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Clinical relevance of IFNγ positive T regulatory cells in renal transplantation

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Transplantation is the last resort therapy of end-stage renal diseases. Currently, scientists and clinicians are discovering the role of Treg in transplantation. Because of Treg's great heterogeneity, it is difficult to determine the subset most appropriate for clinical use. However, there is growing evidence that IFNγ+ Treg with their immunosuppressive capacity could be a potential subset that protects the graft from immunological attack and maintains its long-term function. It was shown that IFNγ+ Treg were increased in the blood of patients with good long-term graft function compared to patients with impaired graft function and that patients with impaired graft function were able to form IFNγ+ Treg pre-transplant. Based on these findings it was tried to answer the question: "What happens with IFNγ+ Treg in patients with impaired graft function post-transplant? Do they remain stable as Foxp3+IFNγ+ Treg, do they further differentiate into Th1 lymphocytes or do they die?
Blood samples were obtained from one hundred and thirty-six renal transplant patients from Giessen (n=85) and Heidelberg (n=51) and from 52 healthy controls. Patients were transplanted a minimum of five months before blood donation. IFNγ+ and IFNγ- CD4+CD25+CD127- Treg were enriched using antibody-coated magnetic beads and investigated in cell culture experiments. Methylation status of CD4+CD25+CD127-IFNγ+/- Treg was determined using real-time PCR. Lymphocytes and Treg subsets were determined in the blood using 4- and 8-color fluorescence flow cytometry. Subset counts and Foxp3 methylation status were correlated with graft outcome.
First, proliferative capacity and phenotype stability of different Treg subsets were investigated *in vitro* in the presence of PMA/Ionomycin and rIFNγ. PMA/Ionomycin in combination with rIFNγ decreased Helios+IFNγ- and increased Helios-IFNγ+ Treg. This effect was more pronounced in cell cultures with enriched CD4+CD25- than in cell cultures with CD4+CD25+ PBL. After the removal of a short-term polyclonal stimulus, IFNγ- as well as IFNγ+ Treg continued to increase during the 96-hour observation period suggesting that Foxp3 expression remained stable in IFNγ+ Treg after elimination of the stimulus (no reprogramming or differentiation of Treg into Th1).
Second, cell cultures stimulated with PMA/Ionomycin only were compared with cell cultures stimulated with PMA/Ionomycin in the presence of different immunosuppressive drugs. Cyclosporine and 6α-methylprednisolone had an inhibiting effect on the induction of Helios+IFNy+ and Helios-IFNy+ Treg whereas azathioprine, mycophenolate mofetil and mycophenolic acid tended to support the induction of Helios-IFNy+ Treg. Higher concentrations of cyclosporine inhibited the induction of

CD119+ and CD119- Tbet+ Treg subsets stronger than lower concentrations. Higher azathioprine concentrations showed a suppressive effect on IFNy-expressing Helios+ and Helios-, CD119-expressing Tbet+ and Tbet- as well as total Treg. Mycophenolate mofetil suppressed the induction of CD119+ and CD119- Tbet+ Treg as well as Helios+IFNy+ and total Treg in higher concentrations. The data suggest that inappropriate immunosuppression might inhibit induction of IFNγ+ Treg *in vitro* and potentially also in transplant recipients with the result of impaired long-term graft outcome.

Third, lymphocytes and Treg counts of renal transplant patients were compared with those of healthy controls. Renal transplant patients showed reduced CD45+, CD3+, and CD4+ T-lymphocyte, CD16+CD56+NK cells, and CD19+ B-lymphocyte counts as well as lower CD4+CD25+Foxp3+CD127- total Treg, Helios+IFNγ-, Helios+IFNγ+, CD183+CD62L+, CD183-CD62L+, CD252-CD152+, CD28+HLADR-, CD28+HLADR+, CD95+CD178-, CD152-CD154+, CD279+CD152+, and CD279-CD152+ Treg counts compared to healthy controls. Interestingly patients with higher glomerular filtration rate (GFR) showed higher CD45+, CD3+, CD4+, CD19+, CD16+56+ cells counts, and higher Helios+IFNγ-, CD28+HLADR+, CD252-CD152+, CD183+CD62L+ Treg counts than patients with lower GFR suggesting a relationship of high effector cell counts with high Treg subsets in patients with good long-term graft outcome. Comparing lymphocyte and Treg subsets in patients ≤1.5 vs. >1.5 years post-transplant, only CD16+CD56+ NK cells and CD28-HLADR+ Treg count increased with time suggesting stability of most effector cell and Treg subsets in stable transplant recipients. Patients on steroids had higher CD28+HLADR- and lower CD28-HLADR+ and CD279+CD152- Treg than steroid-free patients suggesting decreased activation of certain Treg subsets during steroid treatment.

Fourth, Treg phenotype stability depends on methylation status of Foxp3. Low methylation indicates stable, high methylation transient Foxp3 expression. Because the Foxp3 gene is located on the X chromosome, methylation status was determined in females and males separately. Male as well as female renal transplant patients showed strong Foxp3 methylation of enriched IFNγ+ as well as IFNγ- Treg whereas healthy controls provided intermediate Foxp3 methylation in IFNγ+ and low Foxp3 methylation in IFNγ- Treg preparations indicating a more transient Foxp3 expression in Treg of long-term stable transplant recipients. High numbers of total Treg, as well as Helios+IFNγ-, CD183+CD62L+, CD183-CD62L+, and CD28+CDHLADR+ Treg were associated with strong methylation in female patients, high numbers of IFNy+ CD95+CD178+ and IL10-TGFß+ Treg were associated with intermediate methylation in male patients. Obviously, transient Foxp3 expression was associated with IFNγ- activated thymus-derived Treg in secondary lymphatic tissues whereas more stable Foxp3 expression was detected in Treg subsets inhibiting effector cells by TGFß secretion and apoptosis induction via cell-cell contact. The data provide evidence that low IFNγ+ Treg in patients with impaired graft function late post-transplant might be caused in part by intensified immunosuppressive therapy. IFNγ+ Treg appear to be stable *in vitro* and *in vivo*. There was no indication of further differentiation to Th1 lymphocytes *in vitr*o or Treg loss with time post-transplant

in patients with stable long-term graft function. Patients showed primarily IFNγ+ and IFNγ- Treg with transient Foxp3 expression suggesting that they were induced peripherally. Patients with good long-term graft outcome showed higher numbers of strongly methylated Helios+IFNγ-, CD28+HLADR+, CD252-CD152+, CD183+CD62L+ Treg than patients with impaired graft function suggesting an immunoregulatory role of these particular Treg subsets in long-term graft outcome.