Cyclosporin-A Differentially Affects Apoptosis During *In Vivo* Rat Thymocyte Maturation

J. G. M. C. DAMOISEAUX,* M.-P. DEFRESNE,† C. P. M. REUTELINGSPERGER‡ & P. J. C. VAN BREDA VRIESMAN*

**Department of Immunology, University Maastricht, Maastricht, The Netherlands; †Department of Pathological Anatomy, CHU Liège, Liège, Belgium; and ‡Department of Biochemistry, University Maastricht, Maastricht, The Netherlands*

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Maturation arrest and interference with selection are two well-documented effects of cyclosporin-A (CsA) on the thymus. We recently hypothesized that these effects are related and owing to the reduced T-cell receptor (TCR)–CD3 complex-mediated signal transduction in thymocytes upon CsA treatment. In this hypothesis, the maturation arrest is the result of the additional depletion of thymocytes that normally survive by positive selection, whereas the impaired self-tolerance induction is caused by an increased survival of thymocytes that normally undergo negative selection. In this view, it is anticipated that CsA differentially affects thymocyte apoptosis during *in vivo* thymocyte maturation. Indeed, we report in this study a strong increase in apoptotic cells in the thymic cortex on *in situ* analysis. Simultaneously, the number of apoptotic cells had decreased at the cortico-medullary zone which is held to be the site for negative selection. Rapamycin (Rapa) also interferes with thymocyte maturation by inhibiting cytokine-driven proliferation. Hence, Rapa preferentially affects the early maturational stages of thymocyte development and is considered not to alter thymocyte selection and subsequent apoptotic events. Indeed, the number of apoptotic events appears not to be altered. However, possibly owing to the decrease in cortical macrophages, the apoptotic cells revealed an atypical enumeration around blood vessels. Taken together, our results favour the hypothesis that the dominant effect of CsA on the thymus is the reduction of the TCR–CD3 complex-mediated signal transduction in thymocytes upon interaction with stromal cells. Furthermore, the preferential localization of apoptotic cells next to blood vessels upon Rapa administration may indicate that endothelial cells are a back-up system for the removal of apoptotic cells.

Dr J. G. M. C. Damoiseaux, Department of Immunology, University Maastricht, P.O. Box 616, 6200 MD Maastricht, The Netherlands. E-mail: jdam@limm.azm.nl

INTRODUCTION

Cyclosporin-A (CsA) and Rapamycin (Rapa) are two distinct immunosuppressive reagents that interfere with T-cell activation. Together with FK506, these reagents belong to a class of agents whose cellular activity depends upon their binding to immunophilins. CsA forms a complex with cyclophilin, whereas Rapa and FK506 interact, owing to their structural similarity, with the so-called FK506-binding proteins (FKBP). The complexes of FK506 and CsA with their respective immunophilins inhibit calcineurin, subsequently block the translocation of the cytoplasmic component of the transcription factor NF-AT to the nucleus and eventually result in the inhibition of cytokine synthesis [1, 2]. The target of the Rapa–FKBP complex is the phosphatidylinositol-3 kinase homologue FKBP–Rapa-associated protein (FRAP)/RAFT1/mTOR. The current model holds that FRAP resides at a branchpoint in the signalling pathway, leading to two pathways of translational control by mitogenic

stimuli – one leading to the activation of $p70^{S6k}$ and the other to the phosphorylation of PHAS-I. These pathways eventually prevent cell cycle progression from G_1 to S phase [3, 4]. Therefore, unlike CsA, Rapa is considered a more general inhibitor of cytokine-driven proliferation.

Because the molecular mechanisms that are involved in the activation and proliferation of mature T cells are also essential for the maturation and selection of immature T cells, it is not surprising that both reagents have strong effects on T-cell development. Indeed, CsA inhibits the development of mature single-positive (SP) thymocytes and also inhibits positive and negative selection [5–8]. The impaired maturation and self-tolerance induction are not the result of stromal alterations [9, 10]. We recently hypothesized that the dominant effect of CsA on the thymus is the reduction of the T-cell receptor (TCR)–CD3 complex-mediated signal transduction in thymocytes upon interaction with stromal cells [11]. In this view, CsA will cause more apoptosis in cortical thymocytes owing to the inhibition of positive selection, whereas a reduced number of cortico-medullary thymocytes will become apoptotic because CsA also hampers negative selection. With respect to the effect of Rapa on T-cell development, it is evident that Rapa causes significant thymus atrophy and interferes with thymic recovery after lethal X-irradiation and subsequent syngeneic bone marrow transplantation [12, 13]. As Rapa is known to inhibit cytokine-driven proliferation, it is expected that these observations are the result of repressed thymocyte proliferation. Although, in contrast to CsA, Rapa seems not to interfere with negative selection in mice [12], both Rapa and CsA are able to induce autoimmune-like immunopathology in rat [13, 14].

