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ORIGINAL ARTICLE

Impact of *Basiliximab* on regulatory T-cells early after kidney transplantation: down-regulation of CD25 by receptor modulation

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Summary

Monoclonal anti-CD25-antibodies are successfully applied in organ transplantation to reduce the incidence of acute graft rejection. However, targeting the CD25 molecule might not only affect activated T-cells but also regulatory T-cells (T_{regs}) constitutively expressing the $CD4^+CD25^+CD127^{low}FoxP3^+$ phenotype. In this study, we investigated the influence of the anti-CD25-antibody *Basiliximab* on the frequency of T_{regs} early after kidney transplantation comparing individuals receiving/not receiving induction therapy ($n = 14$ and $n = 7$). Following *Basiliximab* administration, a distinct loss of $CD4^+CD25^{high}$ T-cells was observed lasting for at least 6 weeks. This was not accompanied by a disappearance of the entire $CD4^+CD25^+FoxP3^+$ T_{regs} but rather a decreased expression density of CD25 on the latter. In addition, a transient rise in $CD4^+CD25^-FoxP3^+$ T-cells was found which expressed the $CD127^{low}$ phenotype. Thus, a phenotypic shift of T_{regs} from the $CD25^+$ to the $CD25^-$ compartment was suggested. This was supported by *in vitro* findings showing that the disappearance of $CD4^+CD25^{high}$ cells in the presence of *Basiliximab* was due to down-regulation of CD25 expression meanwhile the suppressive function of these cells was maintained. In conclusion, *Basiliximab* therapy directly affects $CD4^+CD25^+CD127^{low}FoxP3^+$ T_{regs} but does not seem to be associated with functional consequences. Thus, it is unlikely that *Basiliximab* treatment negatively influences strategies involving T_{regs} to promote tolerance after organ transplantation.

Introduction

Acute graft rejection (AR) is a well-known immunological phenomenon following solid organ transplantation (Tx). Proliferation of activated T lymphocytes in response to the transplant is an essential step in the development of AR and usually is triggered by binding of interleukin-2 (IL-2) to the multi-subunit IL-2 receptor (IL-2R). The α-chain of the IL-2R is known as CD25 and is expressed on the surface of T-cells upon activation. Blocking of this receptor specifically interferes with the cellular immune response. In consequence, anti-CD25-antibodies have been developed as a novel immunosuppressive strategy [1–4].

However, the CD25 molecule is not solely expressed on activated/effector T-cells (T_{eff}), but also on regulatory T-cells (T_{regs}) constitutively expressing the $CD4^+CD25^{high}$ phenotype [5]. T_{regs} have been shown to play a major role in the development and maintenance of transplantation tolerance [6–8] and adoptive transfer of T_{regs} can lead to enhanced graft acceptance [9]. Thus, much effort is spent on research of specific cell markers for regulatory T-cells

to allow their phenotypic discrimination from activated T_{eff}. Identification of the transcription factor forkhead box P3 (FoxP3) [10] and more recently the down-regulation of the IL-7 receptor (CD127) on T_{regs} [11,12] have provided adequate tools to do so. Nevertheless, due to high expression of CD25, questions are raised whether the treatment with anti-CD25-antibodies might not only affect activated T effector cells, but also T_{regs} of the $CD4^+CD25^+CD127^{low}FoxP3^+$ phenotype. As IL-2 has been shown to be essential for the peripheral generation of T_{regs} [13], blocking of the IL-2R might even decide on the development of transplantation tolerance and thus the long-term outcome of Tx.

Following kidney transplantation, acute rejection episodes can efficiently be prevented by induction therapy with the anti-CD25-antibody *Basiliximab* [14,15]. *Basiliximab* is a chimeric mouse-human monoclonal antibody (mAb) which binds with high specificity and affinity to the α-subunit of the IL-2R. Its application has been reported to be associated with at least a transient reduction of peripheral regulatory T-cells [16,17]. Apart from inhibition of IL-2 binding with subsequent interference of downstream cascades mediated by IL-2/IL-2R signaling [18], the exact mechanism of action of *Basiliximab* is still incompletely understood. Antibody-directed cellular cytolysis and complement-mediated cytotoxicity in T-cells are possible mechanisms induced by antibody therapy, but remain questionable in this context [3].

The aim of the following study thus was to investigate the influence of *Basiliximab* administration on the frequency of regulatory T-cells early after living donor kidney transplantation. For this, transplanted patients who obtained induction therapy were compared to patients similarly receiving kidney allografts but without *Basiliximab* treatment as part of the initial immunosuppressive regimen. *In vitro* experiments were performed to further elucidate how *Basiliximab* affects regulatory T-cells.

