proliferation of human T cells (19,33). As indicated, in some experiments a higher dose is used to be absolutely certain of maximal drug effect. Clinically, RAD is dosed to achieve a serum concentration of 3–15 nM and basiliximab as two 20-mg doses, one pre-transplant, the other at day 4.

RAD and basiliximab permit the suppression of proliferation to allogeneic antigen-presenting cells

In order to test the suppressive properties of CD25⁺ cells on CD25⁻ cells exposed to a more physiological stimulus, mixed lymphocyte cultures were set up with autologous CD25⁺ and CD25⁻ cells together with irradiated allogeneic antigen-presenting cells (DCs or adherent cells). Comparable data to that of bead stimulation were obtained: collated data derived a median ratio of suppression of 0.48 for drug-free coculture, 0.43 with RAD and 0.47 with basiliximab and no significant difference (Figure 2A). Representative data from mixed lymphocyte cultures with CFSE staining are shown in Figure 2B. As predicted, the division of all cells was less than that stimulated by the antibody-coated beads; CD25⁺ cells were much less likely to divide, particularly in the presence of drugs and yet they were unlikely to have been killed by the drugs because suppression of proliferation of CD25⁻ cells was permitted (Figure 2B, lower 2 rows). Variability in the suppressive potency of CD4⁺CD25⁺ cells in these assays may, in part, be explained by different HLA mismatches between experiments (32).

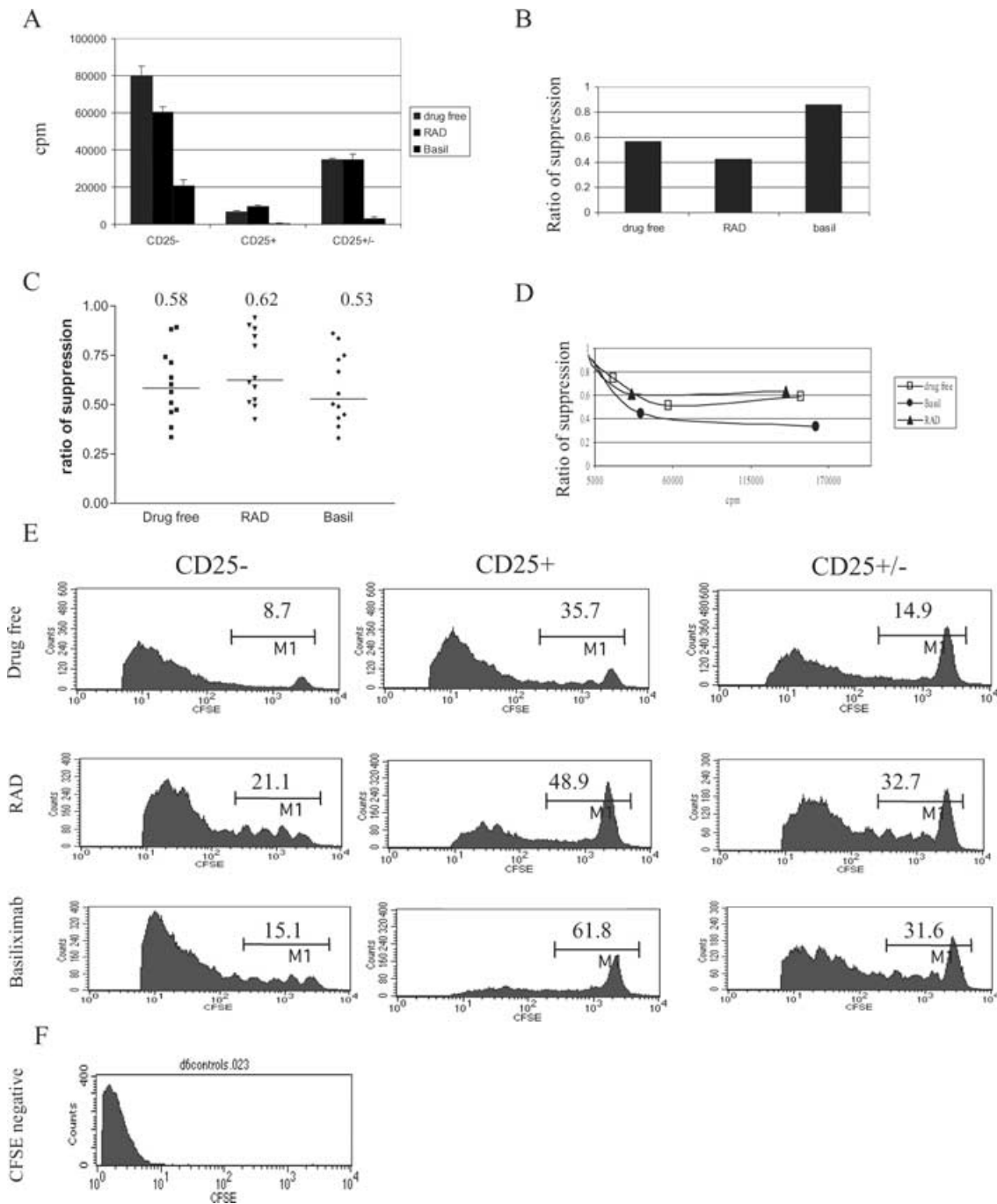


Figure 1: RAD and basiliximab permit suppression by CD25⁺ cells on CD25⁻ cells stimulated with anti-CD3/anti-CD28 beads; suppression is dose-dependent. CD4⁺CD25⁻ cells, CD4⁺CD25⁺ cells or a 1:1 mixture both were stimulated by 0.1μL/mL of anti-CD3/anti-CD28-coated beads. Triplicates of drug free, 5-nM RAD or 10-μg/mL basiliximab were set up. Cellular proliferation was assessed by measuring the incorporation of tritiated thymidine at day 5 (A). The values are mean data with standard errors. From the proliferation counts, the ratio of suppression can be calculated as described in materials and methods (B). Accumulated data of suppression ratios from 12 different experiments (RAD doses 5 nM, basiliximab doses 10 μg/mL) together with median values were obtained (C). In a similar experiment, if the cell cultures were stimulated with serial dilutions of beads (0.001–1μL/mL), plotting the suppression ratio against the cpm of the assay showed that suppression was superior at lower bead concentrations. As an alternative measure of proliferation, using the same drug concentrations, cells were stained with CFSE. To assess CD25⁺-mediated suppression, CD25⁻ cells were stained with CFSE and mixed with unstained CD25⁺ cells. The percentage of cells within three divisions is shown (E). The unstained cells were excluded by gating (F).

