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Costimulatory blockade with mTor inhibition abrogates effector T-cell responses allowing regulatory T-cell survival in renal transplantation

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The authors with the exception of Dr Joseph Grinyó have no conflict of interest.

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Summary

The advent of novel immunosuppressive strategies in renal transplantation, with immunomodulatory properties, might facilitate long-term allograft survival. T-cell depletion, costimulation-blockade and mTor inhibition have been shown to favour anti-donor hyporesponsiveness. Recently, the combination of rATG, belatacept (Bela) and sirolimus (SRL) has been used in kidney transplantation, showing very low incidence of acute rejection and excellent 12-month graft and patient survival. Herein, we have analysed the 1-year evolution of memory/effector and regulatory T cells and assessed the donor-specific T-cell alloimmune response in a group of these patients and compared with others treated with a calcineurin-inhibitor(CNI)-based (rATG/tacrolimus/MMF), and two other Bela-based regimens (rATG/Bela/MMF and basiliximab/Bela/MMF/steroids). During the first year after transplantation, patients receiving rATG/Bela/SRL had significantly higher percentage of Tregs upon the memory T-cell compartment and showed a potent anti-donor suppressive activity. In an *in vitro* naive and memory/effector T-cell co-culture, the combination of costimulation-blockade and SRL could abrogate both antigen-specific T-cell responses as efficiently as using a CNI drug. The combination of T-cell depletion, costimulation-blockade and mTor inhibition seems to be able to allow Treg survival and inhibit donor-specific alloreactive effector immune responses after kidney transplantation in humans.

Introduction

The advent of novel immunosuppressive strategies in renal transplantation, with immunomodulatory properties, might facilitate longer-term allograft survival. It is well documented that T-cell depletion, costimulation-blockade and mTor inhibition favour tolerogenic mechanisms inducing anti-donor hyporesponsiveness both in animal models and in humans [1–4]. Likewise, regulatory T cells (Tregs) have been shown to be the hallmark of self-tolerance and a crucial mechanism for regulation of alloimmune responses in organ transplantation. Indeed, adoptive transfer of Tregs in murine models of allotransplantation has shown to be able to increase allograft acceptance [5]. In humans, enhanced presence of Tregs in renal transplant patients both in peripheral blood and directly within graft infiltrates seems to be favoured by some specific immunosuppressants such as mTor inhibitors and T-cell depletion agents [4–6].

During the last few years, novel immunosuppressants with new mechanisms of action have come out. Among them, belatacept (Bela) or LEA29Y has been developed as an immunosuppressive agent for solid organ transplantation in order to provide comparable efficacy to the

current standard cornerstone immunosuppressants, the calcineurin-inhibitors, but with an improved safety profile. This agent was developed as a modified molecule from CTLA4-Ig (abatacept) in order to increase the efficacy to efficiently abrogate the costimulatory signal between antigen presenting cells (APC) and T cells, mandatory for T-cell activation at the CD28-B7 (CD80/CD86) costimulatory level. In fact, the use of costimulatory blockade has already been performed using Bela combined with induction therapy with an anti-IL-2 receptor monoclonal antibody (basiliximab), mofetil mycophenolate (MMF) and steroids (ST) in clinical trials in renal transplantation. This immunosuppressive combination has shown to be safe for the prevention of acute rejection and allows better graft function and preserved graft parenchyma as compared with a cyclosporine-based regimen 1 year after transplantation [7]. Interestingly, although the blockade of the costimulatory interaction between CD28-CD80/CD86 results in T-cell anergy and transplantation tolerance in some animal models [1,2], it has also been demonstrated that costimulatory blockade at such level leads to a deleterious effect on Treg survival and function [8]. Recently, Bluestone *et al.* [9] have shown that Tregs from patients receiving belatacept and basiliximab (bxmab) suffer from a transient loss in the short-term after transplantation, but can maintain their suppressive function and numbers in the long term, similarly as baseline and as compared with patients treated with a calcineurin-inhibitor (CNI) agent such as cyclosporine, thus suggesting that this immunosuppressive combination would not be the most eligible one in tolerance-promoting immunosuppressive strategies.

Currently, a phase II exploratory study, CNI and steroid-free Bela-based immunosuppressive regimen is being performed in *de novo* renal transplant patients. In this study, Bela is combined with either MMF or sirolimus (SRL) using induction therapy with a T-cell depletion agent such as rATG (Thymoglobulin). These two groups (rATG/Bela/SRL and rATG/Bela/MMF) are compared with a control one based on a CNI-based regimen with tacrolimus (TAC) and MMF (rATG/TAC/MMF). Outstandingly, the 12-month reported evolution has shown a very low incidence of acute rejection in all groups (4%, 12% and 3%, respectively,) as well as an excellent 6-month graft function and patient survival [10]. Herein, we have immune-monitored a group of these patients and also compared it with the former Bela-based trial. We show that the immunosuppressive combination of T-cell depletion, costimulatory blockade and mTor inhibition seems to favour regulatory T-cell expansion in peripheral blood and efficiently abrogate anti-donor T-cell alloreactive immune responses *in vitro*. Thus, this new immunosuppressive approach might conciliate both abrogation of alloreactivity and promotion of alloimmune regulation.

