Arch. Immunol. Ther. Exp. (2016) 64:409–416
DOI 10.1007/s00005-016-0388-8

ORIGINAL ARTICLE

Suppressor Properties of Human $CD8^+CD28^-$ T Cells in Mixed Leukocyte Reaction are not Affected by CsA and RAPA

Anna Korecka-Polak[1] · Katarzyna Bocian[1] · Maria Pachówka[1] · Agnieszka Jałbrzykowska[2] · Grażyna Korczak-Kowalska[1,2]

Received: 18 August 2015 / Accepted: 11 January 2016 / Published online: 26 February 2016

Abstract Human $CD8^+CD28^-$ T suppressor cells were previously shown to be involved in the control of the immune response to transplanted allografts. It seems essential to examine how immunosuppressive drugs influence these cells. However, the $CD8^+CD28^-$ population contains both suppressor (Ts) and cytotoxic (Tc) T cells, and the phenotype of the Ts subpopulation has not been identified explicitly. It is proposed that the transcription factor FOXP3 may be helpful in distinguishing the Ts and Tc subpopulations. The aim of this study was to evaluate the influence of the immunosuppressive drugs cyclosporine A (CsA) and rapamycin (RAPA) on the level, suppressor properties, and phenotype of human $CD8^+CD28^-$ T cells in vitro. The model used was the mixed leukocyte reaction performed with peripheral blood mononuclear cells from healthy volunteers. It was observed that $CD8^+CD28^-$ T cells from cultures with CsA or RAPA had similar suppressor properties to cells from control cultures, although the drugs influenced the expression of FOXP3. CsA and RAPA did not interfere with the suppressor properties of human $CD8^+CD28^-$ T cells in vitro, although they affected the expression of the FOXP3 molecule.

Keywords Cyclosporine A · $CD8^+CD28^-$ · FOXP3 · Rapamycin · Suppressor T cells

✉ Grażyna Korczak-Kowalska
gkorczak-k@biol.uw.edu.pl

[1] Department of Immunology, Faculty of Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland

[2] Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland

Introduction

Patients with organ allograft require lifelong immunosuppressive therapy to inhibit the host's immune response to alloantigens. Among immune cells are not only cells responsible for graft rejection, but also regulatory cells that potentially play essential roles in inducing and maintaining transplant tolerance. Human $CD8^+CD28^-$ suppressor cells were previously shown to be involved in the control of the immune response to transplanted allografts (Colovai et al. 2003; Strioga et al. 2011). The percentage of $CD8^+CD28^-$ cells in the peripheral blood of renal allograft recipients is probably associated with graft function (Assadiasl et al. 2014; Baeten et al. 2006). It seems essential to examine how immunosuppressive therapy influences $CD8^+CD28^-$ suppressor cells. The commonly used immunosuppressive drugs, such as cyclosporine A (CsA) and rapamycin (RAPA), have different mechanisms of action, and therefore their effect on the level and function of suppressor cells can differ. CsA belongs to calcineurin inhibitors and it is responsible for blocking the T cell receptor signal which prevents activation of T lymphocytes. This prevents the action of the main nuclear factor of activated T lymphocytes (Almawi and Melemedjian 2000). Rapamycin in cytosol is bound by the FKBP protein and such a complex prevents mTOR kinase. As a result, the cell cycle and proliferation of T and B lymphocytes are blocked (Abraham 1998).

The main drawback of in vivo studies is the lack of a possibility to investigate the effect of a separate drug because transplant recipients receive CsA or RAPA combined with anti-inflammatory drugs. Therefore in vivo studies should be supplemented by in vitro studies, especially as it was shown that $CD8^+CD28^-$ Ts can be generated in vitro (Allez et al. 2002; Arosa et al. 1998;

Ciubotariu et al. 1998; Colovai et al. 2001; Liu et al. 1998; Suciu-Foca et al. 2005).

Unfortunately, evaluation of the effect of immunosuppressive drugs at the level of the whole $CD8^+CD28^-$ population cannot provide unequivocal results. $CD8^+$-$CD28^-$ cells are a heterogeneous population (Arosa 2002; Colovai et al. 2001). It contains not only suppressor cells (Ts) but also cells that may mediate cytotoxicity (Tc). $CD8^+CD28^-$ Ts cells suppress the activation and proliferation of alloreactive $CD4^+$ lymphocytes (Colovai et al. 2001; Cortesini et al. 2001; Liu et al. 1998; Suciu-Foca et al. 2003), but $CD8^+CD28^-$ Tc cells destroy allograft cells (Zavazava and Kabelitz 2000). Thus there is a need to identify the phenotype of the Ts subset of $CD8^+CD28^-$ cells. A few studies have demonstrated the presence of FOXP3 mRNA in $CD8^+CD28^-$ cells with suppressive properties and revealed that the Ts subset is $FOXP3^+$ (Suciu-Foca et al. 2005; Vlad et al. 2011). Considering the fact that the transcription factor FOXP3 acts as a protein (Hu et al. 2007; Rudensky et al. 2006; Schubert et al. 2001), both forms of this marker should be examined to confirm that hypothesis.