In the present study, we have tested our hypothesis of the differential effect of CsA on apoptosis during thymocyte maturation. We have included Rapa as another immunosuppressive reagent, because in contrast to CsA, Rapa strongly affects the number of thymocytes. Although it is anticipated that this is the result of decreased proliferation, the thymic involution may alternatively be owing to increased apoptosis. In light of these suspected effects of CsA and Rapa on apoptosis and proliferation, respectively, we have analysed in the present study the cell cycle progression as well as the rate and in particular the site of apoptotic events in the rat thymus upon *in vivo* administration of the respective drugs.

MATERIALS AND METHODS

Animals. Specific pathogen-free Lewis rats were obtained from the Central Animal Facility of the University Maastricht. All rats were female and 6 weeks of age at the start of the experiment. All procedures were in accordance with national regulations on animal experiments.

Experimental design. CsA, a gift from Novartis Pharma Inc. (Basel, Switzerland), was dissolved in olive oil at concentration of 5, 10 and 20 mg/ml. Olive oil is used as a solvent and has a depot function without adjuvant effect. Rats were weighed daily and received 5, 10 or 20 mg/kg (=1 ml/kg) subcutaneously for 0, 3, 7 or 14 consecutive days. Rapa, also kindly provided by Novartis Pharma Inc., was dissolved in Solvent G (i.e. Sandimmune Placebo – Novartis Pharma Inc.) plus corn oil (Sigma, St. Louis, MO, USA) at concentration of 2.5, 5 and 10 mg/ml. Rats were weighed daily and received 2.5, 5 or 10 mg/kg (=1 ml/kg) orally for 0, 3, 7 or 14 consecutive days.

Control animals daily received solvent only (1 ml/kg). Three animals were examined for each time-point and drug concentration. At the time of sacrifice, the rats were exsanguinated under ether anaesthesia, and the thymus was removed and weighed; one thymic lobe was snap-frozen in isopentane and used for immunohistochemistry, the other lobe was used for determining the cellularity and for flow cytometry.

Flow cytometry. One thymic lobe was teased apart and passed through a 100 μm mesh nylon gauze and collected in balanced salt solution supplemented with 2% heat-inactivated foetal calf serum. The cells were washed twice by centrifugation ($316 \times g$, 10 min, 4 °C) and resuspended in phosphate-buffered saline (PBS) containing 0.5% w/v bovine serum albumin (PBS–BSA – Sigma). Nucleated cells were counted in Türk solution with a Bürker Haemocytometer, while viability was assessed by Trypan Blue exclusion.

Tricolour flow cytometry was used for the concomitant expression of CD4, CD8 and TCRαβ using flourescein isothiocyanate (FITC)-conjugated OX-35 (CD4), phycoerythrin (PE)-conjugated OX-8 (CD8α) and biotin-conjugated R73 (TCRαβ) – all are commercially available (Pharmingen, San Diego, CA, USA). Cells (5×10^5 cells/sample) were centrifuged in a 96-well microtitre plate ($236 \times g$, 3 min, 4 °C) and resuspended in 20 μl of PBS containing 0.5% BSA and 10 mM NaN_3. The biotin-conjugated monoclonal antibody (MoAb) was stained in second step with streptavidin–Cy-chrome (Pharmingen) [8].

For the detection of apoptosis in cell suspensions, thymocytes were resuspended into HEPES buffer (137 mM NaCl, 2.68 mM KCl, 10 mM HEPES, 1.7 mM $MgCl_2$, pH 7.4) containing 3 mM calcium. Annexin V–FITC was added at a final concentration of 250 ng/ml and propidium iodide (PI) at a final concentration of 2.5 μg/ml [15].

For cell cycle analysis, thymocytes were fixed in 70% ice-cold ethanol for 60 min, washed twice with PBS and stained at room temperature for 60 min with a solution of 100 μg of PI/ml (Sigma) and 200 Kunitz RNase (Serva, Heidelberg, Germany).

Flow cytometry was performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) equipped with an argon laser (excitation at 488 nm). The cells were run using the CELLQUEST software package (Becton Dickinson), and 10,000 events were acquired in list mode. Analysis was performed off line using the same software for the detection of phenotype and apoptosis and using ModFit LT (Becton Dickinson) for cell cycle analysis.

