Clinically-relevant cyclosporin and rapamycin concentrations enhance regulatory T cell function to a similar extent but with different mechanisms: An *in-vitro* study in healthy humans☆,☆☆

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ABSTRACT

Evidence indicates that regulatory T cells (Tregs) are profoundly involved in promoting allograft tolerance after organ transplantation. Since a successful transplantation currently still requires a long-term immunosuppressive treatment, clarifying the specific impact of these drugs on Tregs may be of high clinical relevance. Conflicting results arise from the literature, particularly as concerns cyclosporine (CsA). The specific aim of this work was to evaluate *in-vitro* the direct effects of clinically-relevant drug concentrations of three widely used immunosuppressive drugs, i.e. CsA, rapamycin (RAPA) and mycophenolic acid (MPA), on Treg activity, number and forkhead/winged helix transcription factor (FoxP3) expression in humans.

Tregs ($CD4^+CD25^+$) isolated from healthy donors were cultured in the presence of different concentrations of CsA, RAPA or MPA. The suppressive activity of Tregs was evaluated in mixed lymphocyte reactions with $CD4^+CD25^-$ T cells. Phenotype analysis and FoxP3 expression were assessed by flow cytometry.

Clinically-relevant CsA and RAPA concentrations significantly enhanced to a similar extent the suppressive activity of Tregs. Although this effect was associated with an increase in Treg number as well as in FoxP3 expression with both drugs, the driving mechanism seemed to be primarily quantitative (i.e. increase of the cell number) for RAPA, whereas mainly qualitative (i.e. increase in FoxP3 levels) for CsA, respectively. Conversely, MPA did not show any effect on Treg function and number.

These findings suggest that both RAPA and CsA may be beneficial in promoting Treg-dependent allograft tolerance after organ transplantation.

1. Introduction

Increasing evidence indicates that regulatory T cells (Tregs) are profoundly involved in the development of allograft tolerance after organ transplantation. In fact, after the seminal study of Taylor and Noelle [1] in a murine model of graft-versus-host-disease (GVHD), a large number of studies clearly indicated Tregs as a mainstay actor in achieving allograft tolerance after bone marrow as well as solid organ transplantation [2].

Based on these data, the enhancement of the Treg response may represent an attractive target for a future tolerance-based strategy potentially able to prevent allograft rejection without the need for the current heavy immunosuppression. In fact, successful organ transplantation presently still requires a long-term treatment with a combination of immunosuppressive drugs. At the moment, the effects of these drugs on the Treg cell population are under intensive investigation in order to identify those agents having the best immunomodulating profile, i.e. a strong inhibitory activity towards

the alloimmune response together with a stimulatory effect on the allograft tolerance process.

Many studies on the effects of immunosuppressive drugs on Tregs are already available, although providing conflicting results, particularly where cyclosporine A (CsA) is concerned. Even though the dominant idea is that CsA negatively affects Tregs, an accurate dissection of the literature indicates that the effect of CsA on these cells is actually not clear, as it probably depends on the specific drug concentrations used (in many cases very high and thus absolutely not clinically relevant), the cell origin (animals or humans), the parameters evaluated (Treg number, suppressive activity or forkhead/winged helix transcription factor, FoxP3, expression), as well as the experimental conditions employed [2,3]. In particular, in the largest part of the in-vitro/ex-vivo studies reporting an inhibitory activity of CsA, the effects on Tregs were evaluated by adding the drug at the moment of the mixed lymphocyte reaction (rather than a selective pre-treatment of these cells), thus not allowing to rule out an in-vitro indirect effect of CsA on Tregs, due to the inhibition of T effector cell-dependent IL-2 production, an essential stimulus for Treg proliferation and function [4,5].

On the basis of these premises, the specific aim of this work was to evaluate in-vitro the direct effects of clinically-relevant drug concentrations of three widely used immunosuppressive drugs, i.e. CsA, rapamycin (RAPA) and mycophenolic acid (MPA), on Treg activity, number and FoxP3 expression in healthy humans.

2. Materials and methods

2.1. Donor cells

Experiments were performed on buffy coats obtained from anonymous healthy blood donors, provided by the donor bank of the immune-hematology transfusion center of the Policlinico Le Scotte of Siena, Italy.

2.2. Purification of CD4$^+$CD25$^+$ Treg cells from peripheral blood

CD4$^+$CD25$^+$ Tregs were purified from peripheral blood mononuclear cells (PBMCs) isolated from healthy blood donors. PBMCs were isolated by density gradient centrifugation on Lympholyte (Cedarlane, Burlington, Ontario, Canada) using a Beckman GS-6R tabletop centrifuge (Beckman Coulter SpA, Milan, Italy). Cells were washed 2× in phosphate buffered saline (PBS), resuspended in RPMI 1640 (Sigma-Aldrich, Milan, Italy) (buffered with sodium bicarbonate to pH 7.2) supplemented with 7.5% bovine calf serum (BCS) (Hyclone, Thermofischer Scientific Inc., South Logan, UT), plated in plastic flasks (Sarstedt AG, Numbrecht, Germany) and incubated overnight at 37 °C in a humidified atmosphere with 5% CO$_2$. Non-adherent cells, which consisted principally of peripheral blood lymphocytes (PBL) and of which >90% were T cells (CD3$^+$), were centrifuged at 800 ×g for 5 min at room temperature in the Beckman GS-6R tabletop centrifuge and resuspended in fresh RPMI 1640 supplemented with 7.5% BCS. CD4$^+$ T cells were isolated by negative selection using a CD4 isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). CD4$^+$ T cells were incubated with anti-CD25 Micro Beads (Miltenyi Biotech) and separated into CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T-cell subsets on a positive selection column. This separation resulted in less than 1% CD25$^+$ cells in the CD4$^+$CD25$^-$ fraction and approximately 80% purity in the CD25$^+$ T-cell subset. As shown in Fig. 1A, CD4$^+$ cells expressing high levels of CD25 (CD4$^+$CD25^{high}) represented 70% of live-gated cells and CD4$^+$CD25$^{low/intermediate}$ T cells represented 20% of live-gated cells.