In another study, we evaluated whether there is any correlation between the expression of FOXP3 in the $CD8^+CD28^-$ population and renal allograft function or type of immunosuppression (Korecka-Polak et al. 2011). The present study refers to results obtained in vitro from the mixed leukocyte reaction (MLR). The MLR is a model used to mimic the situation after organ transplantation when passenger leukocytes from the donor, received with the grafted organ, meet and interact with the recipient's leukocytes.

The aim of this study was to evaluate the influence of the immunosuppressive drugs CsA and RAPA on the level, suppressor properties, and phenotype of human $CD8^+$-$CD28^-$ cells in terms of the FOXP3 marker in the MLR.

Materials and Methods

Allogenic MLR

Peripheral blood samples were obtained from healthy volunteers aged 18–60 years. Peripheral blood mononuclear cells (PBMCs) were separated from their buffy coats by Ficoll-Hypaque centrifugation. PBMCs (1×10^6 cells/ml) were stimulated in 96-well plates: with allogenic PBMCs (1×10^6 cells/ml) to perform two-way MLR or with allogenic PBMCs (0.5×10^6 cells/ml) previously blocked by mitomycin (50 ng/10^6 cells/ml; Sigma, USA) and washed, to perform one-way MLR. The cells were co-cultured for 6 days in complete medium (RPMI 1640) with 10 % fetal bovine serum, 1 % HEPES, 100 mM pyruvate, 50 mM 2-mercaptoethanol, 50 mg/ml gentamycin (all Gibco, USA), and 20 mM L-glutamine (Sigma, USA) in the presence or absence of CsA (200 ng/ml/10^6 cells; Sandimmum, Novartis, Switzerland) or RAPA (20 ng/ml/10^6 cells; Rapamune, Wyeth, USA).

Cell Phenotyping

The phenotypes of the cells were examined by standard flow cytometric procedures, usingFACSCalibur (Becton–Dickinson, USA). This involved immunofluorescence staining of cell samples using different combinations of FITC-, PerCP-, PE-, and APC-conjugated mouse anti-human antibodies anti-CD8 (Pharmingen, USA), anti-CD28 (Becton–Dickinson, USA), CD25 (Pharmingen, USA), CD69 (Pharmingen, USA), and a PE anti-human FOXP3 Kit (eBioscence, USA). The all of T cells (activated and resting) were distinguished by FSC/SSC parameters based on our previous studies that confirmed the presence of activation markers (CD25 and CD69) on large granular cells. In these population we evaluated the $CD8^+CD28^-CD69^+$ cells.

Isolation of $CD8^+CD28^-$ Cells

After 6 days, cells from the one-way allogenic MLR were collected and washed, and $CD8^+$ cells were isolated from the PBMCs by negative selection using a Magnetic Particle Concentrator and CD8-Negative Isolation Kit (Dynal, USA) according to the manufacturer's protocol. To separate $CD28^-$ cells from the $CD8^+$ cell suspensions, the isolated $CD8^+$ cells (1×10^7 cells/ml) were incubated with goat anti-mouse beads (Dynal, USA) previously coupled with anti-CD28 monoclonal antibody (Becton–Dickinson, USA); then isolation was performed according to the manufacturer's protocol. Flow cytometric analysis showed that the purity of the $CD8^+CD28^-$ cell suspensions was >85 %. These cells were used to evaluate their suppressor properties or to isolate total mRNA.

Suppressor Properties of $CD8^+CD28^-$ Cells

Freshly isolated PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace CFSE Cell Proliferation Kit; Molecular Probes/Invitrogen, USA) according to the manufacturer's protocol with one exception: CFSE from stock solution was added to a final concentration of 1 μM per 1×10^7 cells/ml. Than the secondary MLR was performed with the CFSE-labeled PBMCs, freshly isolated non-staining allogenic PBMCs, and $CD8^+CD28^-$ cells isolated from the primary one-way MLR. After 4 days the cells were collected, washed, stained for CD4 according to the standard procedure, and the proliferation of the $CD4^+$ cells was determined.

The scheme of cultures was: (a) primary culture: donor A PBMCs (responder cells) plus donor B PBMCs (blocked, stimulator cells), ratio 2:1, and (b) secondary culture: donor A PBMCs (CFSE-labeled, responder cells) plus donor B PBMCs (stimulator cells) plus $CD8^+CD28^-$ cells (isolated from the primary culture), ratio 2:1:0.5.

Suppressive action of T lymphocytes $CD8^+CD28^-$ was evaluated as the ability of these cells to inhibit the proliferation of T $CD4^+$ lymphocytes in MLR. On the last day of culture, viability of lymphocytes was determined using trypan blue.

Total RNA Isolation

Total RNA was isolated from 1 to 2×10^6 $CD8^+CD28^-$ cells using an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol (RNeasy Mini Handbook, Fourth Edition, April 2006). mRNA was kept at −80 °C.