Histology. For the identification of apoptosis, frozen sections (4 μm) were fixed in 10% neutral-buffered formalin and postfixed in ethanol–acetic acid for 5 min at −20 °C. Tissue-fragmented DNA was nick-end labelled using an *in situ* apoptosis detection kit (Boehringer Mannheim, Mannheim, Germany). Negative controls were represented by tissue incubated with distilled water instead of TdT enzyme in the reaction buffer.

The MoAb ED2 was used for the identification of thymic macrophages by immunohistology [16]. Frozen sections (4–6 μm) were air-dried and fixed in acetone for 10 min and air-dried for at least 30 min. Sections were then incubated for 60 min with antibody-containing

culture supernatant and diluted in PBS–BSA. After washing in PBS, sections were incubated with rabbit antimouse immunoglobulin peroxidase (Dakopatts, Copenhagen, Denmark) in PBS–BSA containing 3% normal rat serum for 30 min. After washing with PBS, the slides were stained for peroxidase activity with 3,3′-diaminobenzidine–tetrahydrochloride (Sigma). Control sections were incubated in the same way using an isotype-matched irrelevant control mouse antibody. Sections were counterstained with Mayer's haematoxylin.

RESULTS

CsA and Rapa differentially affect thymocyte maturation

The effects of CsA and Rapa on thymocyte development in rats have been extensively examined [8, 13]. In the experimental design of the present study, we first confirmed the effects of both immunosuppressive reagents on overall thymic weight and on the described maturation arrest. Indeed, when comparing the thymic weight of CsA- and Rapa-treated rats with vehicle-treated control rats, it appeared that CsA did not affect the thymic weight at all. On the other hand, Rapa treatment resulted in a 60% reduction in thymic weight. This degree of reduction was reached after 7 days of treatment (Fig. 1A), even with the lowest concentration of Rapa (2.5 mg/kg – data not shown). Similar results were obtained when the thymi were analysed by thymic cellularity (data not shown).

With respect to thymocyte maturation, as determined by the expression of TCRαβ, it was confirmed that CsA and Rapa induce a maturation arrest at different stages of thymocyte development. Upon treatment with CsA, only a relative and absolute decrease was observed in the thymocyte subset which already had upregulated TCRαβ expression (Figs 1D and 2). Therefore, it can be concluded that CsA blocks the maturation of TCRαβ^{int} (intermediate-positive) to TCRαβ^{high} (high-positive) thymocytes. Although upon treatment with Rapa almost a threefold relative increase was observed in the early maturational stage that does not yet express TCRαβ, the absolute number of this thymocyte subset was not affected. Both TCRαβ-expressing thymocyte subsets (TCRαβ^{int} and TCRαβ^{high}) were strongly reduced in terms of absolute (Fig. 1C,D) but not relative number (Fig. 2), indicating that Rapa induces a maturation arrest at the TCRαβ^{neg} thymocyte stage.