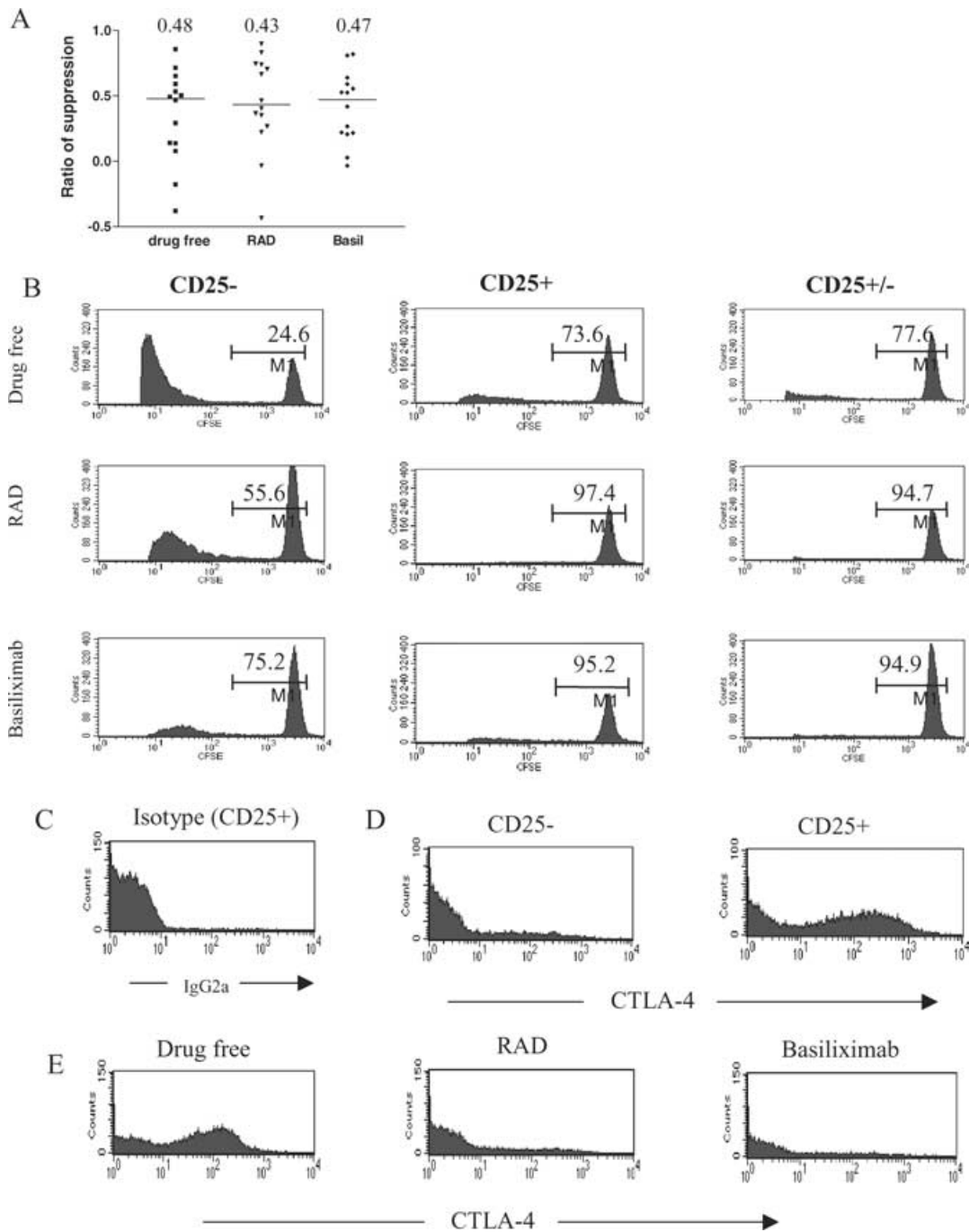


Figure 2: RAD and basiliximab permit suppression by CD25⁺ cells on CD25⁻ cells stimulated with allogeneic APCs; suppression is independent of CTLA-4 expression. CD4⁺CD25⁻ cells, CD4⁺CD25⁺ cells or a 1:1 mixture of both were stimulated by allogeneic APCs (DCs or 2 × 10⁵ adherent cells). Triplicates of drug free, 5-nM RAD or 10-μg/mL basiliximab were set up. Cellular proliferation was assessed by measuring the incorporation of tritiated thymidine at day 7. Accumulated data of suppression ratios together with median values were obtained (A). In a different experiment, the cell populations were stained with CFSE under similar conditions. To assess CD25⁺-mediated suppression, CD25⁻ cells were stained with CFSE and mixed with unstained CD25⁺ cells. The percentage of cells within three divisions is shown (B). CFSE negative cells were gated out as before. In a different experiment, CD4⁺CD25⁻ cells and CD4⁺CD25⁺ cells were purified and some stained for total CTLA-4 as described in the ‘materials and methods’ section. Staining of the isotype on CD4⁺CD25⁻ cells (C) and CTLA-4 in CD4⁺CD25⁻ and CD4⁺CD25⁺ cells (D) is shown. The remaining cells were then stimulated by 0.1 μL/mL anti-CD3/anti-CD28-coated beads in drug free, 10-nM RAD or 25-μg/mL basiliximab conditions for 5 days, then stained for total CTLA-4 (E).