Materials and methods

Patients and immunosuppression

Fourteen patients from our transplant centre receiving a primary renal transplant from two different phase II and III clinical trials of Bela were evaluated. Patients included in this study were all the enrolled participants from our transplant unit in each trial that biological samples from both donor and recipients could be obtained. The two different clinical protocols are summarized in Fig. 1 (protocol schema). Five patients of the first phase III trial receiving Bela-less intensive regimen (bxmab/Bela/MMF/ST) and nine patients of the CNI and steroid-free phase II Bela exploratory study were evaluated in this study; three patients of the SRL group (rATG/Bela/SRL), three patients receiving MMF (rATG/Bela/MMF) and three more patients of the control group receiving tacrolimus were included (rATG/TAC/MMF). As patients included in the phase II, CNI and steroid-free clinical trial had only achieved the first year after transplantation, we focused the immunemonitoring study in all patents until month 12 after transplantation. All patients were included and assessed by flow cytometry analysis evaluating the proportion of Tregs and memory T cells in peripheral blood. Also, the pretransplant anti-donor T-cell immune response as well as the suppressive function of Tregs of all these patients was assessed using an IFN-γ Elispot assay. Moreover, in order to evaluate the inhibitory effect of the different immunosuppressive drugs at the memory/effector and naïve T-cell compartments, an antigen-

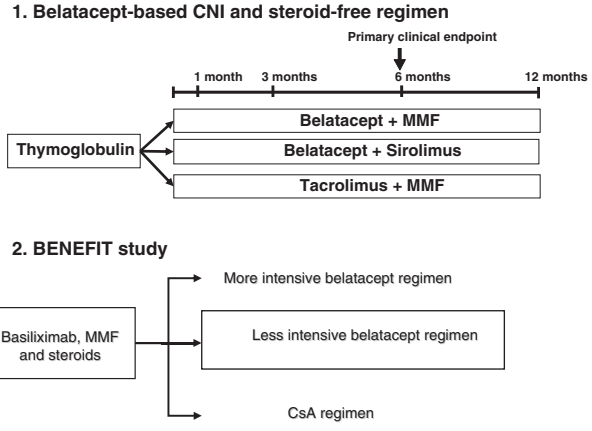

Figure 1 The two belatacept (Bela) clinical trials evaluated. We evaluated nine patients from the Bela-based CNI and steroid-free pilot study. Three patients of each group (rATG/Bela/MMF, rATG/Bela/SRL and rATG/TAC/MMF) were analysed. Also five patients of the less intensive arm of the Benefit study were studied.

specific memory/effector and naïve T-cell line co-culture was performed using PBMCs from healthy volunteers and analysed in an IFN-γ Elispot assay by adding in the mixed lymphocyte co-cultures the different immunosuppressants that these patients had received.

All patients agreed to participate in the study and signed an informed consent. The Ethics Committee of our Institution did also approve the study.

Donor and recipient cell isolation and FACS sorting

Donor splenocytes were frozen in liquid nitrogen and subsequently used as stimulator cells, after immunomagnetic depletion of CD2+ cells (Easysep human CD2 selection kit; Stem Cell Technologies, Grenoble, France). Recipient peripheral heparinized blood samples were collected and PBMC were obtained by standard Ficoll density gradient centrifugation, pretransplantation and at months 3, 6, and 12 after transplantation and then were frozen in liquid nitrogen. $CD4^{+}CD25^{high}CD127^{low/neg}$ Treg cells were isolated by cell sorting [Moflo (Dako-Diagnostico, Barcelona, Spain); purity >98%] and were used for functional assays.

Flow cytometric analysis of T-cell subsets

Recipient PBMC were stained with the following monoclonal Abs: PE anti-CD2 (RPA-2.10), APC anti-CD3 (UCHT1), PerCP-Cy5.5 anti-CD8 (RPA-T8), PE anti-CD20 (2H7), FITC anti-CD4 (RPA-T4), APC anti-CD25 (M-BC96), PerCP-Cy5.5 anti-CD127 (eBioRDR5), PE anti-Foxp3 (PCH101), APC anti-CD45RA (HI100), all stainings were carried out following the instructions of the manufacturer. All reagents belong to eBioscience (Barcelona, Spain). The samples were analysed by FACSCalibur (BD Bioscience, Barcelona, Spain). As negative control, samples were incubated with corresponding isotype controls. A representative dot plot is shown in Fig. 2.

IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay was performed as described previously in detail [4]. Briefly, 3×10^5 pretransplant recipient PBMC were stimulated with irradiated (40 Gy) donor and complete HLA class I and II mismatch third-party CD2-depleted splenocytes and placed in triplicate wells. Responder and stimulator cells were also tested with medium alone and PHA (Sigma-Aldrich, Madrid, Spain) in duplicate wells as negative and positive controls, respectively.

For the suppression assay, PBMC from healthy subjects were cultured as responder cells together with donor and third-party irradiated CD2-depleted splenocytes. FACS-sorted $CD4^{+}CD25^{high}CD127^{low/neg}$ Treg from patients were added to the culture at 1:1 ratio (Treg/control PBMC).