Reverse Transcription

Two μg of RNA, 1 μl of 10 mM dNTP, and 1 μl of 50 ng/μl random hexamers (Invitrogen/USA Life Technologies) was used for the synthesis of complementary strand DNA. The suspension was brought to a total volume of 10 μl with DEPC-treated water. Ten μl of reaction mixture, which consisted of 2 μl 10 × RT buffer, 2 μl of 0.1 mM DTT, 4 μl of 25 mM $MgCl_2$, 1 μl of RNaseOUT (40 U/μl), and 1 μl of SuperScript III RT (200 U/μl), was then added to each tube. The conditions for incubating the tubes in the heating block were 10 min at 25 °C, 50 min at 50 °C, and 5 min at 85 °C. For the last 20 min, 1 μl of *E. coli* RNase H (2 U/μl) was added to each tube and the tubes were incubated at 37 °C.

Real-Time Polymerase Chain Reaction

The single PCR mixture consisted of 10 μl of TaqMan Universal PCR Master Mix, 9 μl of template cDNA, and 1 μl of TaqMan Gene Expression Assays for FOXP3 (study gene) or GAPDH (control gene). PCR mixtures were then placed in a 96-well Optical Reaction Plate with Barcode (Applied Biosystems, USA). The reaction was carried out in an ABI PRISM 7700 Sequence Detection System. The thermocycling conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 15 s at 95 °C, and 1 min at 60 °C. The gene expression level for FOXP3 was defined by the formula: $e = 2^{-\Delta\Delta Ct}$. GAPDH was used as the housekeeping gene.

Experiments were performed in the Department of Immunology, Transplant Medicine and Internal Diseases, Transplantation Institute, Medical University of Warsaw.

Statistical Analysis

Statistical analysis was performed by Wilcoxon's matched pairs test and Spearman's rank order correlations. Value of $p < 0.05$ was regarded as significant.

Results

Influence of CsA and RAPA on the Level of Human $CD8^+CD28^-$ Cells in MLR

We observed strong individual differences in the change in percentage of $CD8^+CD28^-$ cells under the influence of CsA and RAPA. In some cultures with the drugs, the level of $CD8^+CD28^-$ cells decreased significantly, but there was no decline in other cultures or even an increase in the percentage of $CD8^+CD28^-$ cells (Fig. 1a). The investigated drugs reduced the level of activated $CD8^+CD28^-$ cells in all cultures, with an average of 26 % in the control, 5 % in cultures with RAPA and 6 % in cultures with CsA (Fig. 1b), and had no significant effect on resting $CD8^+CD28^-$ cells (data not shown). In cultures with a higher level of activated cells we observed a significant decrease in the percentage of $CD8^+CD28^-$, whereas in cultures with a lower level of activated cells there were three possible situations: a decrease, no change, or an increase in the percentage of $CD8^+CD28^-$ cells (Fig. 1c).

The influence of the drugs on the percentage of $CD8^+CD28^-$ cells was also connected with the presence of human leukocyte antigen (HLA) class I mismatches between the donors of the PBMCs. In control cultures without HLA class I mismatches, the percentage of $CD8^+CD28^-$ cells was higher (64 %) than in cultures with one common HLA class I (47 %) (Fig. 1d) due to the higher level of activated lymphocytes (data not shown), and the drugs reduced the percentage of $CD8^+CD28^-$ cells (Fig. 1d). In Fig. 1d, the horizontal line marks the percentage of $CD8^+CD28^-$ cells in the population of lymphocytes T $CD8^+$ on day 0 of the culture.

Influence of CsA and RAPA on Suppressor Properties of $CD8^+CD28^-$ Cells in MLR

We observed that $CD8^+CD28^-$ cells derived from the primary culture reduced the proliferation of alloreactive $CD4^+$ lymphocytes in the secondary culture (Fig. 2a). The presence of CsA or RAPA in the primary culture did not affect the suppressor properties of $CD8^+CD28^-$ cells shown in the secondary culture.

The viability of cells was >90 % in all variants of cultures.