Material and methods

Patients and immunosuppression

All patients ($n = 21$) received primary kidney allografts from living related (LRD) and unrelated (LUD) donors at Hannover Medical School, Germany. Detailed patient characteristics and transplantation information are summarized in Table 1. The *Basiliximab*-group consisted of $n = 14$ patients (LRD: five, LUD: nine) and obtained induction therapy with two 20 mg doses of *Basiliximab* (Simulect®; Novartis, Basel, Switzerland) on days 0 and 4 of transplantation combined with a triple immunosuppressive regimen made up of a calcineurin inhibitor (CNI), mycophenolate mofetil (MMF) and steroids. The

Table 1. Demographic and clinical data of transplanted patients.

#	Age (years)	Sex	Grafts	Donor	Mismatch	Acute rejection (time after Tx)	Immunosuppression
1	35	F	1	LRD	1-1-1	no	Bas, CyA, MMF, Pred
2	68	M	1	LUD	2-2-2	no	Bas, CyA, MMF, Pred
3	24	M	1	LRD	1-1-0	no	Bas, CyA, MMF, Pred
4	42	M	1	LUD	1-1-2	no	Bas, CyA, MMF, Pred
5	57	F	1	LUD	1-2-2	BL (day 10)	Bas, Tacro, MMF, Pred
6	56	M	1	LUD	1-1-2	Banff 2a (day 64)	Bas, Tacro, MMF, Pred
7	42	M	1	LRD	0-0-0	no	Bas, Tacro, MMF, Pred
8	43	F	1	LUD	2-2-1	no	Bas, Tacro, MMF, Pred
9	44	M	1	LUD	1-1-2	no	Bas, Tacro, MMF, Pred
10	60	M	1	LUD	1-2-0	no	Bas, Tacro, MMF, Pred
11	25	M	1	LRD	1-1-1	BL (day 9)	Bas, Tacro, MMF, Pred
12	43	M	1	LRD	0-1-1	BL (day 88)	Bas, Tacro, MMF, Pred
13	49	M	1	LUD	1-1-2	no	Bas, Tacro, MMF, Pred
14	39	M	1	LUD	1-2-2	no	Bas, Tacro, MMF, Pred
15	21	F	1	LRD	1-2-2	no	Tacro, MMF, Pred
16	23	M	1	LRD	1-1-1	BL (day 7)	Tacro, MMF, Pred
17	29	F	1	LRD	1-1-0	no	Tacro, MMF, Pred
18	53	M	1	LUD	1-2-1	Banff 1a (day 12)	Tacro, MMF, Pred
19	41	M	1	LRD	1-2-2	BL (day 7)	Tacro, MMF, Pred
20	67	M	1	LUD	2-2-2	BL (day 6)	Tacro, MMF, Pred
21	43	M	1	LUD	1-1-2	no	Tacro, MMF, Pred

LRD, living related donor; LUD, living unrelated donor; BL, borderline; Bas, basiliximab; CyA, cyclosporine; Tacro, tacrolimus; MMF, mycophenolat mofetil; Pred, prednisolon.

transplant control group of $n = 7$ patients (non-*Basiliximab*; LRD: four, LUD: three) received triple immunosuppression, but induction therapy was not performed. All patients gave written informed consent to participate in this study. The protocol was approved by the local ethical commission.

Kidney biopsy and acute rejection

All patients received kidney biopsy upon clinical presumption of AR such as insufficient decline or sudden rise of serum creatinine. Furthermore, all patients were included in the protocol biopsy program with additional biopsies taken 6 weeks and 3 months after transplantation. Biopsy-proven AR was diagnosed histologically according to the latest Banff classification [19]. Borderline (BL) changes were regarded and treated as rejection if associated with a creatinine increase of ≥20%. In all cases of clinical and subclinical rejection, treatment of AR and BL was successfully performed by three high-dose steroid pulses.