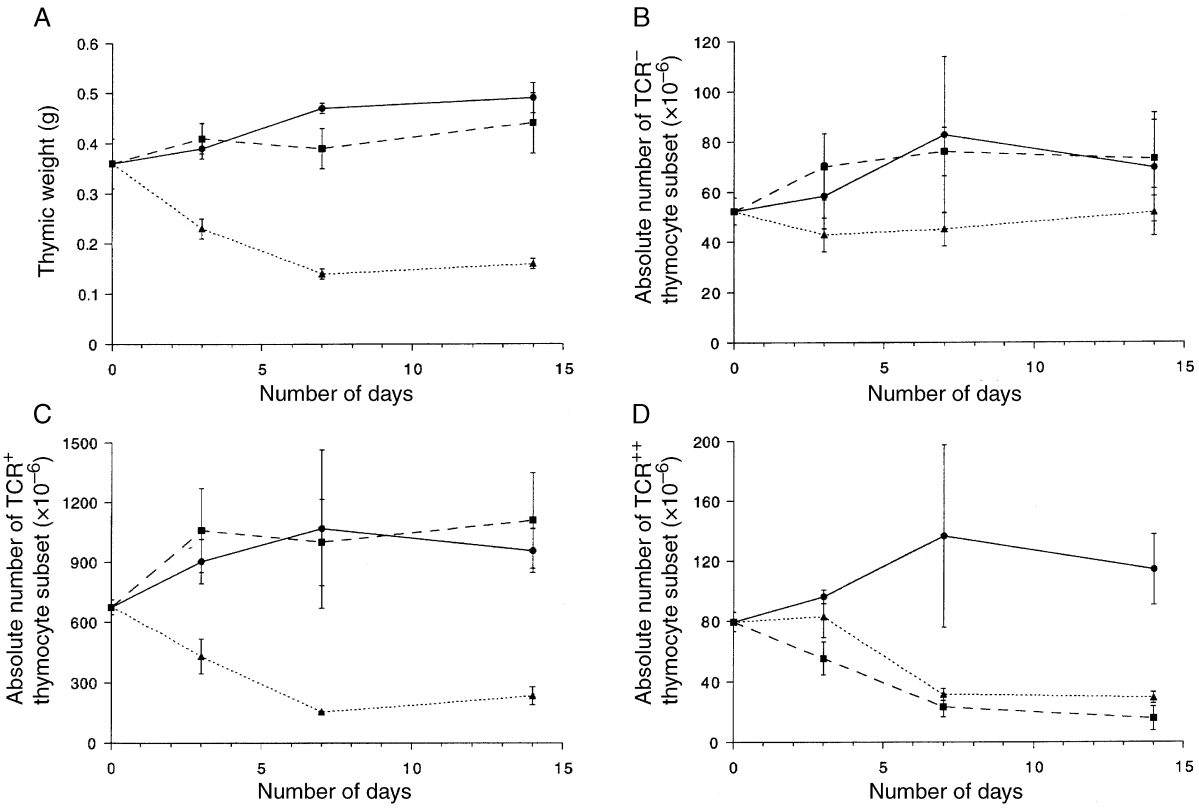

Fig. 1. Rapamycin (Rapa) and cyclosporin-A (CsA) inhibit thymocyte development at distinct maturational stages. *In vivo* administration of Rapa, but not CsA, strongly reduces thymic weight (A). Thymocyte maturation as determined by the expression of T-cell receptor (TCR)αβ enables the distinction of three consecutive maturational stages (negative, intermediate-positive and high-positive) – TCRαβ^{neg} (B), TCRαβ^{int} (C) and TCRαβ^{high} (D). The thymic weight and the phenotype were examined after 0, 3, 7 and 14 days of daily treatment of rats with control vehicle (—●—), 10 mg/kg of CsA (– –■– –) or 5 mg/kg of Rapa (···▲···). The results are represented as the mean of the thymic weight or of the absolute number of the respective thymocyte subset (±standard deviation (SD)) of three rats for each time-point and treatment.

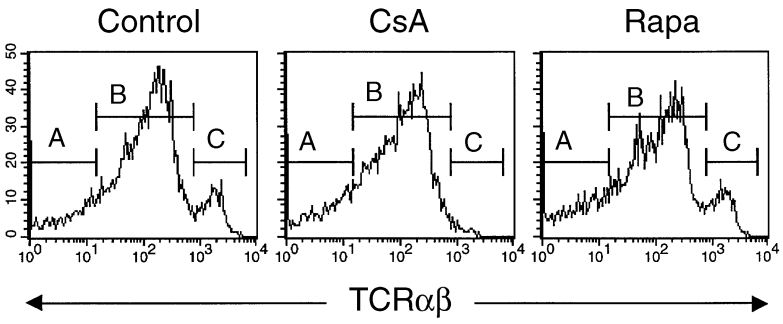

Fig. 2. Distinction of thymocyte subsets, based on the expression level of T-cell receptor (TCR)αβ. Flow cytometric histograms represent the TCRαβ expression level of thymocytes from day 14 control rats (left panel), day 14 cyclosporin-A (CsA)-treated rats (middle panel) and day 14 Rapamycin (Rapa)-treated rats (right panel). Marker A indicates the TCRαβ^{neg} (negative) population, marker B the TCRαβ^{int} (intermediate-positive) population and marker C the TCRαβ^{high} (high-positive) population. The results show that there is a relative decrease in the TCRαβ^{high} thymocyte population upon CsA administration but not upon Rapa.