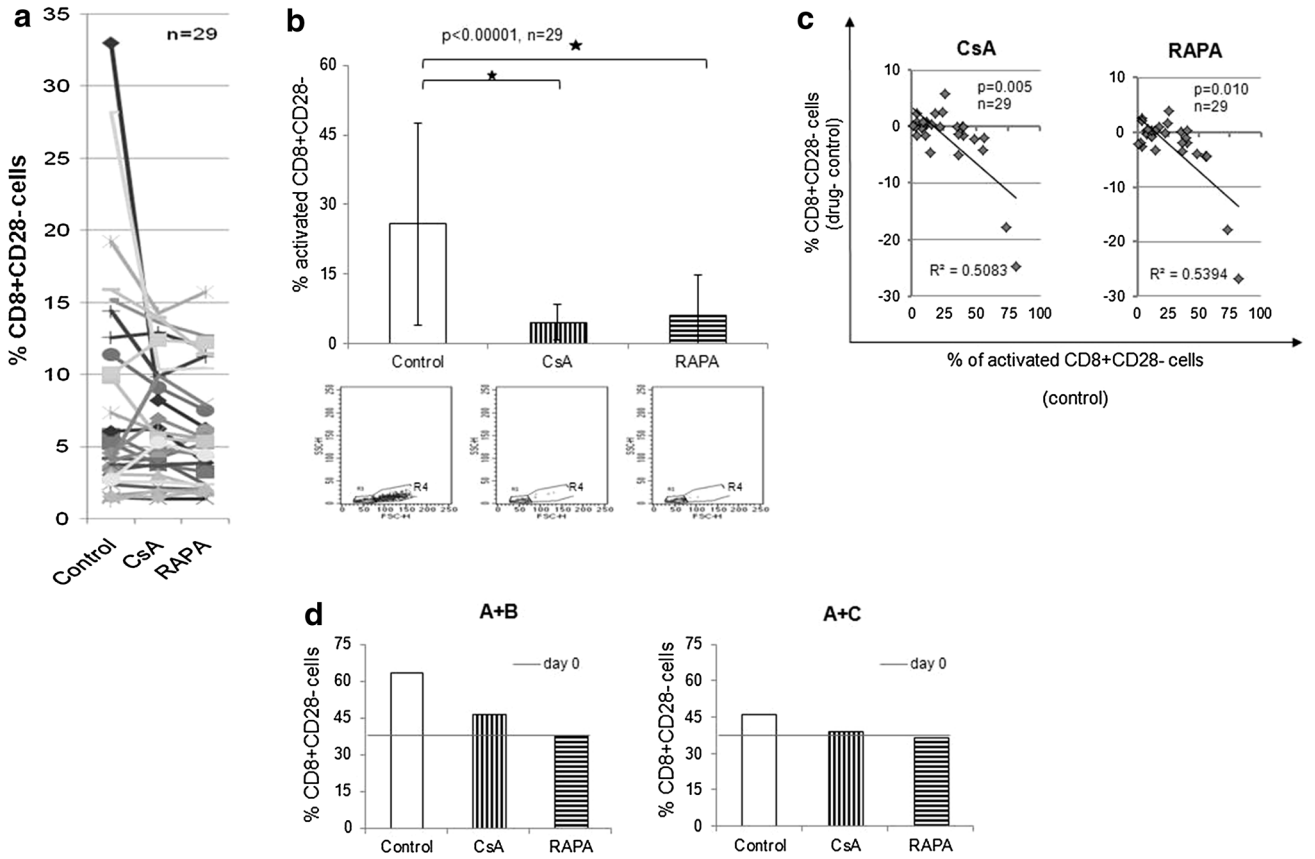

Fig. 1 The effects of CsA and RAPA on the percentage of $CD8^+CD28^-$ cells generated in MLR cultures. The influence of the drugs on the percentage of $CD8^+CD28^-$ cells (**a**); the influence of the drugs on the percentage of activated cells in the $CD8^+CD28^-$ population, Wilcoxon matched pairs test: CsA $p = 0.000007$, RAPA $p = 0.000003$. The *dot-plots* contains a representative experiment (**b**); the correlation between the activation of $CD8^+CD28^-$ cells and the influence of the drugs on the percentage of $CD8^+CD28^-$ cells, Spearman's rank order correlation (**c**); the influence of CsA and RAPA on the percentage of $CD8^+CD28^-$ cells in cultures with (A + C) or without (A + B) HLA I mismatches, HLA− donor A: A1, 26; B37, 56; DR 1, 16(2); HLA- donor B: A24, 32; B 62(15), 61(40); DR 11(5), 11(36); HLA-donor C: A1, 25; B 13, 18; DR 7, 15(2) (d). $p < 0.00001$ compared with the control

Comparison of FOXP3 mRNA and Protein Levels in Human $CD8^+CD28^-$ Cells in MLR with CsA and RAPA

$CD8^+CD28^-$ cells from cultures with RAPA and cells from control cultures had similar amounts of FOXP3 mRNA, but in the presence of RAPA there was a smaller percentage of cells with FOXP3 protein in the $CD8^+CD28^-$ population. The level of FOXP3 mRNA in $CD8^+CD28^-$ cells from cultures with CsA was half that of the cells from control cultures, and no FOXP3 protein was detected (Fig. 2b). The percentages of $CD8^+CD28^-$ cells with FOXP3 protein differed between individual cultures, but this did not correlate with the level of FOXP3 mRNA. Most of the $CD8^{+-}$ $CD28^-$ cells with FOXP3 protein were activated (data not shown).

Discussion

The influence of CsA and RAPA on the level of $CD8^{+-}$ $CD28^-$ cells seems to be dependent on the percentage of activated $CD8^+CD28^-$ cells. The influence of the drugs on the percentage of $CD8^+CD28^-$ cells is probably also connected with HLA class I mismatches in MLR. $CD8^{+-}$ $CD28^-$ cells recognize antigens in the context of MHC class I (Hohn et al. 2003), so the presence or lack of HLA class I mismatches in MLR may be essential for the level of activated lymphocytes and therefore the influence of CsA and RAPA on the percentage of $CD8^+CD28^-$ lymphocytes. This should be taken into consideration in studies evaluating the effect of immunosuppressive drugs on the level of $CD8^+CD28^-$ cells in vitro.