Transplant information

Successful kidney transplantation was performed in all cases with initial function, no grafts were lost. The mean cold ischaemic time for living donation was 2 h 15 min ± 22 min. with no significant differences between the two groups (2 h 17 min ± 21 min vs. 2 h 11 min ± 27 min). Postoperatively, the patients' serum creatinine declined continually (Table 2). For all patients, the creatinine levels 3 months post-Tx were significantly reduced compared to the pretransplant status (174 ± 51 vs. 715 ± 206, $P < 0.001$). There were no significant differences in the total number of HLA mismatches (HLA-A, -B, -DR loci) between *Basiliximab* versus non-*Basiliximab* treated patients (3.6 ± 1.6 vs. 4.1 ± 1.3). The incidence of biopsy proven AR and BL was increased in the non-*Basiliximab* group (14% vs. 7% and 43% vs. 21% for AR and BL, respectively), but not regarded statistically significant due to the small number of cases. The course of peripheral lymphocytes in transplanted patients was comparable in both groups (Table 2).

Table 2. Creatinine levels and lymphocyte counts.

Time after Tx	Serum creatinine (μmol/l)		Blood lymphocytes (cells/μl × 10^3)	
	Basiliximab	Non-*Basiliximab*	*Basiliximab*	Non-*Basiliximab*
Pre-Tx	702 ± 245	750 ± 67	1.4 ± 0.5	1.6 ± 0.6
Day 1	385 ± 162	305 ± 204	0.6 ± 0.3	0.7 ± 0.5
Week 2	154 ± 64	136 ± 42	1.3 ± 0.7	1.3 ± 0.9
Week 4	150 ± 40	136 ± 43	1.5 ± 1.0	1.9 ± 1.6
Week 6	156 ± 60	143 ± 39	1.8 ± 1.1	2.3 ± 1.7
Month 3	173 ± 57	176 ± 38	1.4 ± 0.5	1.7 ± 0.9

Basiliximab group with $n = 14$, non-*Basiliximab* group with $n = 7$; Data presented as mean ± SD.

Sample collection and cell isolation

Heparinized blood samples were collected preoperatively as well as 1 day, 2 weeks, 4 weeks, 6 weeks and 3 months following transplantation. All samples were processed within 3 h. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation. PBMCs were frozen in RPMI 1640 containing 20% FCS and 10% DMSO and stored in liquid nitrogen.

Flow cytometry

Frozen PBMCs were thawed, cultured overnight and analysed by multi-colour flow cytometry on a BD FACSCalibur. Cell surface staining was performed using the following mAb: CD4-FITC (clone: RPA-T4), CD25-PE (clone: M-A251, both BD Biosciences, San Jose, CA, USA) and CD127-PE-Cy5 (clone: eBioRDR5; eBioscience, San Diego, CA, USA). Intracellular staining was performed according to the manufacturer's instructions using the FoxP3 Flow Kit (clone: 206D, isotype control: MOPC-21; BioLegend, San Diego, CA, USA).

Basiliximab in vitro assays

For investigation of the underlying mechanism of action of *Basiliximab*, freshly isolated PBMCs from healthy volunteers were stained for CD4 and CD25 (see above). The $CD4^+CD25^{high}$ subpopulation was separated from PBMCs by cell-sorting on a BD FACSAria. Sorted $CD4^+CD25^{high}$ cells were dye-labeled to allow cell-tracking (CellVue Maroon Kit; Polysciences, Warrington, PA, USA) and resuspended into the original PBMC pool at the initial concentration. Cells were then cultured in supplemented RPMI 1640 containing 1.25 μg/ml of *Basiliximab* at 37 °C. Following 48 h of incubation, cells were stained for CD4 and CD25.

For further functional testing of modulated T_{regs} cells were sorted following *Basiliximab* preincubation as described above. Suppression assays were set up with 2×10^4 $CD4^+CD25^-$ responder cells stimulated using CD3/CD28 beads and cultured for 4 days with *Basiliximab* treated/untreated T_{regs}. Proliferation was then measured by incorporation of ^{3}H-Thymidin within 16 h. Furthermore, CD25 mRNA expression within these T_{regs} was analysed by Real-time-PCR.

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Statistical analysis

Statistical analysis of clinical and experimental data was performed between the groups and/or to pretransplant levels using SPSS 16.0.1 (SPSS Inc., Chicago, IL, USA). The unpaired t-test and Mann–Whitney *U*-test were applied as appropriate. Differences were regarded statistically significant with $P < 0.05$.