The resulting spots were counted using a computer-assisted ELISPOT reader (AID Elispot Reader 4-HR, Autoimmun Diagnostika, GmbH, Strassberg, Germany). Results were given as mean number of IFN-γ spots per 3×10^5 responder cells and were calculated by subtracting both the responder and the stimulator control wells. We considered 25 spots/3×10^5 responder cells as threshold to define a positive test.

Naive and donor-specific memory T-cell line co-cultures under different immunosuppressants

Although in *in vitro* co-cultures there is no protein binding and therefore the concentration of free drug is much higher than that observed *in vivo*, we tried to investigate the impact of these different immunosuppressant in inhibiting memory and naïve T-cell responses. For this purpose a short-term antigen-specific memory T-cell line was produced by mixing 2×10^6 PBMC from a responder individual (A), with 2×10^6 stimulator T-cell depleted PBMCs obtained from an allogeneic individual B (complete HLA A, B and DR mismatch) in 2 ml of complete medium (RPMI with 10% de-complemented FCS with antibiotics and L-glutamine) in a six-well cell culture plate. Negative and positive controls using only complete medium or phytohemagglutinin as stimulators were run in parallel. After incubation at 37 °C and 5% CO2 for 6 days, 3×10^5 of the resulting primed responder PBMCs were tested for alloreactivity against 3×10^5 T-cell-depleted PBMCs from the same donor (B) in an IFN-γ ELISPOT assay. In parallel wells, different immunosuppressive drugs such as TAC, SRL, CTLA4-Ig and its combinations were added in the co-culture at the equivalent doses from those received by the patients *in vivo*. Also, the effect of such immunosuppressants was assessed in a naive T-cell co-culture by adding the same immunosuppressive drugs to a co-culture of the same previously used healthy individuals A and B in an IFN-γ ELISPOT assay but without any previous sensitization. As Bela is not available yet, its homologue CTLA4-Ig was used in order to assess the costimulatory blockade effect. Results of this experiment are shown by means and standard deviation of three different responder and stimulator individuals.

Limitation of the study

The main limitation of this study is the very low number of subjects that could be evaluated among these different Bela-based immunosuppressive regimens.

Statisitics

Data are presented as median and range and mean and standard deviation. Groups were compared using the

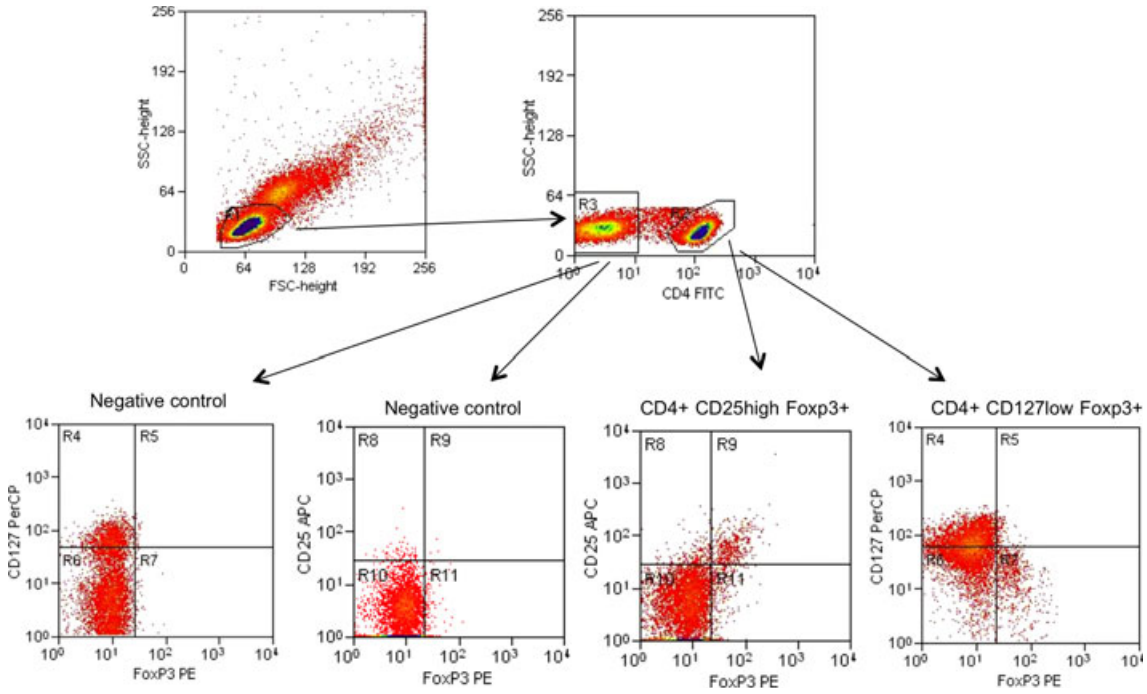

Figure 2 Representative dot plot used for the cytometric analysis of Foxp3+Tregs. Negative controls are also shown.

chi-squared test for categorical variables, the Kruskal–Wallis or Mann–Whitney *U*-test for nonparametric and non-normally distributed variables. The statistical significance level was defined as $P < 0.05$.

Results

Patient baseline characteristics

Demographic characteristics are shown in Table 1. None of the evaluated patients experienced biopsy-proven acute rejection. According to the 12-month reported results of the whole study (10), here the rATG/Bela/SRL and rATG/Bela/MMF groups did also show numerically better graft function than the TAC group. Interestingly, within this small group of patients no major adverse events were reported during the 1-year follow-up such as cardiovascular events, infectious or malignancies.