$CD8^+CD28^-$ cells from the cultures with drugs had regulatory properties similar to the cells from control cul-

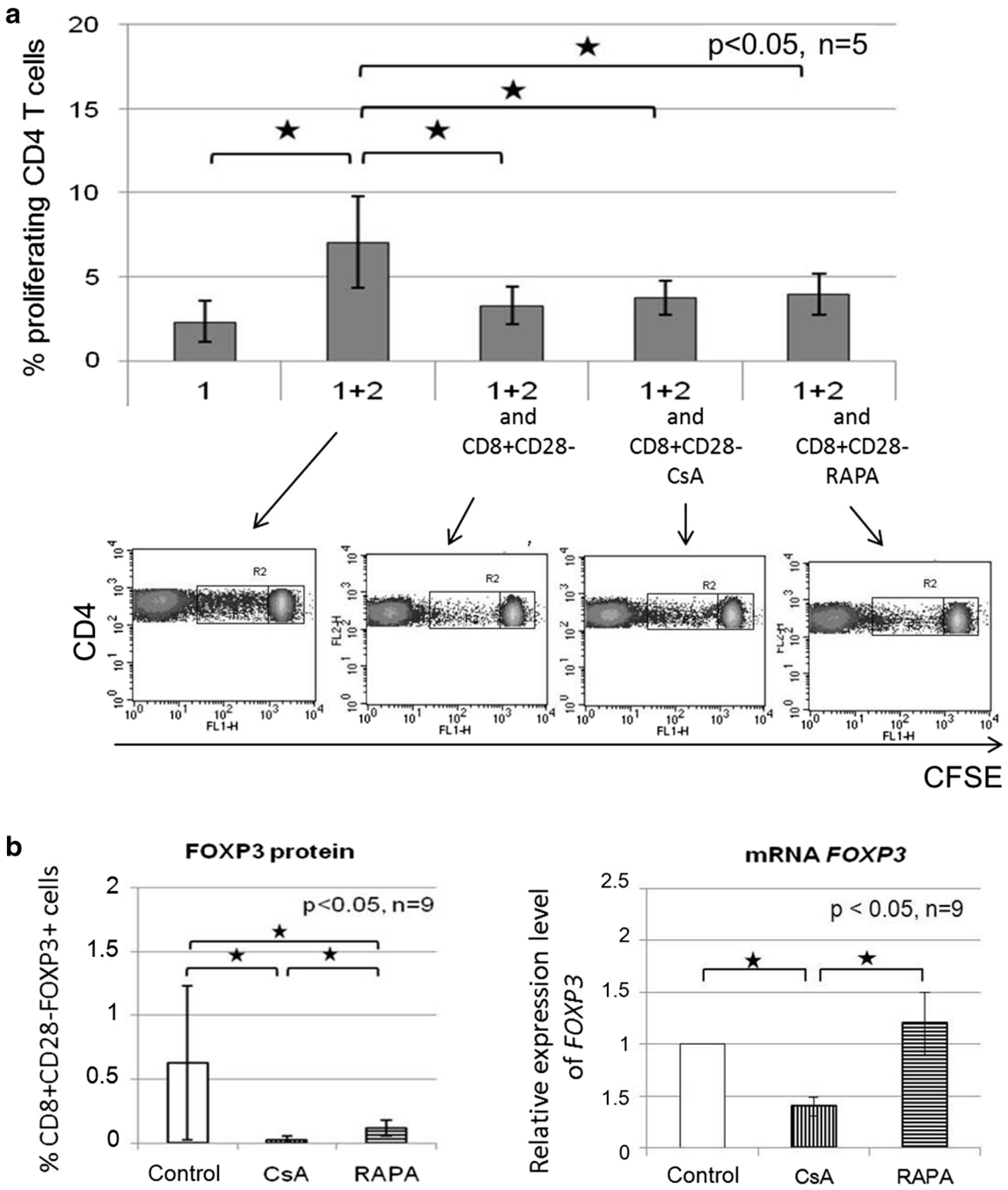

Fig. 2 The influence of CsA and RAPA on the properties of $CD8^+CD28^-$ cells in MLR. Inhibition of $CD4^+$ T cell proliferation in the secondary MLR culture by $CD8^+CD28^-$ cells derived from the primary culture; 1: PBMCs from donor 1; 2: PBMCs from donor 2; $CD8^+CD28^-$: $CD8^+CD28^-$ cells from the primary culture without any drug; $CD8^+CD28^-$CsA: $CD8^+CD28^-$ cells from the primary culture with CsA; $CD8^+CD28^-$RAPA $CD8^+CD28^-$ cells from the primary culture with RAPA. The *dot-plots* contains a representative experiment (**a**). $p < 0.05$ compared with the control culture: 1 + 2; the influence of CsA and RAPA on the expression of FOXP3 in the $CD8^+CD28^-$ population: mean percentage of $CD8^+CD28^-$ cells with FOXP3 protein; mean expression of FOXP3 mRNA (**b**). $p < 0.05$ compared with the control or drug

tures. Currently, there is considerable evidence indicating that RAPA promotes the generative and suppressive properties of $CD4^+CD25^+$ cells, which are other regulatory T cells, whereas CsA inhibits this process (Battaglia et al. 2005; Bocian et al. 2010; Chen et al. 2000; Coenen et al. 2006; Gao et al. 2007). CsA and RAPA affected the level of $CD8^+CD28^-$ cells, but these drugs did not interfere with the suppressor properties of $CD8^+CD28^-$ cells.

