Ex vivo expanded natural regulatory T cells from patients with end-stage renal disease or kidney transplantation are useful for autologous cell therapy

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Novel concepts employing autologous, *ex vivo* expanded natural regulatory T cells (nTreg) for adoptive transfer has potential to prevent organ rejection after kidney transplantation. However, the impact of dialysis and maintenance immunosuppression on the nTreg phenotype and peripheral survival is not well understood, but essential when assessing patient eligibility. The current study investigates regulatory T-cells in dialysis and kidney transplanted patients and the feasibility of generating a clinically useful nTreg product from these patients. Heparinized blood from 200 individuals including healthy controls, dialysis patients with end stage renal disease and patients 1, 5, 10, 15, 20 years after kidney transplantation were analyzed. Differentiation and maturation of nTregs were studied by flow cytometry in order to compare dialysis patients and kidney transplanted patients under maintenance immunosuppression to healthy controls. CD127 expressing $CD4^+CD25^{high}FoxP3^+$ nTregs were detectable at increased frequencies in dialysis patients with no negative impact on the nTreg end product quality and therapeutic usefulness of the *ex vivo* expanded nTregs. Further, despite that immunosuppression mildly altered nTreg maturation, neither dialysis nor pharmacological immunosuppression or previous acute rejection episodes impeded nTreg survival *in vivo*. Accordingly, the generation of autologous, highly pure nTreg products is feasible and qualifies patients awaiting or having received allogenic kidney transplantation for adoptive nTreg therapy. Thus, our novel treatment approach may enable us to reduce the incidence of organ rejection and reduce the need of long-term immunosuppression.

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Over the past decades, renal allotransplantation has become the treatment of choice in patients with end-stage renal disease (ESRD). With the implementation of lifelong immunosuppression, we have succeeded in preventing early rejection and have significantly improved the quality of life and short-term survival.[1–3] These achievements, however, are a Janus-faced success, and we have traded the short-term benefits for the significant risk of opportunistic or severe courses of viral infections, malignancies, and toxicity-associated graft failure[4–6] but failed to prevent chronic rejection effectively.

With the rapidly growing knowledge on regulatory T cells (Tregs) as mediators of immune homeostasis, there is now increasing confidence that Tregs can serve as a rational target for a new generation of immune-modulatory therapy. Over the past years, Tregs have been characterized as a key mediator of tolerance in the context of autoinflammation,[7,8] infection,[9,10] transplantation,[11,12] and tumor entities.[13] Conversely, absolute or relative superiority of conventional T cells (Tconv) over Tregs is associated with autoinflammation and graft rejection. Despite the progress made so far and although pilot studies are currently ongoing, broad therapeutic implementation of Tregs has not yet been achieved.

The group of Tregs has grown to a large family with multiple subsets.[14,15] Each subset is characterized by a unique, although partially overlapping, composition of phenotypic markers and shows a specific endogenous cytokine profile. The classification as a regulatory cell, however, is based on shared functional characteristics including T-cell receptor (TCR)–dependent suppression of proliferation and cytokine release in Tconv.

Among CD4+ T cells, 4 Treg subsets have been defined: (i) natural Tregs (nTregs) are congenitally present and thymically matured, whereas (ii) induced Tregs, which originate from Tconv, can mature upon specific TCR activation and transiently express both FoxP3 and functional Treg characteristics. (iii) Interleukin (IL)-10–secreting type 1 Tregs[16] and (iv) transforming growth factor-ß–producing T-helper cell type 3 cells[17] are derived from the same precursors as induced Tregs and show comparable suppressive capacities but remain negative for FoxP3.[16–20] In the following discussion, we focus on nTregs as a stable, thymically derived regulatory T-cell population.

A few studies recently addressed nTregs in patients with ESRD and after kidney transplantation (KTx).[21–24] Although there is significant evidence that human nTregs can control alloresponsiveness in KTx,[25–28] it is yet unclear if patients with ESRD are eligible for autologous nTreg therapy; in line, the impact of persistent alloactivation and standard maintenance immunosuppression on phenotypic and functional characteristics of circulating nTregs remains to be elucidated.[24,29–31]