Results

Effect of Basiliximab therapy on the frequency of peripheral CD4$^+$ T-cells co-expressing CD25

Monitoring of CD4$^+$CD25$^+$ T-cells in the peripheral blood of kidney transplanted patients receiving *Basiliximab* therapy revealed a significant reduction in the frequency of this subset already 1 day after the first application of anti-CD25-mAb (31.5% vs. 21.2% of CD4$^+$ T-cells for pre-Tx and day 1, respectively, $P = 0.007$). The frequency of these cells continued to decrease for up to 4 weeks with a continuous reconstitution thereafter. 3 months after Tx the frequency of CD4$^+$CD25$^+$ T-cells was almost re-established to pre-Tx counts (Fig. 1a). In comparison, this effect was not observed in the non-*Basiliximab* treated individuals: apart from a slight drop on day 1 after Tx, a stable frequency of the CD4$^+$CD25$^+$ T-cells was noted during the entire follow-up. The transient difference in the frequency of CD4$^+$CD25$^+$ T-cells between the two groups was statistically significant ($P < 0.001$). The reduced frequency of CD4$^+$CD25$^+$ T-cells in *Basiliximab* treated individuals might be due to blocking of the diagnostic anti-CD25-mAb in the presence of the therapeutic antibody. However, this possibility was excluded by *in vitro* blocking experiments revealing no influence of *Basiliximab* bound to CD25 on subsequent staining using the CD25-PE mAb (data not shown).

Recipients receiving *Basiliximab*-therapy showed an almost complete disappearance of CD4$^+$CD25^{high} T-cells within 4 weeks after anti-CD25-mAb application (Fig. 1b). This effect was not seen in patients of the non-*Basiliximab* group (data not shown). Thereafter, in accordance with the reconstitution of the CD4$^+$CD25$^+$ T-cell frequency, a reappearance of this CD25^{high} expressing subpopulation was found. Thus, the reduction of CD4$^+$CD25$^+$ T-cells in patients of the *Basiliximab* group for the most part was addressed to a loss of regulatory T-cells of the CD4$^+$CD25^{high} phenotype.

Basiliximab therapy decreases the expression density of CD25 on regulatory T-cells

To further elucidate whether the transient disappearance of the CD4$^+$CD25^{high} T-cells was due to elimination,

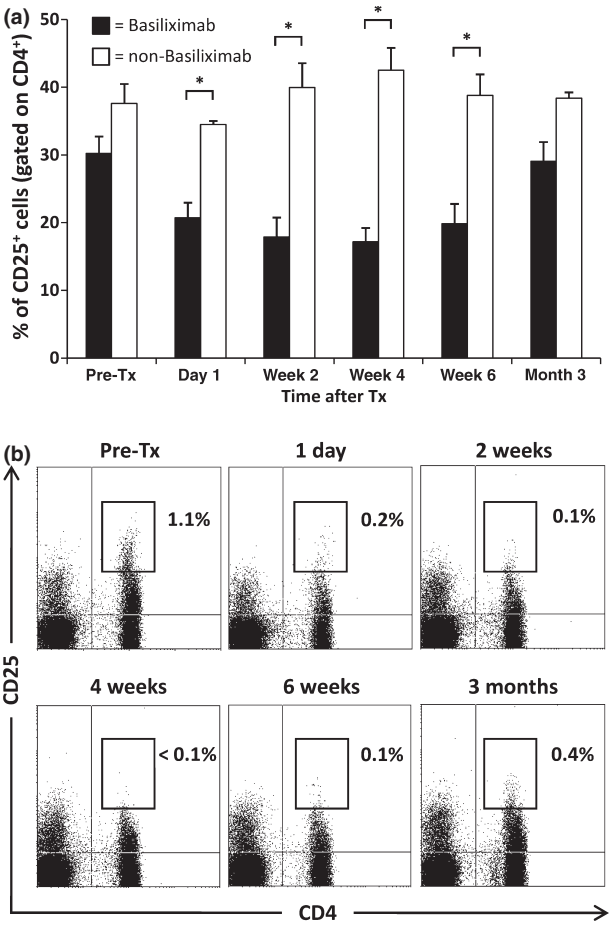

Figure 1 Effect of *Basiliximab* on the frequency of CD4$^+$CD25$^+$ T-cells. PBMCs were stained with mAb to CD4 and CD25. Analysis was performed on gated lymphocytes defined by forward/sideward scatter. (a) Comparison of the frequencies of CD4$^+$ cells co-expressing CD25 in the course of transplantation between individuals receiving *Basiliximab* treatment (black bars, $n = 14$) and the non-*Basiliximab* group (white bars, $n = 7$). Differences were statistically significant with $P < 0.001$ (*). Data are presented as mean ± SEM. (b) Transient disappearance of CD4$^+$CD25^{high} T-cells in a patient receiving *Basiliximab* therapy. Depicted dotblots are representative for all individuals treated with *Basiliximab*. This effect was not observed in the non-*Basiliximab* group (data not shown). Numbers represent the percentage of cells within the total lymphocytes.

