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Immunosuppressive drugs affect induction of IFNy+ Treg in vitro

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ABSTRACT

Background: We reported previously that patients with poor long-term graft function are able to form IFNy+ Treg in vitro pretransplant, but late posttransplant have more frequently undetectable or lower levels of IFNy+ Treg in the peripheral blood than patients with good long-term graft outcome. In the present study, we investigated the induction of IFNy+ and Tbet+ Treg subsets in the presence of immunosuppressants in vitro.

Methods: PBL of 10 healthy individuals were stimulated with PMA/Ionomycin in the presence of different immunosuppressive drugs at 2 different concentrations that were chosen to approximately mirror the blood levels in renal transplant recipients. IFNy+, Tbet+, CD119+, and Helios+ CD4+CD25+CD127−Foxp3+ Treg subsets were analyzed using 8-color-fluorescence-flow-cytometry.

Results: Cyclosporine ($p < 0.01$) and 6α-methylprednisolone ($p < 0.05$) at both concentrations as well as high doses of azathioprine ($p < 0.05$) and mycophenolate mofetil ($p < 0.05$) inhibited the induction of IFNy+ and Tbet+ Treg, whereas lower concentrations of azathioprine and mycophenolate mofetil tended to increase IFNy+, Tbet+ and CD119+ Treg ($p \leqslant 0.05$).

Conclusions: Drug-induced inhibition of Treg induction might result in low IFNy+ Treg levels with the consequence of T effector activation and impaired graft function. Further studies will show whether monitoring of IFNy+ Treg might help to prevent clinical complications provoked by an inappropriate immunosuppressive protocol.

1. Background

In a previous study we showed that patients with poor long-term graft outcome are able to form IFNy+ Treg in MLCs with pretransplant obtained patient and donor lymphocytes [1], indicating that these patients have the capacity to generate IFNy+ Treg during an immune response with donor lymphocytes. However, their impaired posttransplant graft outcome suggests that these patients either do not form IFNy+ Treg in vivo or, alternatively, lose the property to continuously generate IFNy+ Treg. More than 4 years posttransplant, patients with impaired graft function were shown to possess significantly lower numbers of IFNy+ Treg in the peripheral blood than patients with good graft function [2] and we suspect that intensified immunosuppressive treatment during periods of rejection or inappropriately high dosages of immunosuppressive drugs administered for maintenance immunosuppression interfered with the patients' ability to form IFNy+ Treg [2]. A reduction of IFNy+, and conceivably other Treg subsets within the heterogenous Treg pool, might result in an imbalance of stimulating and suppressive T cells leading to acute or chronic rejection. To test whether immunosuppressive drugs are able to affect the induction of IFNy+ Treg, we stimulated PBL of healthy individuals with PMA/Ionomycin in the presence of immunosuppressive drugs and measured the induction of CD4+CD25+CD127−Foxp3+ Treg subsets co-expressing IFNy, Helios, CD119, and Tbet. Thymic-derived natural Treg co-express Helios whereas peripherally induced adaptive Treg lack Helios expression [3]. Moreover, IFNy+ Treg co-express the transcription factor for Th1 cytokines Tbet as well as IFNy receptor CD119 on the cell surface [4–6] We analyzed whether immunosuppressive drugs interfere with these Treg markers and inhibit their co-expression resulting in decreased or ineffective Treg.

Abbreviations: aTreg, adaptive T regulator cell; nTreg, natural T regulator cell; MLC, mixed lymphocyte culture; FCS, fetal calf serum; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; Tbet, T-box expressed in T cells; TCR, T cell receptor; Th1, T helper type 1; IL, interleukin; IFNy, interferon gamma; GVHD, graft versus host disease; mTOR, mechanistic target of rapamycin; CTLA4, cytotoxic T-lymphocyte-associated protein 4; SEM, standard error of mean.

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2. Methods

2.1. Healthy controls

Laboratory staff served as healthy controls ($n = 10$). All controls gave informed consent for the tests performed within this study and the study was approved by the local ethical committee. The study was conducted in adherence to the Declaration of Helsinki.

2.2. Stimulation of PBL using PMA/Ionomycin

PBL were separated from heparinized whole blood by Ficoll density gradient centrifugation and stimulated for different time intervals using a mixture of phorbol 12-myristate 13-acetate (PMA; final concentration in medium: 10 ng/ml; Sigma Aldrich, Munich, Germany) and ionomycin (1 μg/ml; Sigma Aldrich, Munich, Germany) in RPMI medium containing 10% FCS, L-Glutamin, and Penicillin/ Streptomycin (all from Invitrogen Gibco, Paisley, Scotland) as described previously [5]. Lymphocytes were cultured in the presence of different immunosuppressive drugs in 2 different concentrations: cyclosporine in final concentrations of 300 ng/ml and 600 ng/ml, everolimus 60 ng/ml and 120 ng/ml, rapamycin 60 ng/ml and 120 ng/ml, azathioprine 300 ng/ml and 600 ng/ml, 6α-methylprednisolone 300 ng/ml and 600 ng/ml, mycophenolate mofetil 5 μg/ml and 10 μg/ml, and mycophenolic acid 5 μg/ml and 10 μg/ml. Drug concentrations were similar to the blood levels in renal transplant recipients.