Various protocols for adoptive nTreg therapy exist from stem-cell transplantation settings, in which usually a matched healthy donor is available and can serve as an eligible nTreg source. In contrast, in solid organ transplantation matched healthy Treg donors are usually not available, and several additional hurdles need to be overcome in order to manufacture autologous nTregs from chronically ill or immunosuppressed patients. Most importantly, although a third-party nTreg product is principally possible, the feasibility of finding a human leukocyte antigen–matched nTreg donor is structurally not guaranteed. Accordingly, protocols for nTreg therapy in solid organ transplantation are currently based on an autologous regimen, using *ex vivo* expanded nTregs. Therefore, we aimed to investigate whether patients with chronic ESRD are eligible for autologous nTreg therapy and how nTregs develop under immunosuppressive therapy. The latter is of particular importance in order to evaluate how adoptively transferred nTregs behave in immunosuppressed hosts and whether autologous nTreg therapy is also feasible at later stages post-transplantation. To this end, we first investigated circulating nTregs in patients with ESRD and further addressed the course of peripheral nTreg subsets in patients on immunosuppression. Prospectively, the implementation of adoptive nTreg therapy may enable us to significantly reduce or completely withdraw maintenance immunosuppressive therapy and minimize the risk of graft rejection, drug toxicity, and susceptibility to infections.

RESULTS

Despite CD4+ lymphocytosis in ESRD and posttransplantation, $CD4^+CD25^{high}FoxP3^+$ nTreg frequencies remain stable during disease and under maintenance immunosuppression

Lymphocyte analysis was performed in freshly collected blood from healthy controls (HCs), patients with ESRD, and KTx patients between 18 and 87 years of age (Figures 1 and 2a–l). Patient characteristics are listed in Table 1. All KTx patients received combination therapy for long-term maintenance immunosuppression. Two representative patients were chosen as examples to show the gating strategy (Figure 1) used for the results depicted in Figures 2–4. Total CD3+ lymphocyte frequencies were comparable between HCs and all patients groups (Figure 2a), whereas both ESRD and KTx patients showed mild CD4+ lymphopenia (Figure 2b). nTreg frequencies appeared to be slightly elevated in ESRD and KTx patients, although this effect was not statistically significant (Figure 2c). Because mTor inhibitors were previously postulated to increase the amount of circulating nTregs, we were further interested in the drug-specific effects of the immunosuppressive agents used for maintenance therapy. Interestingly, no drug-specific differences were observed. In particular, neither mTOR inhibitors nor calcineurin inhibitors, mycophenolate mofetil, steroids, nor azathioprine altered the frequencies of CD3+ (Figure 2e), CD4+ (Fig. 2f), or regulatory (Figure 2g) T cells *in vivo*. We then went on to assess nTreg maturation using CD45RA as a marker expressed by naïve, unresponsive T cells that have not yet encountered their TCR-specific antigen.[32] Here, KTx patients with maintenance immunosuppression tend to have increased naïve $CD45RA^+$ nTregs, indicating partially impaired nTreg maturation (Figure 2d). This phenomenon was again independent of the immunosuppressive agent (Figure 2h) and the number of previous acute rejection episodes (Figure 2i–l).

KTx patients show increased frequencies of naïve and effector memory nTregs, whereas central memory nTregs are reduced

To further investigate nTreg maturation, we assessed additional naïve and memory maturation states (Figure 3a–j). CD62L is expressed on naïve T cells; upon activation, CD62L mediates T-cell recruitment to peripheral lymphoid organs through high endothelial venules.[33] Subsequently, CD62L expression fades and CD62L- cells are considered as "antigen-experienced." Accordingly, whereas naïve, unresponsive cells are $CD45RA^+CD62L^+$ (T_{NAIVE}), central memory T cells have lost CD45RA expression and shifted to a $CD45RA^-CD62L^+$ (T_{CM}) phenotype. These cells can be recruited to lymph nodes where they mature to effector memory T cells, defined as $CD45RA^-CD62L^-$ (T_{EM}).[34,35] In our KTx patient cohorts, $nTreg_{NAIVE}$ (Figure 3a) and $nTreg_{EM}$ (Figure 3c) were elevated compared with HC and ESRD patients, whereas $nTreg_{CM}$ were substantially reduced (Figure 3b). These findings were independent of the maintenance immunosuppressive regimen (Figure 3d–f) and previous rejection episodes (Figure 3g–i). Drug-specific alterations were not observed, and although it seemed that the amount of $nTreg_{EM}$ correlates with the frequency of rejection episodes, this effect was not statistically significant (Figure 3i). Figure 3j summarizes T-cell subset markers and specific distributions of $nTreg_{NAIVE}$, $nTreg_{CM}$, and $nTreg_{EM}$ in our cohorts. In order to exclude that the observed maturation shifts of nTreg subsets in KTx patients are secondary, transplantation-associated effects due to