additional staining for FoxP3 to further define the regulatory subset and to allow differentiation of T_{regs} from activated/effector T-cells was performed. Analysis of CD4$^+$CD25$^+$FoxP3$^+$ T-cells revealed that this subpopulation was not entirely lost as might have been suggested by the disappearance of the CD4$^+$CD25^{high} subpopulation. Nevertheless, individuals receiving *Basiliximab* showed a moderate reduction of the frequency of CD4$^+$FoxP3$^+$ T-cells following Tx (6.0 ± 0.6% and 5.0 ± 0.6%, pre-Tx and 3 months, respectively), but this was also observed in

the non-*Basiliximab* group (5.2 ± 0.8% and 4.6 ± 1.0%, pre-Tx and month 3, respectively). However, the mean fluorescence intensity (MFI) of CD25 on T_{regs} was markedly reduced in *Basiliximab* treated patients (Fig. 2a). Due to a decrease in the expression density of CD25, the T_{reg} subpopulation shifted from the $CD25^{high}$ to the $CD25^{low}$ compartment. This effect lasted for at least 6 weeks. Thereafter, a continuous increase in expression density of CD25 was observed. Focusing on $CD25^{low}$ expressing cells, this phenotypic shift of T_{regs} could be traced even better: the proportion of $CD25^{low}$ cells co-expressing FoxP3 was found to transiently increase after kidney Tx lasting for at least 4 weeks (Fig. 2b). By month 3, FoxP3 expression returned to pre-Tx level. This phenomenon was not observed in transplanted individuals of the control group.

The assumption that *Basiliximab* treatment results in down-regulation of CD25 on T_{regs} was also supported by measuring the mean fluorescence intensity (MFI) of CD25 on $CD4^+CD25^+FoxP3^+$ T_{regs}. Initially expressing the same density of CD25 as found on regulatory T-cells of the controls, the MFI of CD25 on T_{regs} was already significantly reduced after the first injection of *Basiliximab* in most patients (MFI: 49 vs. 89 at day 1 after Tx for *Basiliximab* and non-*Basiliximab* group, respectively; $P = 0.026$; Fig. 2c). Following the second administration on day 4, the MFI in these recipients continued to decrease with the lowest levels observed at 1 month. Thereafter, continuous reconstitution of CD25 expression on $CD4^+FoxP3^+$ T-cells was observed. Meanwhile, following a slight increase on day 1 after Tx, the MFI of CD25 in individuals not receiving *Basiliximab* constantly decreased at a slow rate until a MFI around 50 was reached. Three months postapplication of induction treatment the MFI of CD25 was almost equal in both patient groups (MFI: 47 vs. 52 for *Basiliximab* and non-*Basiliximab* group, respectively). All transplanted patients showed a significantly reduced MFI of CD25 on T_{regs} 3 months following Tx compared to baseline (77 ± 7.3 vs. 49 ± 3.9, $P < 0.001$).

To gain further evidence that T_{regs} were not entirely eliminated due to *Basiliximab* treatment but rather their expression of CD25 modulated, the frequency of $CD4^+FoxP3^+$ T-cells was monitored. Regulatory T-cells constitute only a small subset of the $CD4^+$ T-cells but usually account for most part of the $CD4^+FoxP3^+$ population. Thus, calculating the ratio of $CD4^+CD25^+FoxP3^+$ T_{regs} to $CD4^+FoxP3^+$ T-cells, a loss in T_{regs} should equally reduce the $CD4^+FoxP3^+$ population – the ratio would remain stable. Nevertheless, the decrease of the $CD4^+CD25^+FoxP3^+$ T_{reg} population observed in patients receiving *Basiliximab* was not associated with a pursuant reduction of the frequency of $CD4^+FoxP3^+$ cells. In consequence, the ratio of