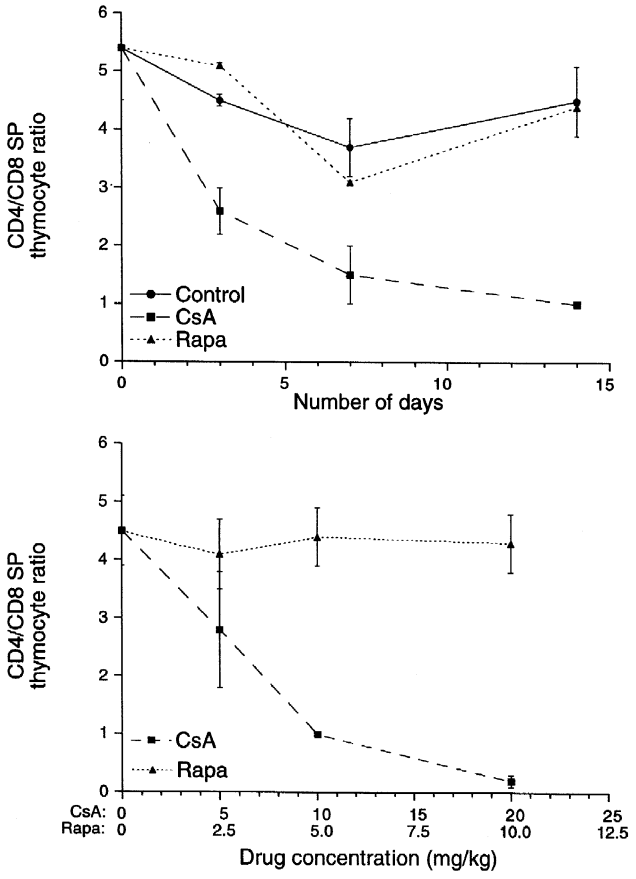

Fig. 3. *In vivo* administration of cyclosporin-A (CsA), but not Rapamycin (Rapa), differentially affects the development of CD4 and CD8 single-positive (SP), mature thymocytes. The number of SP, mature thymocytes is determined by tricolour flow cytometry. The upper panel represents the CD4/CD8 SP thymocyte ratio after 0, 3, 7 and 14 days of daily treatment of rats with control vehicle (—●—), 10 mg/kg of CsA (– –■– –) or 5 mg/kg of Rapa (···▲···). The lower panel represents the CD4/CD8 SP thymocyte ratio after 14 days of daily treatment of rats with 0, 5, 10 and 20 mg/kg of CsA (– –■– –) or 0, 2.5, 5 and 10 mg/kg of Rapa (···▲···). The results are represented as the mean of the CD4/CD8 SP, mature thymocyte ratio (±standard deviation (SD)) of three rats for each time-point and treatment.

Finally, CsA, but not Rapa, differentially affected the development of CD4 SP and CD8 SP mature thymocytes, as illustrated by the analysis of the CD4/CD8 SP TCRαβ^{high} thymocyte ratio (Fig. 3). A time and concentration-dependent decline in this ratio was observed owing to a more severe reduction in the development of CD4 SP thymocytes.

Effect of CsA and Rapa on thymocyte apoptosis

In order to test our hypothesis that CsA reduces the TCR–CD3 complex-mediated signal transduction, i.e. increases the threshold for both positive and negative selection, we have examined the effect of CsA and Rapa on thymocyte apoptosis. We have first examined the effect of CsA and Rapa on apoptosis in an *ex vivo* system. Using annexin V–FITC and PI in two-colour flow cytometry, apoptotic cells were single-positive for annexin V because apoptotic cells characteristically have lost membrane asymmetry but not membrane integrity. Neither Rapa nor CsA had any effect on the frequency of apoptotic cells in the *ex vivo*-prepared thymocyte suspensions. This was independent of the period of drug administration or drug concentration. Thus, even after 3 days and low dose of CsA (5 mg/kg), no increased apoptosis was detectable, although under these conditions the effects of CsA on thymocyte subsets were far from complete (Figs 1 and 3). In all instances, the amount of apoptotic cells was ±5% (Fig. 4).

However, the *ex vivo* presence of apoptotic cells may not be representative of apoptosis frequency *in vivo*. Detection of an altered frequency of apoptosis *in vivo* may be obscured by the efficient removal of apoptotic cells by the mononuclear phagocyte system. Indeed, immunohistological analysis of thymic macrophages revealed that CsA, but not Rapa, administration resulted in a two- to threefold increased density, as well as in an enlarged and more rounded morphology of cortical macrophages (Fig. 5A–C). Therefore, we have further analysed the presence of apoptotic cells *in situ* using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) assay. Our results

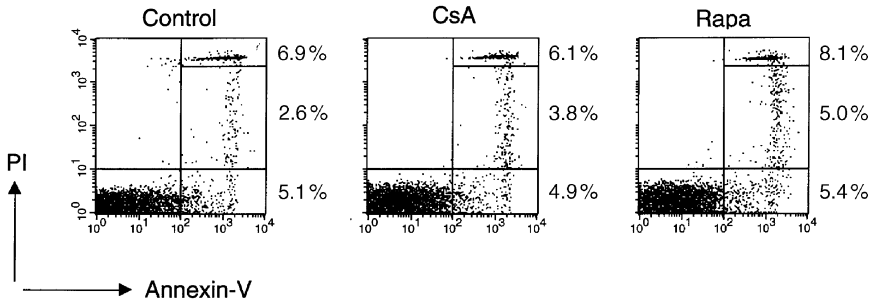

Fig. 4. Effect of *in vivo* administration of cyclosporin-A (CsA) or Rapamycin (Rapa) on apoptosis detection in thymocyte suspensions. Two-colour flow cytometry dot plots represent thymocyte suspensions prepared from animals treated for 14 days with control vehicle (left panel), 10 mg/kg of CsA (middle panel) or 5 mg/kg of Rapa (right panel). Cells were analysed for loss of membrane asymmetry (annexin-V) and loss of membrane integrity (propidium iodide, PI). The lower right regions represent the apoptotic cells. Similar results are obtained for shorter periods of drug administration and other drug concentrations.