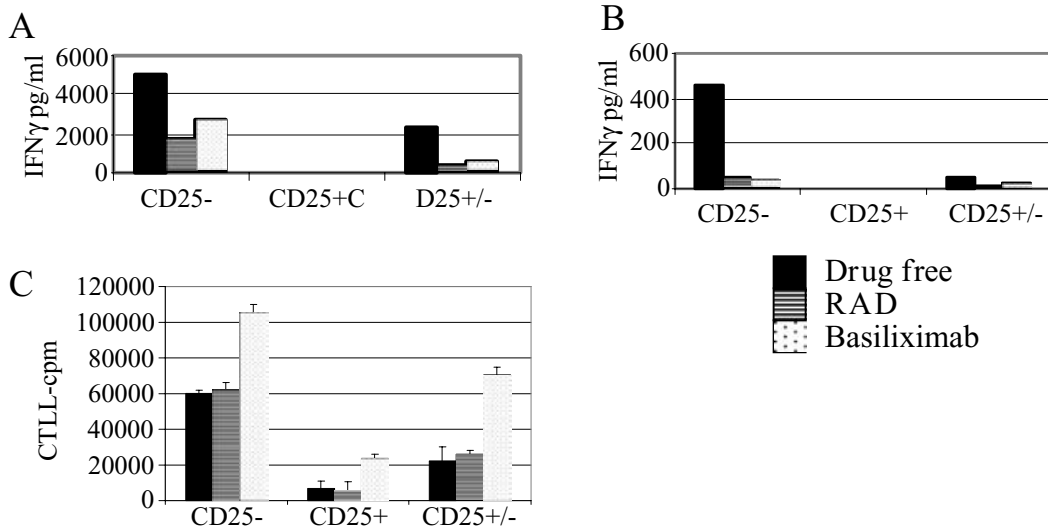


Figure 3: CD4⁺CD25⁺ cells, RAD and basiliximab suppress secretion of IFN- γ before suppression of proliferation. 1×10^6 CD4⁺CD25⁻ cells, 1×10^6 CD4⁺CD25⁺ cells or a mixture of the same number of both were stained with CFSE (CD25⁻ cells only in mixture) and stimulated by 0.1 μ L/mL of anti-CD3/anti-CD28-coated beads or allogeneic APCs (2×10^5 DCs) in a final volume of 500 μ L. Drug doses were 10-nM RAD or 25- μ g/mL basiliximab. Supernatants were harvested at 36 h and ELISA for IFN- γ performed as described in the 'materials and methods' section. The results of a representative experiment for bead stimulation (A) and allo-DC stimulation (B) are shown (n = 4 for each). The values are mean data; standard errors were less than 5% of the mean. At this time, half of the cells were removed and cell division determined by CFSE dilution as discussed. Supernatant was harvested at 48 h from the remaining cultures and IL-2 secretion determined by CTLL bioassay as described in the 'materials and methods' section (C). The values are mean data; standard errors were less than 5% of the mean.

RAD and basiliximab inhibit CTLA-4 expression in CD4⁺CD25⁺ cells without compromising suppressor function

The role of CTLA-4 expression in the suppressor function of CD4⁺CD25⁺ cells is controversial. The effect of RAD and basiliximab on the intra-cellular staining level of CTLA-4 was determined. Upon purification, significant staining of CTLA-4 was detected on CD4⁺CD25⁺ cells but not CD4⁺CD25⁻ cells (Figure 2D) when compared with isotype (Figure 2C). The cells were then stimulated with anti-CD3/anti-CD28 beads with, or without RAD or basiliximab and the intra-cellular CTLA-4 expression determined again at day 5. As seen in Figure 2E, expression of CTLA-4 in drug-free CD4⁺CD25⁺ cells was essentially unchanged whereas CTLA-4 expression was abolished by both RAD and basiliximab. Other surface markers such as HLA-DR and CD62L were also reduced in the presence of RAD and basiliximab (data not shown). As will be clear from the results above, this did not compromise suppressor function.

RAD, basiliximab and CD4⁺CD25⁺ cells suppress the secretion of IFN- γ by CD25⁻ cells before the exertion of anti-proliferative effects

CD4⁺CD25⁺ cells are known to suppress the secretion of IFN- γ by CD25⁻ cells while not secreting any IFN- γ themselves (10): this is confirmed here for bead-stimulated CD25⁻ cells (Figure 3A, black bars) and CD25⁻ cells stim-

ulated by allogeneic DCs (Figure 3B, black bars); representative data of four independent experiments are given. In this study, IFN- γ secretion was determined by ELISA of supernatants taken from cocultures after 36 h. Analysis of CFSE staining of bead-stimulated CD25⁻ cells showed that the inhibition of cytokine secretion was seen before significant proliferation had occurred and with allogeneic stimulation, suppression of IFN- γ preceded all proliferation (data not shown). Therefore CD4⁺CD25⁺ cells were able to suppress effector function before suppression of proliferation. As seen in Figure 3, this relationship also holds true for both RAD and Basiliximab, a novel finding for RAD. Furthermore, IL-2 bioassay reveals that RAD had no effect on IL-2 secretion at 48 h and free IL-2 in the supernatant was increased in the presence of basiliximab, consistent with published data (18) (Figure 3C).