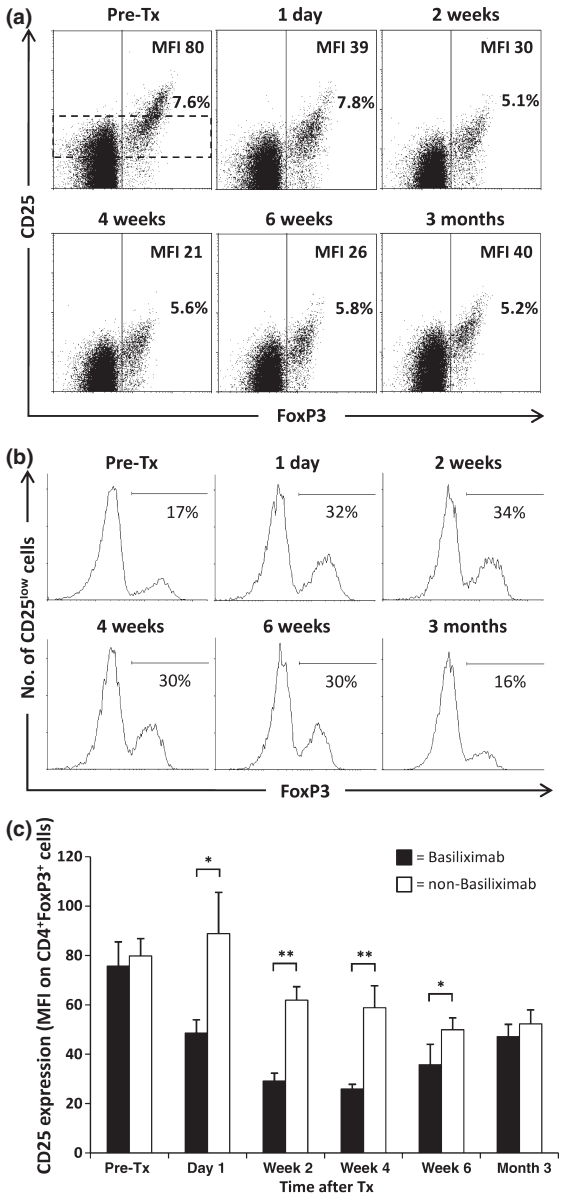

Figure 2 *Basiliximab* reduces the expression density of CD25 on T_{regs}. PBMCs were stained with mAb to CD4, CD25 and FoxP3. Analysis was performed on gated $CD4^+$ lymphocytes. (a) Course of $CD4^+CD25^+FoxP3^+$ T_{regs} in a patient with *Basiliximab* therapy. The mean fluorescence intensity (MFI) of CD25 on $CD4^+CD25^+FoxP3^+$ T_{regs} and the percentage of $FoxP3^+$ cells within the $CD4^+$ population are presented. These findings are representative for all patients with *Basiliximab* treatment. The gate shown in the upper left dotplot defines $CD25^{low}$ cells and was used to calculate the proportion of FoxP3 expressing cells within this subset. (b) The numbers represent the percentage of $FoxP3^+$ cells within the $CD4^+CD25^{low}$ population. (c) Comparison of the expression densities of CD25 on $CD4^+FoxP3^+$ T-cells in the course of transplantation between individuals receiving *Basiliximab* treatment (black bars, $n = 14$) and the non-*Basiliximab* group (white bars, $n = 7$). Differences were statistically significant with $P < 0.05$ (*) and $P < 0.01$ (**). Data is presented as mean ± SEM.

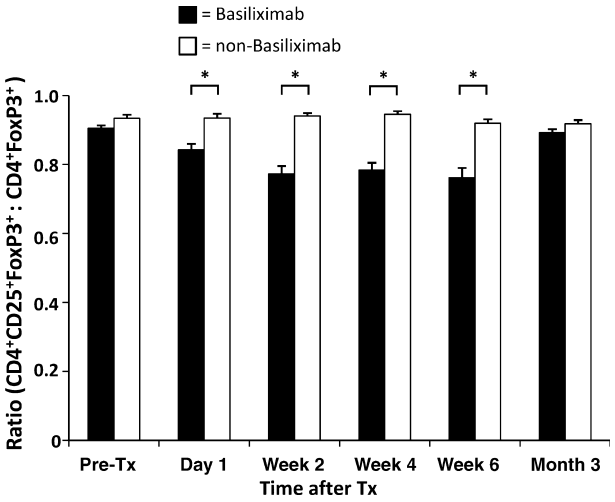

Figure 3 Loss of $CD4^+CD25^+FoxP3^+$ T_{regs} due to *Basiliximab* does not equally reduce the $CD4^+$ $FoxP3^+$ population. PBMCs were stained with mAb to CD4, CD25 and FoxP3. The frequency of $CD4^+$ subsets was determined by flow cytometry and the ratio of $CD4^+CD25^+$ $FoxP3^+$ T_{regs} to $CD4^+$ $FoxP3^+$ T-cells was calculated. Black bars represent data obtained from patients receiving *Basiliximab* treatment (n = 14), white bars represent the non-*Basiliximab* group (n = 7). Differences were statistically significant with $P < 0.01$ (*). Data is presented as mean ± SEM.