One-year effect of different Bela immunosuppressive combinations in circulating FOXP3+ regulatory and memory T cells

We first evaluated the effect of different Bela immunosuppressive combination regimens (bxmab/Bela/MMF/ST, rATG/Bela/SRL, rATG/Bela/MMF and rATG/TAC/MMF) in the number of $CD4^+$Foxp3+ Tregs circulating in peripheral blood during the first year after transplantation. We analysed Foxp3+Tregs by staining the Tregs in the $CD4^+CD25^{high}$ and $CD4^+CD127^{low}$ T-cell compartments at baseline, 3, 6 and 12 months after transplantation. As shown in Fig. 3, the number of Foxp3+Treg, both in the $CD4^+CD127^{low}$ and $CD4^+CD25^{high}$ compartments in the group of patients receiving rATG/Bela/SRL were numerically higher in all time points of follow-up than baseline and as compared with the bxmab/Bela/MMF/ST and the CNI-based treated patients. Interestingly, at 3 months after transplantation the group of patients receiving rATG/Bela/MMF did show similar counts of Tregs than the SRL-treated patients, and were also higher than the other two evaluated groups. Thereafter, in this particular group, Tregs progressively decreased achieving the same numbers than the bxmab/Bela/MMF/ST and the CNI-based treated patients.

We next analysed the number of memory T cells in the $CD4^+$, $CD8^+$ and the $CD3^+$ T-cell subpopulation. As shown in Fig. 4, no differences were observed at any time point between therapies. Nevertheless, when the ratio between Treg/memory T cells was evaluated, a significantly higher ratio was appreciated in the rATG/Bela/SRL as compared both to the baseline and to the other treatment groups during all time points (Fig. 5). Again, at 3 months after transplantation, the rATG/Bela/MMF group did show a higher ratio of Treg/memory T cells as compared with bxmab/Bela/MMF/ST and the TAC-treated patients, returning to the baseline numbers thereafter. Interestingly, it is to note that despite the low

Table 1. Demographic characteristics.

Variables	rATG/TAC/MMF (n = 3)	rATG/Bela/SRL (n = 3)	rATG/Bela/MMF (n = 3)	Bxmab/Bela/MMF/ST (n = 5)	P
Donor age (years)	48 ± 8	47 ± 2	49 ± 4	50 ± 3	NS
Donor gender (M/F)	2/1	1/2	1/2	3/2	NS
Recipient age (years)	54 ± 4	44 ± 8	51 ± 7	44 ± 8	NS
Recipient gender (M/F)	2/1	2/1	1/2	3/2	NS
HLA mismatches A, B, DR (mean ± SD)	3.1 ± 3	2.9 ± 3	3.3 ± 3	2.8 ± 4	NS
DGF (no/yes)	(3/0)	(3/0)	(2/1)	(4/1)	NS
Acute rejection (no/yes)	(3/0)	(3/0)	(3/0)	(5/0)	NS
Mean sCreat (μmol/l)					
Month 3	172 ± 25	103.2 ± 34.2	125.6 ± 25.8	141.6 ± 25.3	NS
Month 6	167 ± 30	87.3 ± 12.6	110 ± 21.1	141.8 ± 18.2	NS
Month 12	157 ± 34	91 ± 23.2	125 ± 16.4	136.6 ± 27.6	NS
Mean eGFR (ml/min)					
Month 3	38 ± 10	60.5 ± 21.3	54.0 ± 24.1	38 ± 4.3	NS
Month 6	39.8 ± 16	65.7 ± 10.3	60.3 ± 20	43.5 ± 2.3	NS
Month 12	41.6 ± 21	66.2 ± 17.9	51.9 ± 18.1	45.1 ± 8.4	NS

rATG, rabbit ATG; TAC, tacrolimus; Bela, belatacept; SRL, sirolimus; MMF, mycophenolate mofetil; Bxmab, basiliximab; ST, steroids; DGF, delayed graft function; sCreat, serum creatinine; eGFR, glomerular filtrate rate.