RAD and basiliximab permit suppression of IFN- γ secretion to bead stimulation

Comparable to the proliferation data, RAD and basiliximab reduced secretion of IFN- γ by CD25⁻ cells, but still permitted the additional suppressive effect of CD25⁺ cells (Figure 3A). Allogeneic stimulation of CD25⁻ cells resulted in much less secretion of IFN- γ , such that CD25⁺ cells, RAD and basiliximab suppressed IFN- γ secretion so markedly that further suppression could not be detected (Figure 3B).

Exogenous IL-2 and IL-15 reverse the suppression of proliferation by CD4⁺CD25⁺ cells and neither RAD nor basiliximab can restore it

Consistent with the literature, the addition of exogenous IL-2 or IL-15 was shown to induce marked proliferation of CD4⁺CD25⁺ cells in the context of allogeneic stimulation (8,34); suppression of proliferation by the CD25⁺ cells was also prevented, at least partially if proliferation of CD25⁺ cells is also considered (Figure 4A). Figure 4A also shows that RAD reduced T-cell proliferation in all conditions by approximately 50%, including the addition of exogenous cytokines. In addition, basiliximab markedly inhibited proliferation; exogenous IL-2 could compete with the basiliximab to promote proliferation whereas 'IL-2-deprived' cells briskly proliferated to IL-15, almost to drug-free levels.

Exogenous IL-2 or IL-15 did not reverse the permissive nature of RAD and basiliximab on the suppression of IFN- γ secretion by CD4⁺CD25⁺ cells

The suppression of IFN- γ secretion in the context of exogenous IL-2 and IL-15 was also tested. Although the secretion was low, addition of IL-2 and IL-15 slightly increased secretion at 36 h (Figure 4B) but unlike the proliferation data, CD25⁺ cells could suppress IFN- γ secretion by CD25⁻ cells in the presence of exogenous IL-2 and IL-15. Furthermore, in this system, suppression of IFN- γ secretion was permitted by RAD and basiliximab.

CD4⁺CD25⁺ cells are resistant to the pro-apoptotic effects of RAD and basiliximab

Cocultures of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells stimulated by anti-CD3/anti-CD28 beads, were performed with either cell population stained with CFSE. Five days later the cells were stained with annexin V to assess the degree of apoptosis in dividing and non-dividing cells. As seen in Figure 5, first column, CD4⁺CD25⁺ cells were more prone to apoptosis than CD25⁻ cells in the drug-free condition. The addition of RAD and basiliximab increased the degree of apoptosis of CD25⁻ cells (Figure 5, top row) but decreased the degree of apoptosis of CD4⁺CD25⁺ cells (Figure 5, bottom row). In all cases, apoptosis was more likely in maximally dividing cells; whether the reduced apoptosis in CD4⁺CD25⁺ cells in the presence of the drugs was simply a function of less proliferation, or whether there is a direct anti-apoptotic effect was not determined.

Discussion

These data demonstrate that CD4⁺CD25⁺ regulatory cells can exert their suppressive effect *in vitro* in the presence of basiliximab or RAD. In fact, the concept that regulatory cells may be 'spared' the inhibitory effects of immunosuppressive agents is not new (35) but whether this is the case for the inhibition of IL-2 pathways in CD4⁺CD25⁺ cells has not previously been addressed in the human system.

The *in vitro* ratio of 1:1 suppressor to effector cell was chosen as this reproducibly demonstrated suppression in this human system albeit with variable potency; any effect of the drugs could therefore be tested. In the human circulation the typical ratio is 1:10 and regulation is difficult to demonstrate at this level (14). Therefore, this article describes a 'proof of principle' although, *in vivo*, it is conceivable that antigen-specific expansion of suppressor cells at the graft or draining lymph node could raise the proportion of suppressor cells.

Our finding that basiliximab and RAD permit suppression by CD4⁺CD25⁺ cells can have several explanations. First, basiliximab may compete poorly with IL-2 for CD25 (36), and the residual IL-2 binding may be sufficient for regulatory cell function. Indeed, IL-2 responsive cells can be detected in the blood of patients treated with basiliximab, even when further basiliximab is added *in vitro* (37). Second, in contrast to the *in vivo* data implying a non-redundant role for IL-2 in the function of CD4⁺CD25⁺ cells (30), it appears that IL-15 is perfectly capable of driving proliferation of CD4⁺CD25⁺ cells *in vitro* (8) and in some systems may actually be superior to IL-2 in this regard (34); furthermore, our data suggest that cells deprived of IL-2 by basiliximab are exquisitely sensitive to IL-15. While this may account for some of the effects in allogeneic cell cultures, clearly there is no source of IL-15 in the APC-free system described here. Third, in general, the suppressive properties of CD4⁺CD25⁺ cells are thought to be more effective when the stimulus to the CD4⁺CD25⁻ cells is weaker (2,31,32). Consistent with this, we found that titration of anti-CD3/anti-CD28 beads revealed greater suppression at lower proliferation counts (Figure 1D). Therefore, the action of the drugs may reduce the cellular reaction to within a more easily regulated magnitude. Finally, the CD4⁺CD25⁺ cells may actually be resistant to the effects of basiliximab and RAD and there is emerging data in support of this contention.

The ability of CD4⁺CD25⁺ cells to operate in the clinical context is supported by the study of Salama et al. in which suppression of the indirect pathway by CD25⁺ cells was demonstrated using lymphocytes isolated from renal transplant recipients: these patients had received a variety of immunosuppressive agents (13). Of note, Salama et al. describe suppression by CD25⁺ cells in patients who had previously received anti-IL-2 receptor antibodies: the antibody is likely to have been cleared by the time of assay; here, we describe suppression by CD4⁺CD25⁺ cells in the presence of basiliximab.