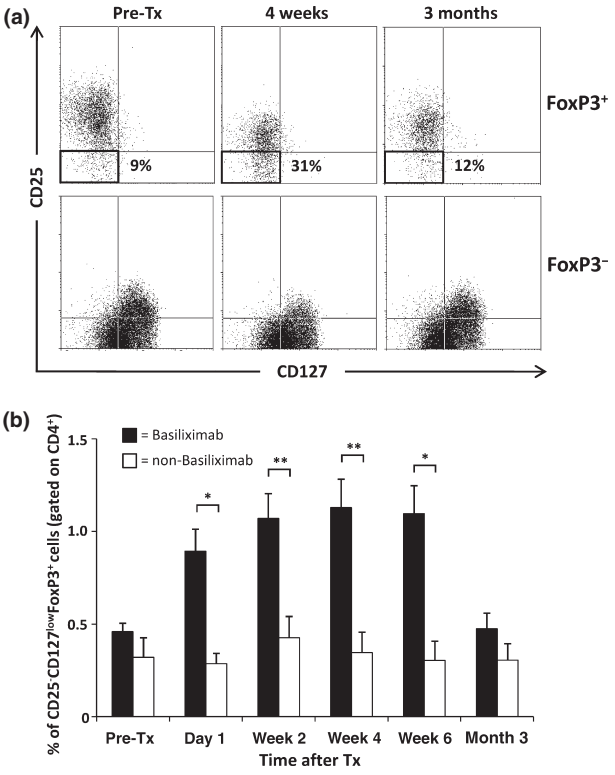

Figure 4 Appearance of $CD25^-$ regulatory T-cells in *Basiliximab* treated patients. PBMCs were stained with mAb to CD4, CD25, CD127 and FoxP3. Analysis was performed on gated $CD4^+$ lymphocytes. (a) CD25/CD127 expression patterns on $FoxP3^+$ (upper row) and $FoxP3^-$ cells (lower row). Numbers represent the percentage of $CD25^-$ cells within the $CD4^+FoxP3^+$ population. (b) Frequencies of $CD4^+CD25^-CD127^{low}FoxP3^+$ cells in the course of transplantation between individuals receiving *Basiliximab* treatment (black bars, n = 14) and the non-*Basiliximab* group (white bars, n = 7). Differences were statistically significant with $P<0.05$ (*) and $P < 0.01$ (**). Data is presented as mean ± SEM.

$CD4^+CD25^+FoxP3^+$ T_{regs} to $CD4^+FoxP3^+$ T-cells was seen to decline following the first anti-CD25-mAb injection (Fig. 3). This reduction lasted for about 6 weeks. With reconstitution of the $CD4^+CD25^+FoxP3^+$ T_{regs} the T_{reg}: $CD4^+FoxP3^+$ ratio normalized to pretransplant levels. No changes of this ratio were observed in the non-*Basiliximab* group. The differences in-between the two groups was statistically significant ($P < 0.001$). These findings revealed a transient increase in the frequency of a $CD4^+FoxP3^+$ subpopulation not expressing CD25 following *Basiliximab* therapy. Down-regulation of CD25 is suggested as the underlying mechanism of action.

Appearance of $CD25^-$ regulatory T-cells in Basiliximab treated patients

In patients receiving *Basiliximab* a population of cells expressing FoxP3 but not CD25 was observed. To further characterize these cells, additional staining for CD127 expression as a complementary T_{reg}-marker was performed. As expected, almost all of the $CD4^+CD25^+FoxP3^+$ T_{regs} presented as $CD127^{low}$ whereas $CD4^+FoxP3^-$ T-cells expressed high levels of CD127 (Fig. 4a). Investigation of CD127 expression on the transiently increasing $CD4^+CD25^-FoxP3^+$ subpopulation revealed that this subset also expressed the $CD127^{low}$ phenotype similar to conventional T_{regs} (Fig. 4a). Following *Basiliximab* administration, a significant increase of the $CD4^+CD25^-$ $CD127^{low}FoxP3^+$ population was observed lasting for at least 6 weeks (Fig. 4b). 3 months after Tx the frequency of this subset had returned to baseline. No alteration of the $CD4^+CD25^-FoxP3^+$ subset was seen throughout the entire follow-up period in individuals of the non-*Basiliximab* group. These findings suggest that *Basiliximab* treatment might not only induce partial down-regulation of CD25 but even can result in a complete loss of CD25 expression on T_{regs}.