Calvo-Turrubiartes writes that calcineurin inhibitors induce, in vivo and in vitro, a diminution in the levels of $CD8^+CD28^-$ lymphocytes, which may correspond to T suppressor or cytotoxic cells (Calvo-Turrubiartes et al. 2009).

We should focus on the change in phenotype of $CD8^+CD28^-$ cells caused by drugs. However, it is difficult to indicate which markers should be taken into consideration.

Our results suggest that FOXP3 cannot help in distinguishing the Ts and Tc subsets of $CD8^+CD28^-$ cells. Although CsA and RAPA influenced the percentage of $FOXP3^+$ cells within the $CD8^+CD28^-$ population, they did not significantly affect the capacity of $CD8^+CD28^-$ cells to reduce the proliferation of alloreactive $CD4^+$ cells in the secondary MLR.

FOXP3 is considered a key marker for regulatory and suppressor cells (Li and Greene 2008; Li et al. 2008; Nakamura et al. 2007). This view is based mainly on indirect evidence. The presence of FOXP3 mRNA was noted in $CD8^+CD28^-$ cells isolated from the peripheral blood of patients with stable graft function (Manavalan et al. 2004; Suciu-Foca et al. 2005; Zhou et al. 2007). It was also observed that the level of FOXP3 mRNA was much lower in $CD8^+CD28^-$ cells from the blood of patients with chronic renal allograft rejection than patients with stable graft function and healthy non-recipients (Baeten et al. 2006). There is also more potent evidence, such as a few observations that FOXP3 mRNA is present in $CD8^+CD28^-$ cells with suppressor properties generated in vitro (Manavalan et al. 2004; Scotto et al. 2004; Suciu-Foca et al. 2005).

Nonetheless, we should remember that there is no possibility to check the suppressor properties of cells that had earlier confirmed presence of FOXP3 mRNA. There is "reverse evidence": researchers examined the presence of FOXP3 mRNA in cells that had shown suppressor properties, for example had inhibited the proliferation of $CD4^+$ cells by more than 80 % (Suciu-Foca et al. 2005). Considering that the $CD8^+CD28^-$ population is very heterogeneous, it is not good evidence for a connection between the FOXP3 marker and the suppressor properties of $CD8^+CD28^-$ cells. What is more, the FOXP3 molecule should be in the form of a protein to act as a transcriptional factor and influence cell function. Data about the presence of FOXP3 protein in $CD8^+CD28^-$ cells or generally in $CD8^+$ cells are very poor. We have found only one study that tried to evaluate the level of FOXP3 protein in $CD8^+CD28^-$ cells. The cells were isolated from the peripheral blood of patients who received immunosuppressive therapy based on CsA or RAPA, and FOXP3 protein was not detected (Segundo et al. 2006). In another study, the percentages of $CD8^+CD28^-FOXP3^+$ T cells were significantly higher in the belatacept group (after renal transplantation) when compared to those under cyclosporine and controls. Moreover, a difference was also found when the cyclosporine group was compared with controls, being higher in the latter group (Furuzawa-Carballeda et al. 2014).

Some literature data suggest that the FOXP3 marker is not restricted to regulatory cells (Gavin et al. 2006) and its expression and the suppressor properties of cells are only a coincidence (Pillai et al. 2007). What is more, FOXP3 protein is detected mainly in proliferating cells (Pillai et al. 2007). $CD8^+CD28^-$ cells do not proliferate (Arosa 2002; Effros et al. 2003; Scheuring et al. 2002) or only a few percent of these cells maintain proliferative capacity (Colovai et al. 2001), so the very low level of $FOXP3^+$ cells in the $CD8^+CD28^-$ population we observed seems to be nothing unexpected. We should probably conclude that FOXP3 is a marker of cell activation and that CsA and RAPA inhibit the activation and proliferation of cells and that is the reason why they reduced the expression of FOXP3 protein. Our results suggest that $CD8^+CD28^-$ cells can be activated in the presence of RAPA, but it is not possible in the presence of CsA.

In our study, we observed that CsA completely inhibits translation of the FOXP3 gene and reduces transcription, whereas RAPA interferes only with translation of this gene in $CD8^+CD28^-$ cells. In the case of FOXP3 mRNA, this is compatible with literature data about the influence of CsA and RAPA on $CD4^+$ cells (Mantel et al. 2006; Zuber et al. 2007). The literature data about the influence of the investigated drugs on FOXP3 protein are more ambiguous. There are studies that show both a lack (Mantel et al. 2006) and the presence (Coenen et al. 2006) of FOXP3 protein in $CD4^+$ cells from cultures with CsA. What is more, it is considered that RAPA does not change the expression of FOXP3 protein in $CD4^+CD25^+$ cells (Coenen et al. 2006) and selectively expands $CD4^+CD25^+FOXP3^+$ cells (Battaglia et al. 2005; Bocian et al. 2010). Wang et al. (2013) demonstrated that only cultivated $CD4^+$ Treg cells expressed the phenotype $CD4^+CD25^+$ $FOXP3^+$, whereas $CD8^+$ Treg cells expressed $CD8^+$ $CD25^+$ without FOXP3 coexpression.