show that in control rats, the $ED2^+$ macrophages were mainly located in the cortex and that the majority of them contained only a few apoptotic cells each. Very few apoptotic cells were observed not to be macrophage-associated in the cortex. Complexes of $ED2^+$ macrophages and apoptotic cells were also observed at the cortico-medullary junction. Upon administration of CsA, the number of apoptotic thymocytes was strongly increased in the cortex, but nearly all apoptotic cells remained macrophage-associated (Fig. 5D,E). Not only the number of cortical macrophages was increased but also the number of apoptotic cells contained by each $ED2^+$ macrophage was increased. The amount of macrophages and apoptotic cells at the cortico-medullary junction was decreased from day 7 onwards (data not shown). On day 14, the medulla had almost completely disappeared, making the evaluation of apoptosis at the cortico-medullary junction impossible.

The strong thymic atrophy upon Rapa administration may be a result of either inhibition of proliferation or increased apoptosis. As the most important phase in thymocyte expansion is at the early maturational stages (i.e. the $TCR\alpha\beta^{neg}$ stages), it was anticipated that the strong thymic atrophy upon *in vivo* Rapa administration was the result of defective thymocyte proliferation. Cell cycle analysis of the total thymocyte population by flow cytometry revealed no differences in the relative number of thymocytes in G_0/G_1, S or G_2/M phase between Rapa- and vehicle-treated animals (Table 1). However, taking into account that thymocyte proliferation occurs predominantly in the $TCR\alpha\beta^{neg}$ stages and that there was a threefold relative increase in this subset upon Rapa treatment, one may expect that indeed Rapa inhibits thymocyte proliferation and thereby causes the observed severe thymic atrophy. In agreement with this observation, we did not observe an increase in apoptosis *in situ*. However, treatment with Rapa resulted in a strong decrease in the amount of cortical macrophages up to day 7, and the remaining macrophages hardly contained any apoptotic cells – upon day 14, the $ED2^+$ macrophages had completely disappeared. Already from day 3 onwards, clusters of apoptotic cells, which were not engulfed by macrophages, were found next to blood vessels at the cortico-medullary junction (Fig. 5F).

Table 1. The effect of *in vivo* cyclosporin-A (CsA) or Rapamycin (Rapa) administration on thymocyte proliferation*

	Control	CsA†	Rapa†
G_0/G_1	91.7% (0.5)	91.7% (0.9)	90.0% (1.6)
S	7.1% (0.6)	7.7% (0.9)	9.0% (1.6)
G_2/M	1.2% (0.2)	0.7% (0.1)	1.3% (0.3)
TCR^-	6.3% (0.9)	6.1% (0.2)	16.2% (0.7)

*Results are presented as the mean percentage (±standard deviation (SD)) of three animals per group and treatment.
†Rats were daily treated with 10 mg/kg of CsA or 5 mg/kg of Rapa for 14 consecutive days.

DISCUSSION

It is well established that CsA exerts multiple effects on the thymus. CsA inhibits the development of mature SP thymocytes, and this maturation arrest is associated with a relative involution of the thymic medulla [8, 10]. Furthermore, in mouse, CsA interferes with both positive selection as well as the deletion of cells bearing self-reactive TCRs [5–7]. We recently hypothesized that the dominant effect of CsA on the thymus is the reduction of the TCR–CD3 complex-mediated signal transduction in thymocytes upon interaction with stromal cells. As a consequence, more cortical thymocytes are expected to undergo apoptosis instead of survival by positive selection, whereas more self-reactive medullary thymocytes will survive negative selection instead of undergoing apoptosis [11]. Although it is evident that potentially all antigen-presenting cells in the thymus can trigger positive or negative