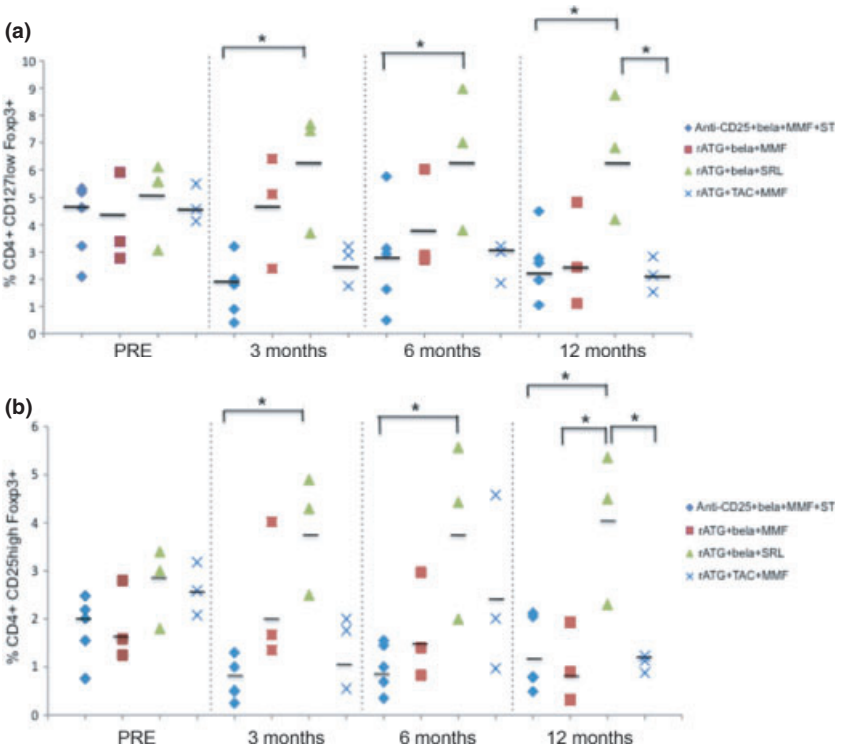

Figure 3 One-year evolution of Tregs in peripheral blood. (a) One-year evolution of $CD4^+CD127^{low}$Foxp3+ Tregs SRL-treated patients. SRL-treated patients showed higher Foxp3+Tregs at the $CD4^+CD127^{low}$ compartment as compared with the other groups after transplantation (at month 3: $P < 0.05$ versus Bxmab and TAC groups; at month 6: $P < 0.09$ versus Bxmab, MMF and TAC; at month 12: $P < 0.07$ versus Bxmab, MMF and TAC). (b) One-year evolution of $CD4^+CD25^{high}$Foxp3+ Tregs. Among the patients, the SRL-treated patients showed higher number of Foxp3+Tregs at the $CD4^+CD25^{high}$ compartment as compared with the other groups after transplantation (at month 3: $P < 0.08$ versus Bxmab and TAC groups; at month 6: $P < 0.079$ versus Bxmab, MMF and TAC; at month 12: $P < 0.04$ versus Bxmab, MMF and TAC). * denotes the P value between the groups is <0.05.

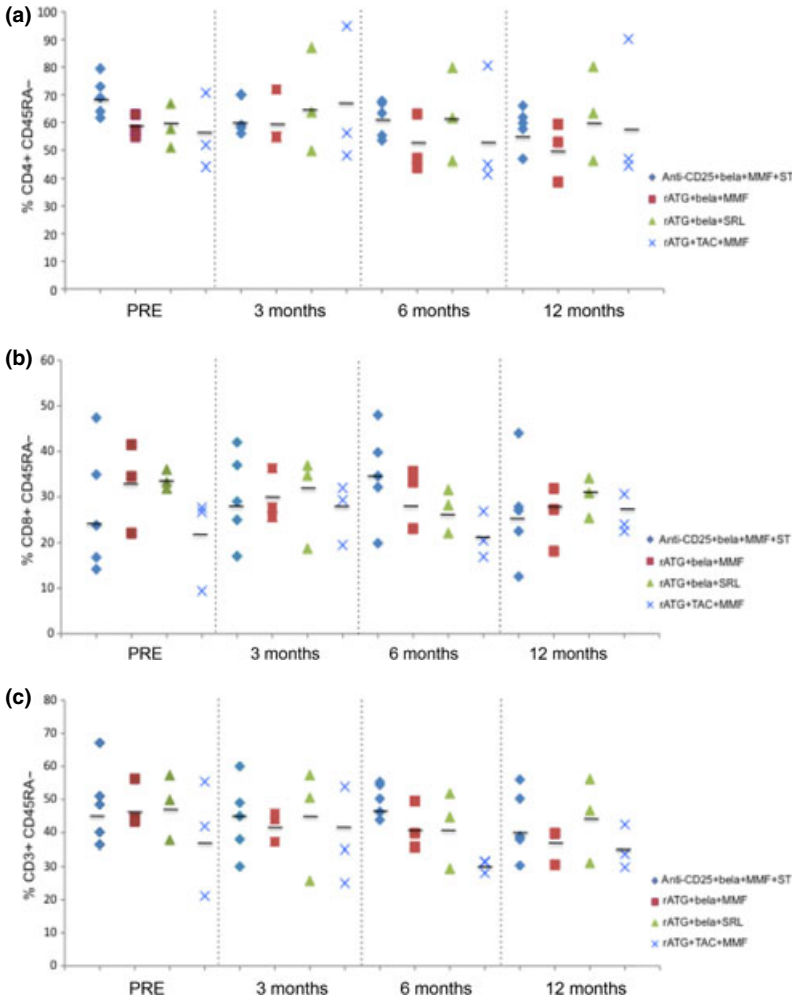

Figure 4 One-year evolution of the memory T-cell compartments in peripheral blood. No differences in the number of circulating memory T-cells within the different immunosuppressive groups were observed. (a) Evolution in the $CD4^+$ T-cell compartment. (b) Evolution in the $CD8^+$ T-cell compartment. (c) Evolution of total memory T cells.

number of patients evaluated, when analysing the ratio between Treg/memory T cells, the differences between groups were remarkably higher than when only measuring the number of Tregs.