We should remember that $CD8^+CD28^-$ Ts and $CD4^+$-$CD25^+$ Treg cells have different origins and mechanisms of action, and they could also differ in their sensitivity to drugs.

To sum up, in our study we found that CsA and RAPA did not interfere with the suppressor properties of human $CD8^+CD28^-$ T cells in vitro, although they affected the expression of the FOXP3 molecule.

Acknowledgments This work was supported in part by grant nos. 3P05B 07,425, N N402 268,036 and N303 015 32/0671 from the Ministry of Science and Higher Education. We thank Anna Duszota and Magdalena Jastrzebska for providing the samples for HLA typing, Marta Zylicz for experimental support regarding the CFSE

proliferation assay, Lidia Malchar and Marcin Cichocki for technical assistance, and the Transplantation Institute of the Medical University of Warsaw for kindly providing the immunosuppressive drugs.

References

Abraham RT (1998) Mammalian target of rapamycin: immunosuppressive drugs uncover a novel pathway of cytokine receptor signaling. Curr Opin Immunol 10:330–336

Allez M, Brimnes J, Dotan I et al (2002) Expansion of CD8+ T cells with regulatory function after interaction with intestinal epithelial cells. Gastroenterology 123:1516–1526

Almawi WY, Melemedjian OK (2000) Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A. Nephrol Dial Transplant 15:1916–1918

Arosa FA (2002) CD8 + CD28− T cells: certainties and uncertainties of a prevalent human T-cell subset. Immunol Cell Biol 80:1–13

Arosa FA, Irwin C, Mayer L et al (1998) Interactions between peripheral blood CD8 T lymphocytes and intestinal epithelial cells (iEC). Clin Exp Immunol 112:226–236

Assadiasl S, Ahmadpoor P, Nafar M et al (2014) Regulatory T cell subtypes and TGF-β1 gene expression in chronic allograft dysfunction. Iran J Immunol 11:139–152

Baeten D, Louis S, Braud C et al (2006) Phenotypically and functionally distinct CD8+ lymphocyte populations in long-term drug-free tolerance and chronic rejection in human kidney graft recipients. J Am Soc Nephrol 17:294–304

Battaglia M, Stabilini A, Roncarolo MG (2005) Rapamycin selectively expands CD4+ CD25+ FoxP3+ regulatory T cells. Blood 105:4743–4748

Bocian K, Borysowski J, Wierzbicki P et al (2010) Rapamycin, unlike cyclosporine A, enhances suppressive functions of in vitro induced $CD4^{+}CD25^{+}$ Tregs. Nephrol Dial Transplant 25:710–717

Calvo-Turrubiartes M, Romano-Moreno S, García-Hernández M et al (2009) Quantitative analysis of regulatory T cells in kidney graft recipients: a relationship with calcineurin inhibitor level. Transpl Immunol 21:43–49

Chen BJ, Morris RE, Chao NJ (2000) Graft-versus-host disease prevention by rapamycin: cellular mechanisms. Biol Blood Marrow Transplant 6:529–536

Ciubotariu R, Colovai AI, Pennesi G et al (1998) Specific suppression of human CD4+ Th cell responses to pig MHC antigens by CD8+ CD28− regulatory T cells. J Immunol 161:5193–5202

Coenen JJ, Koenen HJ, van Rijssen E et al (2006) Rapamycin, and not cyclosporin A, preserves the highly suppressive CD27+ subset of human CD4+ CD25+ regulatory T cells. Blood 107:1018–1023

Colovai AI, Ciubotariu R, Liu Z et al (2001) CD8(+)CD28(−) T suppressor cells represent a distinct subset in a heterogeneous population. Transplant Proc 33:104–107

Colovai AI, Mirza M, Vlad G et al (2003) Regulatory CD8+ CD28− T cells in heart transplant recipients. Hum Immunol 64:31–37

Cortesini R, LeMaoult J, Ciubotariu R et al (2001) CD8+ CD28− T suppressor cells and the induction of antigen-specific, antigen-presenting cell-mediated suppression of Th reactivity. Immunol Rev 182:201–206

Effros RB, Dagarag M, Valenzuela HF (2003) In vitro senescence of immune cells. Exp Gerontol 38:1243–1249

Furuzawa-Carballeda J, Bostock IC, Lima G et al (2014) Immunophenotyping of peripheral immunoregulatory as well as Th17A and Th22 cell subpopulations in kidney transplant recipients under belatacept or cyclosporine treatment. Transpl Immunol 30:107–113

Gao W, Lu Y, El Essawy B et al (2007) Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. Am J Transplant 7:1722–1732