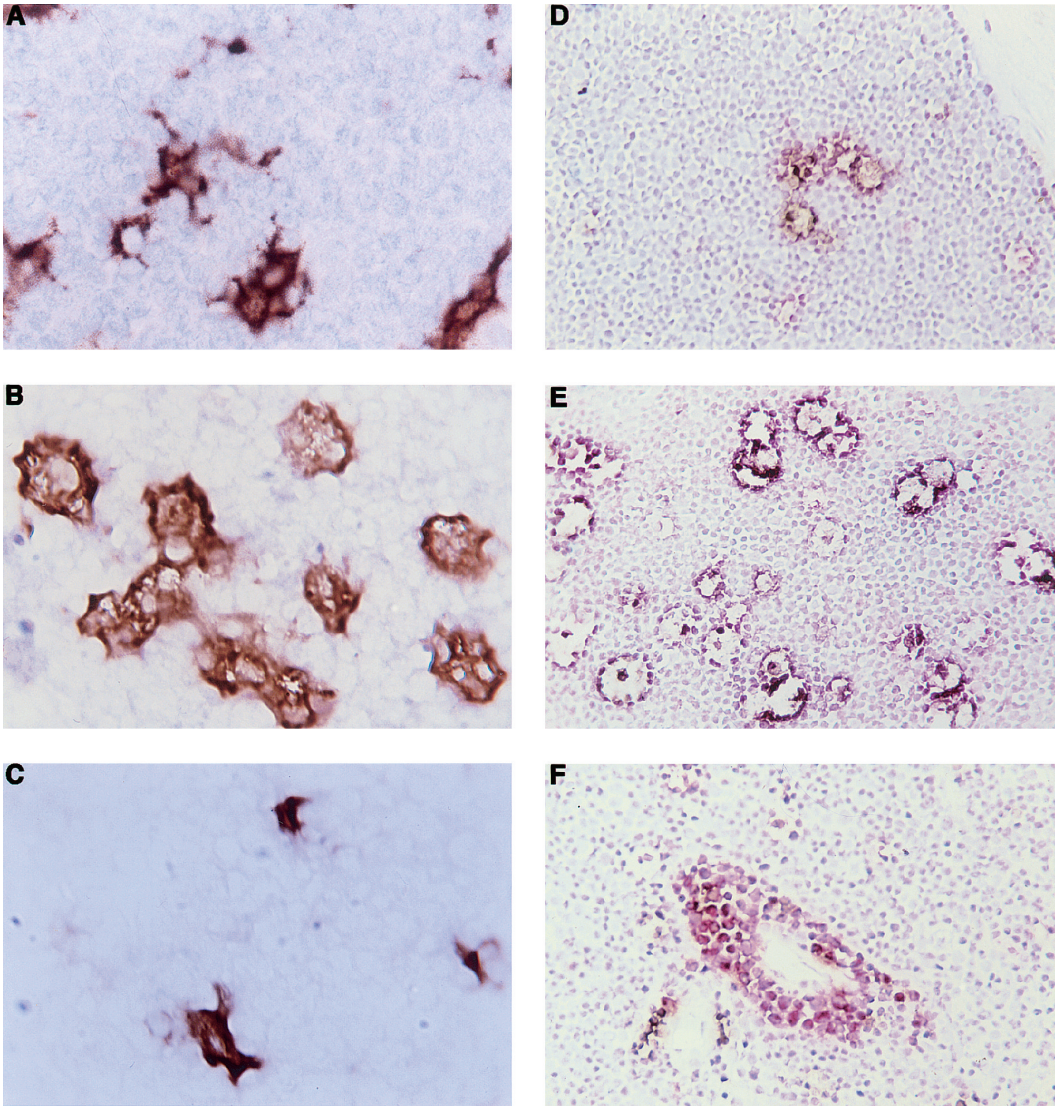

Fig. 5. *In situ* detection of macrophages and apoptotic cells in the thymus of rats treated with cyclosporin-A (CsA) or Rapamycin (Rapa). Thymus sections of rats treated for 14 days with control vehicle (A, D), 10 mg/kg of CsA (B, E) or 5 mg/kg of Rapa (C, F) were immunohistochemically stained by a two-step immunoperoxidase method with the monoclonal antibody ED2, specific for cortical macrophages (A–C). Note the increase in macrophage number and the enlarged, more rounded morphology of the macrophages upon CsA treatment – magnification 400×. Cryostat sections of thymus were additionally stained with the TUNEL method (D–F). E shows the strong increase in apoptotic cells in the cortex upon *in vivo* CsA administration. Note that nearly all apoptotic cells are engulfed by the cortical macrophages. F shows the typical enumeration of apoptotic cells around blood vessels as observed after treatment with Rapa – magnification 160×.