Suppressive Treg function under different Bela immunosuppressive combinations

As shown in Fig. 6, Tregs from all groups were capable to inhibit IFN-γ release in all time points. However, while Tregs from rATG/Bela/SRL and rATG/Bela/MMF groups could inhibit between 40% and 50% of cytokine production, the bxmab/Bela/MMF/ST and the rATG/TAC/MMF regimens suppressed between 20% and 30% of IFN-γ release. In almost all evaluated cases, Treg suppression seemed to be higher at later time points. However, the suppressive activity of Tregs from the CNI and bxmab/Bela/MMF/ST-treated patients appeared to be slightly lower as compared with their pretransplant inhibitory capacity.

Pretransplant donor-specific memory/effector T-cell immune monitoring

As reproduced in Fig. 7, some patients from each group did show the presence of highly alloreactive donor-specific memory/effector T cells circulating in peripheral blood. All patients showed a significant alloresponse against a third-party stimulator (data not shown). Despite the presence of highly alloreactive donor-specific memory/effector T cells circulating in peripheral blood among this group of patients receiving different immunosuppression, none of them did display acute clinical rejection during the first year after transplantation.

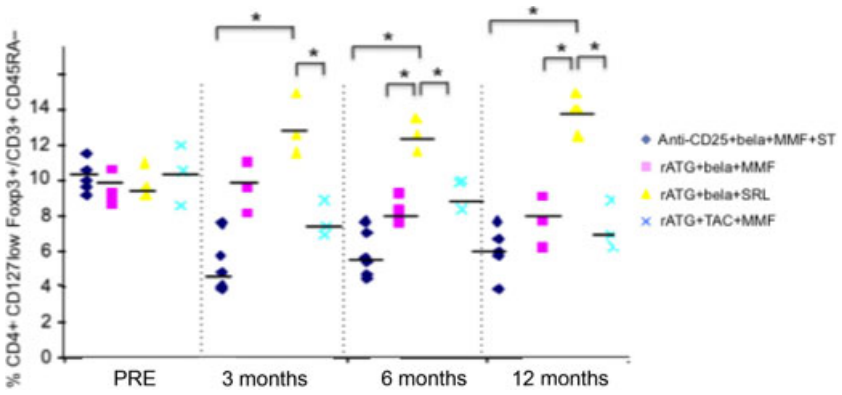

Figure 5 One-year evolution of the ratio Treg/memory T cells in peripheral blood. Among the patients, the SRL-treated patients showed a significantly higher ratio of Treg/memory T cells as compared with the other groups after transplantation (at month 3: $P < 0.013$ versus Bxmab and TAC groups; at month 6: $P < 0.01$ versus Bxmab, MMF and TAC; at month 12: $P < 0.022$ versus Bxmab, MMF and TAC). At 3 months after transplantation, the rATG/Bela/MMF group did show a higher ratio of Treg/memory T cells as compared with bxmab/Bela/MMF/ST and the TAC-treated patients ($P < 0.04$), returning to the baseline numbers and similarly to the Bxmab and TAC-treated patients thereafter. * denotes the *P* value between the groups is <0.05.

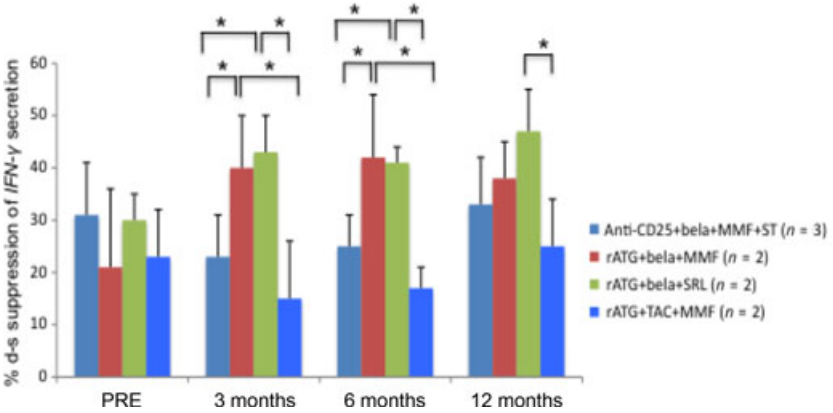

Figure 6 Percentage of anti-donor suppressive activity of Tregs from different immunosuppressive regimens in allogeneics co-culture at different time-points. Tregs from all groups were capable to inhibit IFN-γ release in all time points. Tregs from rATG/Bela/SRL and rATG/Bela/MMF groups could inhibit between 40% and 50% of cytokine production as compared with bxmab/Bela/MMF/ST ($P < 0.05$ at 3 and 6 months, P = NS at 12 months) and especially to rATG/TAC/MMF regimens ($P < 0.05$ versus SRL at all time points and $P < 0.05$ versus MMF at 3 and 6 months but not at 12), which suppressed between 20% and 30% of IFN-γ release. In almost all evaluated cases, Treg suppression seems to be higher at a later time points (P = NS). * denotes the *P* value between the groups is <0.05.