We found that exogenous IL-2 and IL-15 prevented suppression of proliferation by CD4⁺CD25⁺ cells and the drugs did not reverse this. However, IL-2 and IL-15 did not reverse suppression of IFN- γ secretion by CD4⁺CD25⁺ cells and the drugs were permissive in this context. The dichotomy of CD4⁺CD25⁺-mediated suppression of proliferation versus suppression of effector function has been

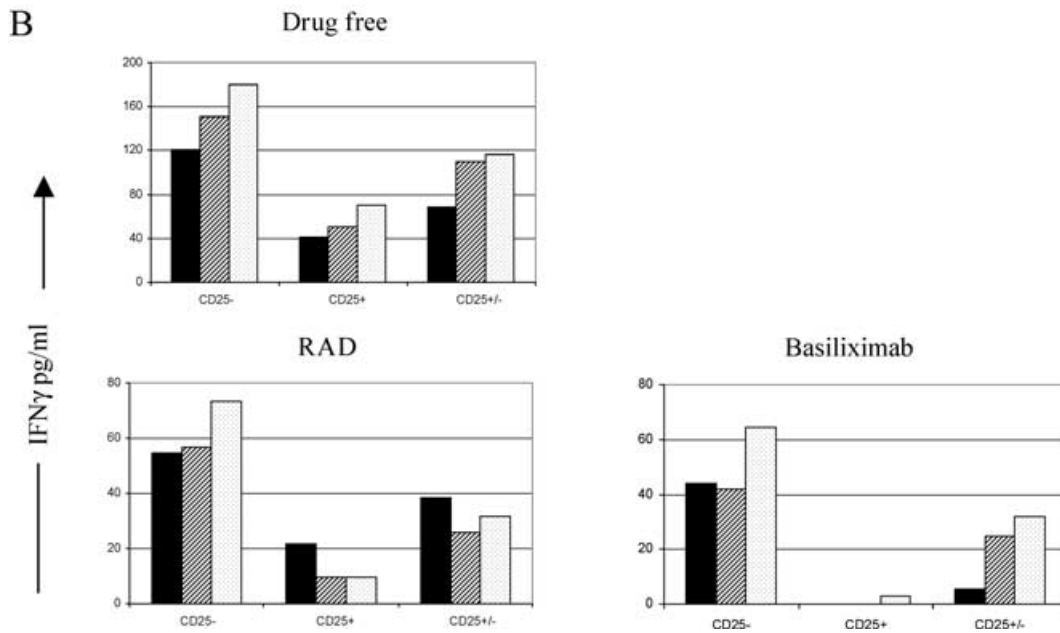
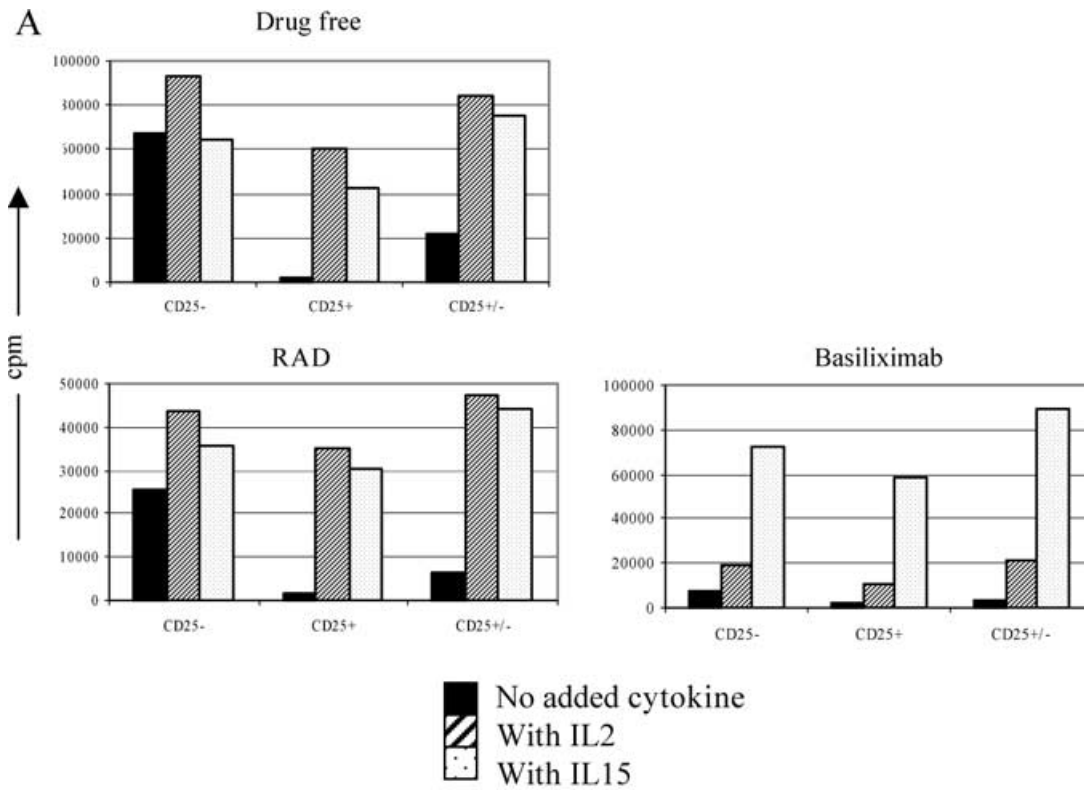


Figure 4: Exogenous IL-2 or IL-15 and suppression by CD4⁺CD25⁺ cells drug free or with RAD or basiliximab. 5×10^4 CD4⁺CD25⁻ cells, 5×10^4 CD4⁺CD25⁺ cells or a mixture of the same number of both were stimulated by allogeneic APCs (1×10^5 DCs). Triplicates of drug free, 25-nM RAD or 25- μ g/mL basiliximab were set up with no added cytokines, 10-U/mL IL-2 or 25-ng/mL IL-15. Supernatants were harvested at 36 h and cell proliferation was assessed by measuring the incorporation of tritiated thymidine at day 7. Representative data for cell proliferation (A) and IFN- γ secretion (B) are shown (n = 3). The values are mean data; standard errors were less than 5% of the mean.