Gavin MA, Torgerson TR, Houston E et al (2006) Single-cell analysis of normal and FOXP3–mutant human T cells: FOXP3 expression without regulatory T cell development. Proc Natl Acad Sci USA 103:6659–6664

Hohn H, Julch M, Pilch H et al (2003) Definition of the HLA-A2 restricted peptides recognized by human CD8+ effector T cells by flow-assisted sorting of the CD8+ CD45RA+ CD28− T cell subpopulation. Clin Exp Immunol 131:102–110

Hu H, Djuretic I, Sundrud MS et al (2007) Transcriptional partners in regulatory T cells: Foxp3, Runx and NFAT. Trends Immunol 28:329–332

Korecka-Polak A, Duszota A, Wierzbicki P et al (2011) Human peripheral blood CD8+ CD28− T cells of renal allograft recipients do not express FOXP3 protein. Transplant Proc 43:2917–2921

Li B, Greene MI (2008) Special regulatory T-cell review: FOXP3 biochemistry in regulatory T cells—how diverse signals regulate suppression. Immunology 123:17–19

Li Y, Zhao X, Cheng D et al (2008) The presence of Foxp3 expressing T cells within grafts of tolerant human liver transplant recipients. Transplantation 86:1837–1843

Liu Z, Tugulea S, Cortesini R et al (1998) Specific suppression of T helper alloreactivity by allo-MHC class I-restricted CD8+ CD28− T cells. Int Immunol 10:775–783

Manavalan JS, Kim-Schulze S, Scotto L et al (2004) Alloantigen specific CD8+ CD28− FOXP3+ T suppressor cells induce ILT3+ ILT4+ tolerogenic endothelial cells, inhibiting alloreactivity. Int Immunol 16:1055–1068

Mantel PY, Ouaked N, Ruckert B et al (2006) Molecular mechanisms underlying FOXP3 induction in human T cells. J Immunol 176:3593–3602

Nakamura S, Suzuki M, Sugimoto A et al (2007) IL-2-independent generation of FOXP3(+)CD4(+)CD8(+)CD25(+) cytotoxic regulatory T cell lines from human umbilical cord blood. Exp Hematol 35:287–296

Pillai V, Ortega SB, Wang CK et al (2007) Transient regulatory T-cells: a state attained by all activated human T-cells. Clin Immunol 123:18–29

Rudensky AY, Gavin M, Zheng Y (2006) FOXP3 and NFAT: partners in tolerance. Cell 126:253–256

Scheuring UJ, Sabzevari H, Theofilopoulos AN (2002) Proliferative arrest and cell cycle regulation in CD8(+)CD28(−) versus CD8(+)CD28(+) T cells. Hum Immunol 63:1000–1009

Schubert LA, Jeffery E, Zhang Y et al (2001) Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem 276:37672–37679

Scotto L, Naiyer AJ, Galluzzo S et al (2004) Overlap between molecular markers expressed by naturally occurring CD4+ CD25+ regulatory T cells and antigen specific CD4+ CD25+ and CD8+ CD28− T suppressor cells. Hum Immunol 65:1297–1306

Segundo DS, Ruiz JC, Izquierdo M et al (2006) Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+ CD25+ FOXP3+ regulatory T cells in renal transplant recipients. Transplantation 82:550–557

Strioga M, Pasukoniene V, Characiejus D (2011) CD8+ CD28− and CD8+ CD57+ T cells and their role in health and disease. Immunology 134:17–32

Suciu-Foca N, Manavalan JS, Cortesini R (2003) Generation and function of antigen-specific suppressor and regulatory T cells. Transpl Immunol 11:235–244

Suciu-Foca N, Manavalan JS, Scotto L et al (2005) Molecular characterization of allospecific T suppressor and tolerogenic dendritic cells: review. Int Immunopharmacol 5:7–11

Vlad G, King J, Chang CC et al (2011) Gene profile analysis of CD8(+) ILT3-Fc induced T suppressor cells. Hum Immunol 72:107–114

Wang H, Daniel V, Sadeghi M et al (2013) Differences in the induction of induced human $CD4^{+}CD25^{+}FoxP3^{+}$ T-regulatory cells and $CD3^{+}CD8^{+}CD28-$ T-suppressor cells subset phenotypes in vitro: comparison of phorbol 12-myristate 13-acetate/ionomycin and phytohemagglutinin stimulation. Transplant Proc 45:1822–1831

Zavazava N, Kabelitz D (2000) Alloreactivity and apoptosis in graft rejection and transplantation tolerance. J Leukoc Biol 68:167–174

Zhou H, Wang ZD, Zhu X et al (2007) CD8 + FOXP3 + T cells from renal transplant recipients in quiescence induce immunoglobulin-like transcripts-3 and -4 on dendritic cells from their respective donors. Transplant Proc 39:3065–3067

Zuber J, Viguier M, Lemaitre F et al (2007) Severe FOXP3 + and naive T lymphopenia in a non-IPEX form of autoimmune enteropathy combined with an immunodeficiency. Gastroenterology 132:1694–1704