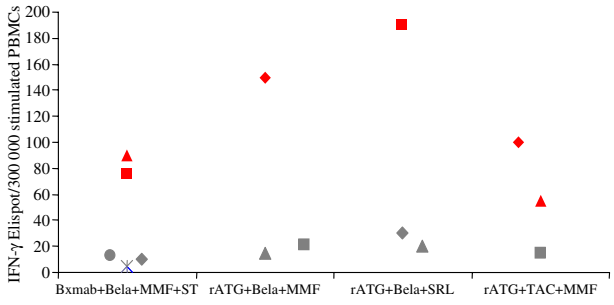

Figure 7 Pretransplant donor-specific and anti-third party IFN-γ ELISPOT responses. Black spots show donor-specific IFN-γ alloresponses and grey spots are respective anti-third-party alloresponses of each patient. Broken line at 25 spots/300 000 stimulated PBMC represents the threshold considered as a significant detectable alloresponse [3].

In vitro evaluation of costimulatory blockade immunosuppressive combinations on circulating memory/effector and naive T cells

As shown in Fig. 8, only TAC and the combination of costimulatory blockade with SRL, were potent enough for an efficient inhibit between 70% and 90% of both naive and antigen-specific memory/effector T cell alloresponses *in vitro*.

Discussion

In this study we show how different Bela-based immunosuppressive combinations may differently impact memory and regulatory T-cell subsets in renal transplant patients.

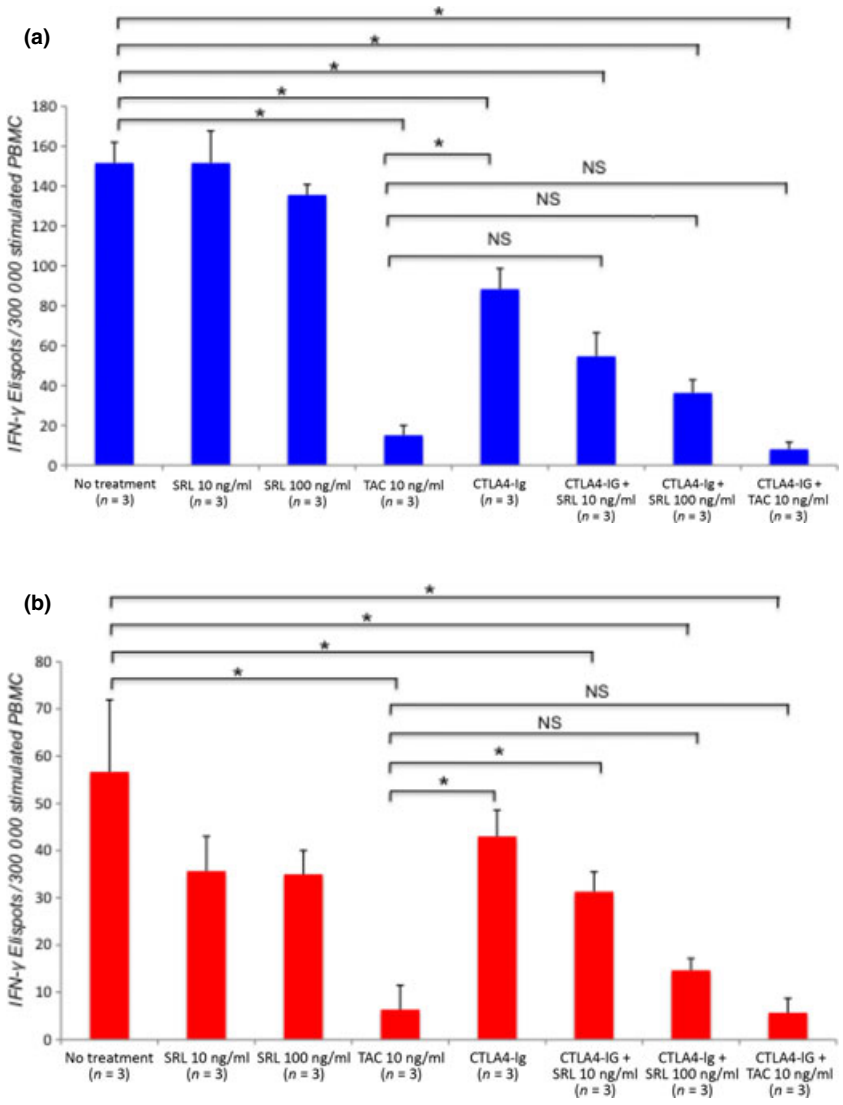

Figure 8 Naive and memory T-cell co-culture under different immunosuppressants. Tacrolimus and the combination of SRL and CTLA4-Ig were able to efficiently suppress both naïve and antigen-specific memory/effector alloresponses. (a) Inhibitory effect of different immunosuppressants to an antigen-specific memory/effector T-cell line co-culture. (b) Inhibitory effect of different immunosuppressants to a naive T-cell co-culture. * denotes the *P* value between the groups is <0.05.

In fact, we confirm previous data showing that Bela given with an anti-CD25 monoclonal antibody as induction therapy impairs Foxp3+Treg survival, especially during the first 6 months after transplantation. Conversely, we illustrate for the first time that the combination of Bela with SRL and rATG induction seems to favour Foxp3+-Treg survival upon the memory T-cell compartment after transplantation. Furthermore, when costimulatory blockade and SRL are tested together in an *in vitro* co-culture, this combination is able to significantly abrogate both donor-specific memory and naïve effector T-cell responses similarly, than a potent CNI drug.