To further confirm the identity of the CD4$^+$CD25$^+$ T-cell fraction deriving from immunomagnetic separation step, cells were stained with PE anti-human FoxP3 antibody, as described more in detail below. As expected, in the CD4$^+$CD25$^+$ T-cell fraction CD4$^+$CD25$^+$ FoxP3$^+$ T cells represented 90% of live-gated cells (Fig. 1B).

Both CD4$^+$CD25^{high}T cells and CD4$^+$CD25$^{low/intermediate}$ T cells were used for experiments described in this report.

2.3. Drug concentration employed

In-vitro studies were conducted using immunosuppressive drugs at different concentrations, also including those relevant in the clinical setting. More in detail: CsA (Sigma-Aldrich, Milan, Italy) 40,100 or 400 ng/ml [therapeutic serum levels in transplanted patients ranging from approximately 50–400 ng/ml] [6,7]; RAPA (Sigma-Aldrich) 20,100 or 1000 ng/ml [5–25 ng/ml] [6,8]; MPA (Sigma-Aldrich) 5, 10 or 25 μg/ml [1–5 μg/ml] [9].

2.4. Antibodies and flow cytometry (FACS)

For flow cytometric analyses, 2×10^5 cells/sample were stained and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Purified CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells were labeled with fluorescein isothiocyanate (FITC) anti-human CD4 (BD Biosciences, San Jose, CA, USA) and Phycoerythrin (PE) anti-human CD25 (BD Biosciences). The CD4$^+$CD25$^+$ T cell fraction was also stained with PE anti-human FoxP3 (BD Biosciences) as subsequently described. Cell proliferation was measured by flow cytometric analysis of carboxyfluorescein succinimidyl ester (CFSE)- and PE anti-human CD3 (eBioscience, San Diego, CA, USA)-labeled cells.

2.5. T cell proliferation and suppression assays

Treg function was studied by evaluating the capacity of Tregs to suppress CD4$^+$CD25$^-$ T cell proliferation. CD4$^+$CD25$^-$ T cell proliferation was measured by flow cytometric analysis of the carboxyfluorescein succinimidyl ester (CFSE)-labeled cells. Cells were resuspended at 20×10^6/ml in PBS and loaded with 10 μM CFSE (Molecular Probes/Invitrogen Life Technologies, CA, USA) for 8 min at room temperature. Cells were subsequently washed twice in RPMI 1640 supplemented with 7.5% BCS and resuspended in the same medium. The suppressive capacity of Tregs was studied in a primary mixed lymphocyte reaction (MLR) assay in which the CD4$^+$CD25$^-$ T cells labeled with CFSE, used as a responder, were added to heterologous Mitomycin C-treated PBMCs, used as a stimulator, in the presence of low concentrations of soluble OKT3 (anti-CD3; American Type Culture Collection, Manassas, VA) able to stimulate Treg activity in-vitro without altering CD4$^+$CD25$^-$ T cell proliferation. More in detail, IgG from OKT3 hybridoma supernatants was purified using Mabtrap (Amersham Biosciences, Inc.) and titrated until a dilution (1:600) was obtained showing no effects on CD4$^+$CD25$^-$ T cell proliferation. Three samples were prepared: a control sample represented by CFSE labeled CD4$^+$CD25$^-$ T cells; a second sample in which 1×10^6 CFSE labeled CD4$^+$CD25$^-$ T cells were stimulated by 1×10^6 heterologous Mitomycin C-treated PBMCs, in the presence of soluble OKT3. The last sample was prepared like the second to which 1×10^6 autologous Tregs were added in a stimulator-to-responder and suppressor-to-effector ratio of 1:1. Proliferation of CD4$^+$CD25$^-$ T cells was evaluated by assessing CFSE dilution using the FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cells were analyzed by flow-cytometry 72, 96 and 120 h after stimulation, gating on CD3$^+$ cells. In particular, a specific gating strategy aimed at excluding dead cells from the analysis (based on the peculiar FSC/SSC profile characterizing live cells) was initially performed. As a result, all the following analyses selectively involved the live lymphocyte population.

As shown in Fig. 1(C–D), when Treg cells were added to MLR in a suppressor-to-effector ratio of 1:1 a very strong suppression of proliferation of CD4$^+$CD25$^-$ by Tregs was obtained (about 80%). Thus, a suppressor-to-effector ratio of 1:4 (resulting in about a 20–30% suppression) was selected as the optimal ratio to detect any potential additional effect of the drug on Treg suppressive activity.