selection if a certain level of avidity is reached [17–19], there is consensus that the majority of thymocytes undergo positive selection in the cortex and negative selection in the cortico-medullary zone or medulla. Therefore, the results obtained in our present study support our hypothesis as outlined above, because the number of apoptotic cells in the cortex is strongly increased, whereas in the cortico-medullary zone, the reversed situation was observed upon *in vivo* CsA administration. The increased apoptosis in the cortex can explain the observed maturation arrest as well as the medullary involution. The consequence of the decreased apoptosis in the cortico-medullary zone is the thymic output of autoreactive T cells that eventually can cause autoimmune disease [14]. The fact that the overall increase in apoptosis was not detectable in thymocyte cell suspensions is explained by the extremely efficient removal of apoptotic cells by thymic macrophages. Indeed, as described before [20], the cortical macrophages exhibited an enlarged and rounded morphology, and furthermore, nearly all apoptotic cells were situated inside macrophages. The concomitant two- to threefold increase in the number of cortical macrophages emphasizes that cross-talk between thymocytes and their

microenvironment is not restricted to the thymic medulla [21] but also occurs in the cortex [22].

The effects of Rapa on the thymus are less well understood. Most prominent is the strong thymic involution accompanied by the relative increase in the early precursor stages. The thymic atrophy caused by Rapa can be a result of enhanced apoptosis or of decreased proliferation of thymocytes. As shown in the present study and by others [12, 23], Rapa does not exhibit substantial apoptosis-augmenting activity. However, a new finding of our study is that the removal of apoptotic cells seems to be somehow deficient, owing to the partial disappearance of the cortical macrophages – small clusters of apoptotic cells enumerated around blood vessels. Whether endothelial cells are a back-up system for the removal of apoptotic cells remains to be determined. Anyway, the thymic atrophy is not a result of increased apoptosis, and as based on the mechanism of action of Rapa, an effect on thymocyte proliferation is more likely. Rapa is known to upregulate $p27^{Kip1}$ expression, and this results in G_1 arrest in exponentially growing T cells [24]. Recently, it has been established that the downregulation of $p27^{Kip1}$ expression is required for the development and function of T cells [25]. The cyclin-dependent kinase inhibitor $p27^{Kip1}$ is abundant throughout development in the cells of T-cell lineage, with the exception of late stage $CD4^-CD8^-$ thymocytes and activated mature T cells, both of which show a high rate of proliferation. In contrast to Luo *et al*. [12], we did not observe any effect on thymocyte cycling in total thymocyte suspensions, possibly owing to relative changes between thymocyte subsets. Especially, the two- to threefold increase in the early maturational stages (i.e. the $TCR\alpha\beta^{neg}$ stages), which are known to undergo the major phase of thymocyte expansion [26], may have obscured the expected effect of Rapa on the cell cycle. Although Rapa, like CsA, is able to induce an autoimmune-like syndrome in mice and rats [13, 27], it remains a matter of debate whether Rapa interferes with clonal deletion in the thymus [12, 27]. However, it is most likely that the Rapa-induced syndrome is owing to effects on mature T cells and peripheral tolerance, as the Rapa syndrome in mice does not require an intact thymus, and the disease cannot be adoptively transferred [28]. This is in agreement with our finding in the present study, showing a maturation arrest before TCR rearrangement and thus before selection taking place. The advent of new immunosuppressive reagents may further unravel the effect of immunosuppression on central and peripheral tolerance. In this respect, the novel immunosuppressant Sanglifehrin seems to be interesting, as it binds to cyclophilin, although it is structurally distinct from CsA, but does not affect calcium-dependent interleukin-2 (IL-2) production. Furthermore, Sanglifehrin blocks T-cell proliferation induced by IL-2 in G_1, with no appreciable effect on IL-2 receptor expression, in a manner similar to that of Rapa [29].

Taken together, CsA and Rapa are potent immunosuppressants, with established clinical applications in transplantation and autoimmune disease, but also interfere with intrathymic T-cell development. CsA disrupts a signalling pathway that couples T-cell antigen receptor stimulation to the transcription of several cytokine genes and thereby interferes with positive and negative selection in the thymus. The present study gives further support to the hypothesis that the multiple effects of CsA on the thymus, like the maturation arrest, medullary involution, changes in macrophage morphology and distribution, are secondary to the CsA-induced reduction in TCR–CD3 complex-mediated signal transduction in thymocytes. As shown in the present study, the inhibition of both positive and negative selection can be visualized by the detection of apoptosis *in situ* and reveals increased apoptosis in the cortex and decreased apoptosis in the cortico-medullary junction. Rapa inhibits biochemical events required for the progression of cytokine-stimulated T cells from G_1 to S phase of the cell cycle and thereby causes a maturation arrest even before rearrangement of TCR occurs. The Rapa studies revealed an interesting point: the disappearance of the dominant cell type involved in the clearance of apoptotic cells, i.e. the cortical macrophage, unmasks endothelial cells as a potential back-up system for the removal of apoptotic cells.

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