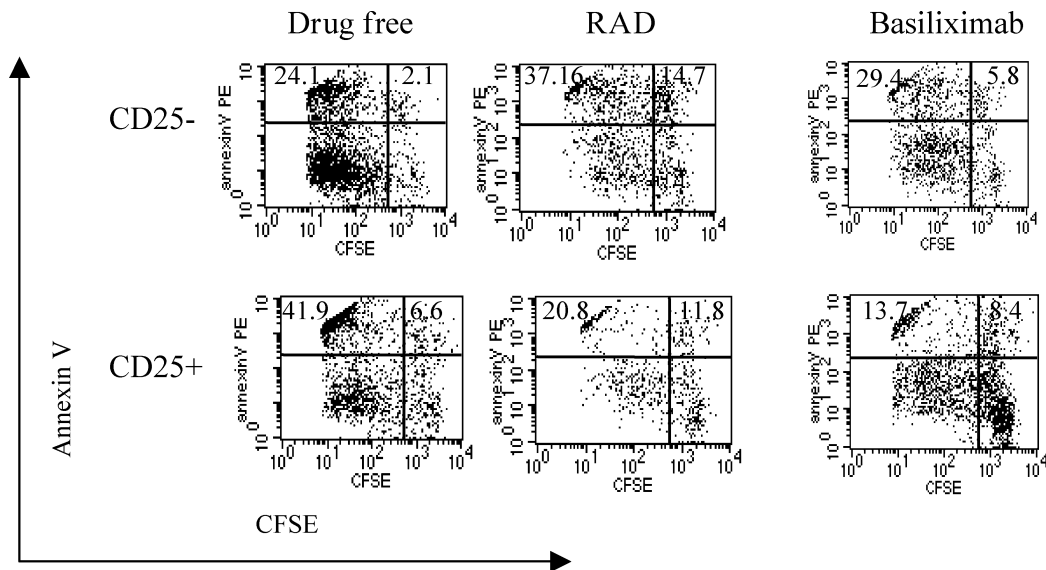


Figure 5: CD4⁺CD25⁺ cells are resistant to the pro-apoptotic effects of RAD and basiliximab. CD4⁺CD25⁻ or CD4⁺CD25⁺ cells were stained with CFSE as described. 5×10^4 stained cells were mixed with an equal number of unstained cells of the alternative population and stimulated with 0.1 μ L/mL of anti-CD3/anti-CD28-coated beads in drug free, 25-nM RAD or 25- μ g/mL basiliximab conditions. At day 5 the cultures were washed and cells stained with Annexin V according to the manufacturer's instructions. Representative data from three independent experiments are given. The results for CD4⁺CD25⁻ cells in coculture is shown in the top row and for CD4⁺CD25⁺ cells in coculture in the bottom row. The numbers given are the percentage of cells in the quadrants, which are undivided, apoptotic (right) and divided, apoptotic (left). CFSE negative cells were gated out as before.

shown for CD8⁺ cells (3) but this is the first explicit description in CD4⁺ cells. In this case, however, there may be a kinetic explanation: the exogenous cytokines may have not yet driven proliferation of both CD25⁺ and CD25⁻ cells when IFN- γ secretion was measured. Whether CD4⁺CD25⁺ cells can suppress while proliferating is under investigation.

Zheng and colleagues generated Fc fusion proteins of IL-2 and IL-15 (38). The rationale was that blockade of IL-15 was required to terminate T-cell proliferative responses, and IL-2 signaling was investigated using either lytic or agonist IL-2 fusion proteins; rapamycin was added to block the early expansion of alloreactive T cells while allowing apoptosis. The initial supposition was that IL-2 signaling was required for pro-apoptotic signals in effector cells and for the survival/expansion of CD4⁺CD25⁺ cells. In fact, CD4⁺CD25⁺ cells were relatively resistant to the lytic IL-2 fusion protein, and tolerance was permitted. In a different study, rapamycin was found to induce thymic atrophy with increased apoptosis of thymocytes *in vivo* but relative sparing of CD4⁺CD25⁺ thymocytes; the number of peripheral CD4⁺CD25⁺ cells was increased with rapamycin (39).

We also found reduced apoptosis of CD4⁺CD25⁺ cells compared with CD4⁺CD25⁻ cells with both RAD and basiliximab but increased apoptosis in CD4⁺CD25⁺ cells not treated with these drugs. Consistent with our drug-free data, Taams et al. found that CD4⁺CD25⁺ cells were more prone to apoptosis than CD4⁺CD25⁻ cells (11) and Banz

et al. found that CD4⁺CD25⁺ cells were more prone to apoptosis than CD4⁺CD25⁻ cells when mixed together (40).

The precise mechanism of the resistance of regulatory cells to apoptosis in the presence of drugs is obscure: it may relate to the relatively lower cell turnover when compared to CD4⁺CD25⁻ effector cells; the expression of CD25 on regulatory cells has been reported to be less than on activated effectors by some (41) but not by others (42)—a lower expression may confer relative resistance to basiliximab. A more recent finding provides a compelling alternative. CD4⁺CD25⁺ cells have been shown to express high levels of the tumor suppressor gene, 'phosphatase and tensin homologue' PTEN; PTEN negatively regulates PI3 kinase expression and may be responsible for the low proliferation of CD4⁺CD25⁺ cells (43). Interestingly, loss of PTEN sensitizes cells to mTOR inhibition in mice (44). Therefore, it is conceivable that the resistance of CD4⁺CD25⁺ cells to interference with IL-2 signaling relates to increased PTEN, although this has not been formally tested.