Despite the small number of patients evaluated, here we show that memory T-cell numbers do not seem to be significantly affected by any of these immunosuppressive strategies. Conversely, the particular combination of rATG induction with Bela and SRL seems to be able to not only maintain but also increase Treg numbers over time, suggesting an SRL-mediated Treg expansion effect. This phenomenon becomes even clearer when the ratio between Treg/memory T cells is analysed. Interestingly, the group of patients receiving rATG/Bela/MMF was also able to show a transient increased numbers of Tregs until month 3 after transplantation. The potential

explanation could be based on the favouring effect of rATG in the generation and expansion of adaptive Treg only during T-cell homeostatic proliferation and reconstitution [11]. However, our findings suggest that maintenance therapy with MMF and Bela does not seem to have the capacity to sustain this Treg expansion with time.

Indeed, and different from mTOR inhibitors and T-cell depletion agents [3,11,12], both the CD28-CD80/86 costimulation and CD25 blockades have been shown in animal models and humans to have a negative effect on Treg survival and function [10,13,14] as both IL-2 and CD28 seem to be relevant factors for Treg survival [8,15,16]. Therefore, induction with rATG in combination with SRL and the less intensive Bela regimen, which allows a not entire saturation of the CD80/CD86 receptors in APC [9], could counterbalance to some extent this deleterious effect on the Treg subset population.

Recently, Bluestone *et al.* [9] evaluated the impact on Tregs of the immunosuppressive combination of basiliximab, Bela, MMF and steroids compared with a CNI-based regimen. They showed a significant loss of $CD4^+CD25^+$Foxp3+Tregs in both groups during the first 3 months after transplantation, with a progressive recovery of this T-cell subset, achieving the same baseline numbers, suggesting an anti-CD25 depletion-mediated effect. Likewise, in our study we observe the same phenomenon in this group of patients, suggesting thus, that Bela does not seem to favour Treg expansion but allows Treg survival, at least at such doses.

Although circulating Tregs from all groups showed preserved anti-donor suppressive activity during the 1-year follow-up, the rATG/Bela/SRL and rATG/Bela/MMF-treated patients seemed to show increased inhibitory capacities than the other two groups when co-cultured at the same ratio with responder cells. These results suggest that human Tregs might potentially be less sensitive to CD28 costimulatory blockade than mice Tregs and as pointed out before, the not so complete saturation of both CD80/CD86 receptors may permit Treg survival. Moreover, the fact that Tregs from SRL-treated patients seems to show an increased ability for alloimmunosuppression and found in higher numbers in peripheral blood, might suggest that the rATG/bela/SRL combination is more likely to favour regulatory functions after transplantation than the other immunosuppressive strategies.

Despite the dramatic impact of the inhibition of CD28 signalling on allograft survival in many animal models [17,18], in which naive T cells are the main T-cell subset population to overcome, the more limited effect of such blockade on effector/memory T cell responses because of the less need of co-stimulation of this T-cell subset for activation, could raise important concerns regarding its efficacy in humans [19]. In fact, it is well known that CNI are the most robust drugs to suppress alloreactive effector/memory T cells [20]. As mentioned before, in the Bela-based, CNI and steroid-free pilot study the incidence of 12-month biopsy-proven acute rejection (BPAR) was remarkably low, and especially within the rATG/Bela/SRL combination [10]. In this direction, none of our 14 analysed patients experienced any BPAR but interestingly, some patients did show highly alloreactive donor-specific memory/effector T cells circulating in peripheral blood pretransplantation. Therefore, we wondered whether an efficient inhibitory effect on alloreactive effector/memory T-cells was produced after the administration of these drug combinations. To answer this question, we performed an *in vitro* antigen-specific naïve and memory/effector T cell line and added such immunosuppressants at the equivalent clinically given doses and assessed the *in vitro* inhibition of such drugs at the two different T-cell subset compartments. As expected, TAC was capable to efficiently abrogate both naive and alloreactive effector/memory T-cell responses. Conversely, SRL and costimulation-blockade alone were not potent enough to inhibit such T-cell alloimmune responses and especially the effector/memory one. Outstandingly, when SRL and costimulation-blockade were given in combination, both the naive and the effector/memory T-cell response were as deeply inhibited as with TAC, suggesting that this potent immunosuppressive effect shown *in vitro* could explain the extremely low incidence of BPAR among this group, and even in highly pre-transplant T-cell sensitized patients.

In summary, the combination of T-cell depletion, costimulation-blockade and mTor inhibition seems to be able to efficiently inhibit alloreactive T-cell immune responses and allowing Treg survival after kidney transplantation, suggesting an interesting immunosuppressive combination for allogeneic organ transplantation. In addition, although we are very conscious that our observations are based on a small cohort of patients thus, potentially influencing the results shown here, we believe that these preliminary data may open a new perspective for further exploring the immunomodulatory effects of such combinations in renal transplantation.

Authorship

OB: participated in research design, in writing the paper and in data analysis. LC, MlaF and ML: participated in performance of the research. JMC: participated in research design and in writing the paper. JT and SG-V: participated in writing the paper.

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