2.3. Determination of different PBL subsets

PBL subsets were determined as described previously [5]. For analysis of determinants on the cell surface, PBL were incubated with fluorochrome-labelled monoclonal antibodies against CD4, CD25, CD119, CD127 (all from BD Biosciences). Intracellular determinants were stained with fluorochrome-labelled monoclonal antibodies against Foxp3, IFNy (clone B27), Helios and T-bet (all BD Biosciences). Briefly, PBL were incubated with combinations of monoclonal antibodies for 30 min as described and eight-color fluorescence was analyzed using a FACSCanto II triple-laser flow cytometer (BD Biosciences) [5]. When, in addition, intracellular proteins were studied, cell membranes were permeabilized using BD Perm/Wash buffer (BD Biosciences). At least 100,000 events were analyzed in the initial FSC/SSC dot plot.

2.4. Statistics

Representative test results with mean ± SEM were depicted in the figures. For statistical analysis PASW Statistics program version 21 (IBM, Chicago, Illinois, USA) and Wilcoxon signed rank test for pairwise comparison were used. *P*-values $\leqslant 0.05$ were considered significant. Further *p*-values of $\leqslant 0.09$ were considered to show a trend.

3. Results

PBL of 10 healthy individuals were stimulated with PMA/Ionomycin in the presence of immunosuppressive drugs at 2 different concentrations. In a first analysis, proportions of Treg subsets in PMA/Ionomycin stimulated cell cultures were compared with Treg proportions in cell cultures with PMA/Ionomycin and immunosuppressive drugs; in a second analysis, Treg proportions were compared between cell cultures with different concentrations of the same immunosuppressive drug.

3.1. PMA/Ionomycin-stimulated cell cultures with vs without immunosuppressant

When cell cultures stimulated with PMA/Ionomycin only were compared with cell cultures stimulated with PMA/Ionomycin in the presence of the immunosuppressive drugs cyclosporine A, everolimus, rapamycin, azathioprine, 6α-methylprednisolone, mycophenolate mofetil, or mycophenolic acid, CD4+CD25+CD127−Foxp3+ Treg were increased in cultures containing immunosuppressants compared with controls (Wilcoxon signed rank test: with vs without drug in cell culture medium; $p \leqslant 0.05$), with the exception of 6α-methylprednisolone (Table 1). Methylprednisolone did not alter the proportion of CD4+CD25+CD127−Foxp3+ Treg in cell cultures compared to cell cultures stimulated with PMA/Ionomycin only. The result indicates that, with the exception of 6α-methylprednisolone, all other drugs support the induction of CD4+CD25+CD127−Foxp3+ Treg in vitro. However, when subsets of CD4+CD25+CD127−Foxp3+ Treg were analyzed, strong suppression of Helios+IFNy+ Treg in the presence of cyclosporine became evident ($p = 0.005$) (Table 1, Fig. 1) and there was mild suppression of this Treg subset in the presence of 6α-methylprednisolone ($p = 0.028$). Incubation with azathioprine resulted in increased induction of Helios+IFNy+ Treg ($p = 0.017$) (Table 1, Fig. 1). It thus appears that cyclosporine and 6α-methylprednisolone have an inhibiting effect on the induction of Helios+IFNy+ Treg. Helios−IFNy+ Treg were likewise inhibited by cyclosporine ($p = 0.051$), whereas azathioprine ($p = 0.005$), mycophenolate mofetil ($p = 0.074$) and mycophenolic acid ($p = 0.086$) tended to support the induction of Helios−IFNy+ Treg (Table 1). IFNy production is initiated by activation of the transcription factor Tbet. A reduction of CD119−Tbet+ Treg was observed in the presence of cyclosporine ($p = 0.007$) and methylprednisolone ($p = 0.021$) whereas incubation in the presence of everolimus ($p = 0.017$) increased this Treg subset (Table 1). CD119+Tbet− Treg were upregulated in the presence of everolimus ($p = 0.012$), azathioprine ($p = 0.009$), 6α-methylprednisolone ($p = 0.051$), mycophenolate mofetil ($p = 0.008$), and mycophenolic acid ($p = 0.012$), whereas CD119+Tbet+ Treg were augmented by mycophenolate mofetil ($p = 0.069$), mycophenolic acid ($p = 0.043$), and, interestingly, by low concentrations of cyclosporine ($p = 0.068$). CD4+CD25+CD127−Foxp3+ Helios+IFNy− represent the classical phenotype of nTreg. Azathioprine ($p = 0.007$), 6α-methylprednisolone ($p = 0.005$), mycophenolate mofetil ($p = 0.037$), and mycophenolic acid ($p = 0.012$) all inhibited the induction of Helios+IFNy− Treg, whereas incubation of PBL in the presence of everolimus ($p = 0.037$) or rapamycin ($p = 0.074$) increased this Treg subset (Table 1). In conclusion, although incubation with cyclosporine and 6α-methylprednisolone increased the total pool of CD4+CD25+CD127−Foxp3+ Treg, these drugs inhibited the induction of Treg subsets with Th1 qualities, such as Helios+ and Helios− IFNy+ Treg, as well as CD119−Tbet+ Treg. In contrast, incubation with azathioprine, mycophenolate mofetil and mycophenolic acid at lower concentrations led to an increase in IFNy+, Tbet+ and CD119+ Treg subsets.

3.2. Comparison of 2 different drug concentrations in vitro

When PMA/Ionomycin stimulated cell cultures with 2 different drug concentrations were compared, higher concentrations of cyclosporine inhibited the induction of CD119+ and CD119− Tbet+ Treg subsets stronger than lower concentrations (Wilcoxon signed rank test: lower vs higher drug concentration in cell culture medium; $p = 0.037$ and $p = 0.013$, respectively) (Table 2, Fig. 2). Higher azathioprine concentrations showed a suppressive effect on IFNy-expressing Helios+ ($p = 0.022$) and Helios− ($p = 0.059$),