We found that CD4⁺CD25⁺ cells and both RAD and basiliximab can suppress the production of IFN- γ before the suppression of proliferation. We, and others, have previously shown that CD4⁺CD25⁺ cells do not produce IFN- γ but can potently inhibit its production by CD4⁺CD25⁻ cells (10,42). Whether this was a result of reduced proliferation (fewer cells to produce IFN- γ) or a direct effect on effector

function was not defined. Returning to the drugs, blockade of CD25 has previously been shown to reduce IFN- γ production in PBMCs at 48 h implying that IL-2 signaling can directly induce IFN- γ production (45). We corroborate these findings and further show that RAD can also directly inhibit IFN- γ production before its anti-proliferative effect; whether this effect of RAD is dependent on mTOR, has not been defined in this study. This is in contrast to Matsue et al., who found that rapamycin did not markedly influence IFN- γ production in DO11.10 T cells stimulated with DCs and ova peptide (46); concentrations of drugs used were comparable and so the discrepancy may reflect a species or drug difference. In support of our data, however, is the recent finding that rapamycin can inhibit IFN- γ production in DCs (47).

The role of CTLA-4 expression in the suppressor function of CD4⁺CD25⁺ cells is controversial: *in vivo* studies have shown that blockade of CTLA-4 can reverse the protective effects of CD4⁺CD25⁺ cells on graft rejection (7). *In vitro* studies, however, have shown that blockade of CTLA-4 did not significantly influence suppression of proliferation (10). We found that CTLA-4 expression was abolished by RAD and basiliximab without compromising suppressor function: the molecular mechanism behind cell-contact-dependent suppression remains obscure.

Overall, these data reassuringly suggest that CD4⁺CD25⁺ regulatory cells can operate in the context of clinically relevant concentrations of basiliximab and rapamycin derivative consistent with the concept that these drugs are 'tolerance promoting' agents.

Acknowledgments

The authors thank Drs. Federica Marelli-Berg, Fabien Sebille, Henrieta Fazekasova and Marina Garin for their helpful discussion and Dr. Nicola Rogers for critically reading the manuscript.

DSG held a Medical Research Council Clinical Training Fellowship and the work was further supported by a Medical Research Council Programme Grant.

References

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; 155: 1151–1164.
2. Thornton A, Shevach EM. CD4⁺CD25⁺ Immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 1998; 188: 287–296.
3. Lin CY, Graca L, Cobbold SP, Waldmann H. Dominant transplantation tolerance impairs CD8⁺ T cell function but not expansion. *Nat Immunol* 2002; 3: 1208–1213.
4. van Maurik A, Herber M, Wood KJ, Jones ND. Cutting edge: CD4⁺CD25⁺ alloantigen-specific immunoregulatory cells that can prevent CD8⁺ T cell-mediated graft rejection: implications for anti-CD154 immunotherapy. *J Immunol* 2002; 169: 5401–5404.

5. Camara NO, Sebille F, Lechler RI. Human CD4⁺CD25⁺ regulatory cells have marked and sustained effects on CD8⁺ T cell activation. *Eur J Immunol* 2003; 33: 3473–3483.
6. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens *in vivo*. *J Immunol* 2001; 166: 3789–3796.
7. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25⁺CD4⁺ Regulatory T cells Prevent Graft Rejection: CTLA-4 and IL10 Dependent Immunoregulation of Alloresponses. *J Immunol* 2002; 18: 1080–1086.
8. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex Vivo Isolation and Characterization of CD4⁺CD25⁺ T cells with Regulatory Properties from Human Blood. *J Exp Med* 2001; 193: 1303–1310.
9. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characteristics of human CD4⁺CD25⁺ T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; 193: 1285–1294.
10. Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD et al. Human CD4⁺CD25⁺ T cells: a naturally occurring population of regulatory T cells. *Blood* 2001; 98: 2736–2744.
11. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human/ anergic suppressive CD4⁺CD25⁺ T cells: a highly differentiated and apoptosis prone population. *Eur J Immunol* 2001; 31: 1122–1131.
12. Taams LS, Vukmanovic-Stejic M, Smith J, Dunne PJ, Fletcher JM, Plunkett FJ et al. Antigen-specific T cell suppression by human CD4⁺CD25⁺ regulatory T cells. *Eur J Immunol* 2002; 32: 1621–1630.
13. Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory CD25⁺ T cells in human kidney transplant recipients. *J Am Soc Nephrol*. 2003; 14: 1643–1651.
14. Game DS, Hernandez-Fuentes MP, Chaudhry AN, Lechler RI. CD4⁺CD25⁺ regulatory T cells do not significantly contribute to direct pathway hyporesponsiveness in stable renal transplant patients. *J Am Soc Nephrol* 2003; 14: 1652–1661.
15. Jiang S, Camara NOS, Lombardi G, Lechler RI. Induction of Allopeptide-Specific Human CD4⁺CD25⁺ Regulatory T cells *Ex vivo*. *Blood* 2003b; 102: 2180–2186.
16. Amlot PL, Rawlings E, Fernando ON, Griffin PJ, Heinrich G, Schreier MH et al. Prolonged action of a chimeric interleukin-2 receptor (CD25) monoclonal antibody used in cadaveric renal transplantation. *Transplantation* 1995; 60: 748–756.
17. Webster AC, Playford GE, Higgins G, Chapman JR, Craig JC. Interleukin 2 receptor antagonists for renal transplant recipients: a meta-analysis of randomised trials. *Transplantation* 2004; 77: 166–176.
18. Dumont FJ, Staruch MJ, Koprak SL, Melino MR, Sigal NH. Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J Immunol* 1990; 144: 251–258.
19. Schuler W, Sedrani R, Cottens S, Haberlin B, Schulz M, Schuurman HJ et al. SDZ RAD, a new rapamycin derivative: pharmacological properties *in vitro* and *in vivo*. *Transplantation* 1997; 64: 36–42.
20. Groth CG, Backman L, Morales JM, Calne R, Kreis H, Lang P et al. Sirolimus (rapamycin)-based therapy in human renal transplantation: similar efficacy and different toxicity compared with cyclosporine. Sirolimus European Renal Transplant Study Group. *Transplantation* 1999; 67: 1036–1042.
21. Johnson RW, Kreis H, Oberbauer R, Brattstrom C, Claesson K, Eris J. Sirolimus allows early cyclosporine withdrawal in renal transplantation resulting in improved renal function and lower blood pressure. *Transplantation* 2001; 72: 777–786.

22. Kahan BD, Kaplan B, Lorber MI, Winkler M, Cambon N, Boger RS. RAD in de novo renal transplantation: comparison of three doses on the incidence and severity of acute rejection. *Transplantation* 2001; 71: 1400–1406.
23. DeSilva DR, Urdahl KB, Jenkins MK. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J Immunol* 1991; 147: 3261–3267.
24. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med* 1999a; 5: 1298–1302.
25. Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med* 1999; 5: 1303–1307.
26. Smiley ST, Csizmadia V, Gao W, Turka LA, Hancock WW. Differential effects of cyclosporine A, methylprednisolone, mycophenolate, and rapamycin on CD154 induction and requirement for NFkappaB: implications for tolerance induction. *Transplantation* 2000; 70: 415–419.
27. Wekerle T, Kurtz J, Sayegh M, Ito H, Wells A, Bensing S et al. Peripheral deletion after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. *J Immunol* 2001; 166: 2311–2316.
28. Macian F, Garcia-Cozar F, Im S-H, Horton HF, Byrne MC, Rao A. Transcriptional Mechanisms Underlying Lymphocyte Tolerance. *Cell* 2002; 109: 719–731.
29. Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Penit C. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. *Int Immunol* 1998; 10: 371–378.
30. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* 2002; 196: 851–857.
31. Baecher-Allan C, Viglietta V, Hafler DA. Inhibition of human CD4(+)CD25(+high) regulatory T cell function. *J Immunol* 2002; 169: 6210–6217.
32. Game DS, Lechler RI. HLA-DR matching and immune regulation. *Am J Transplant* 2004; 4: 1725–1726.
33. Rowshani AT, Uss A, Yong SL, van Lier RA, ten Berge IJ. Effects of CD25 monoclonal antibody on proliferative and effector functions of alloactivated human T cells in vitro. *Eur J Immunol* 2004; 34: 882–899.
34. Koenen HJ, Fasse E, Joosten I. IL-15 and cognate antigen successfully expand de novo-induced human antigen-specific regulatory CD4(+) T cells that require antigen-specific activation for suppression. *J Immunol* 2003; 171: 6431–6441.
35. Kupiec-Weglinski JW, Filho MA, Strom TB, Tilney NL. Sparing of suppressor cells: a critical action of cyclosporine. *Transplantation* 1984; 38: 97–101.
36. Kircher B, Latzer K, Gastl G, Nachbaur D. Comparative in vitro study of the immunomodulatory activity of humanized and chimeric anti-CD25 monoclonal antibodies. *Clin Exp Immunol* 2003; 134: 426–430.
37. Baan CC, van Gelder T, Balk AHMM, Knoop CJ, Holweg CTJ, Maat LPWM et al. Functional responses of T cells blocked by anti-CD25 antibody therapy during cardiac rejection. *Transplantation* 2000; 69: 331–336.
38. Zheng XX, Sanchez-Fueyo A, Sho M, Domenig C, Sayegh MH, Strom TB. Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance. *Immunity* 2003a; 19: 503–514.
39. Tian L, Lu L, Yuan Z, Lamb JR, Tam PK. Acceleration of apoptosis in CD4+CD8+ thymocytes by rapamycin accompanied by increased CD4+CD25+ T cells in the periphery. *Transplantation* 2004; 77: 183–189.
40. Banz A, Pontoux C, Papiernik M. Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. *J Immunol* 2002; 169: 750–757.
41. Waldmann TA. A novel means of favorably tipping the balance between cytopathic and regulatory T cells. *Immunity* 2003; 19: 465–466.
42. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25+high Regulatory Cells in Human Peripheral Blood. *J Immunol* 2001; 167: 1245–1253.
43. Bensing SJ, Walsh PT, Zhang J, Carroll M, Parsons R, Rathmell J et al. Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J Immunol* 2004; 172: 5287–5296.
44. Neshat MS, Mellinghoff IK, Tran C, Stiles B, Thomas G, Petersen R et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc Natl Acad Sci USA* 2001; 98: 10031–10033.
45. McDyer JF, Li Z, John S, Yu X, Wu CY, Ragheb JA. IL-2 receptor blockade inhibits late, but not early, IFN-gamma and CD40 ligand expression in human T cells: disruption of both IL-12-dependent and -independent pathways of IFN-gamma production. *J Immunol* 2002; 169: 2736–2746.
46. Matsue H, Yang C, Matsue K, Edelbaum D, Mummert M, Takashima A. Contrasting impacts of immunosuppressive agents (rapamycin, FK506, cyclosporin A, and dexamethasone) on bidirectional dendritic cell-T cell interaction during antigen presentation. *J Immunol* 2002; 169: 3555–3564.
47. Chiang PH, Wang L, Bonham CA, Liang X, Fung JJ, Lu L et al. Mechanistic insights into impaired dendritic cell function by rapamycin: inhibition of Jak2/Stat4 signaling pathway. *J Immunol* 2004; 172: 1355–1363.