Basiliximab-mediated *in vitro* modulation of CD25 on $CD4^+$ T-cells is not associated with functional consequences

The increase of $CD4^+CD25^{low}$ cells expressing FoxP3 and the appearance of $CD4^+CD25^-FoxP3^+$ cells in patients

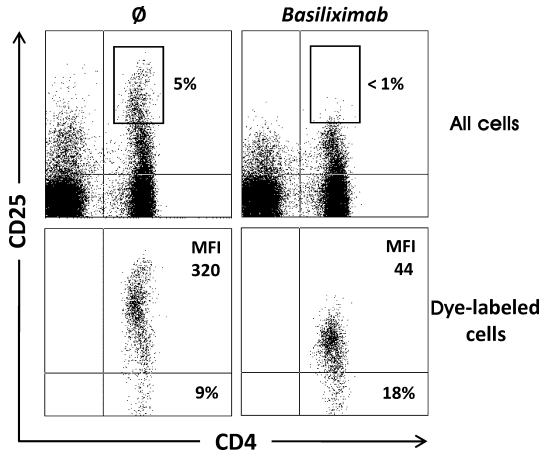

Figure 5 *In vitro* effects of *Basiliximab* on the expression of CD25. Freshly isolated PBMCs from healthy individuals were stained with mAb to CD4 and CD25. The $CD4^+CD25^{high}$ T-cells were separated using a cell sorter and dye-labeled. The labeled cells were returned to the original PBMC pool at the initial concentration. Reconstituted PBMCs were then incubated for 48 h with medium alone or in the presence of *Basiliximab* (1.25 μg/ml). Analysis was performed on gated lymphocytes (all cells, upper panels; numbers indicate the percentage of cells within the $CD4^+$ population) or on gated dye-labeled cells (lower panels; numbers indicate the MFI of CD25 and the percentage of $CD25^-$ cells within the dye-labeled population). Data represent one of three independent experiments revealing similar patterns of reactivity.

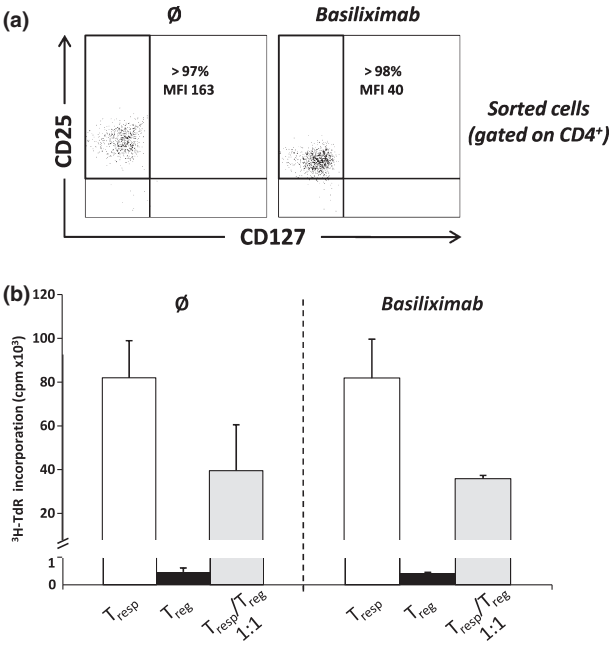

Figure 6 *In vitro* effects of *Basiliximab* on the suppressive function of T_{regs}. PBMCs were cultured in the absence/presence of *Basiliximab* and sorted for regulatory T-cells as previously described. (a) CD25/CD127 expression patterns of sorted T_{regs} which had been untreated or treated with *Basiliximab*. Numbers represent the purity of sorted cells (%) and expression density of CD25 (MFI). (b) Suppressive capacity of untreated and *Basiliximab* treated T_{regs}. $CD4^+CD25^-$ T-cells (T_{resp}) and sorted T_{regs} were stimulated (CD3/CD28 beads) alone or in co-culture containing 2×10^4 cells each. Proliferation was determined after 5 days by [^{3}H]-TdR incorporation. Data present the mean proliferation ± SEM observed in two independent experiments.

receiving *Basiliximab* both highly suggest down-regulation of CD25 on the same cell. However, it could also be possible that a small subset of $CD25^-$ T_{regs} that is already present in patients could expand during the time course of Tx. To distinguish between these possibilities we performed *in vitro* experiments. $CD4^+CD25^{high}$ cells were separated from PBMCs, dye-labeled and then returned to the T_{reg}-depleted cell population. Reconstituted PBMCs were cultured for 48 h with *Basiliximab* or medium alone. As previously observed in *Basiliximab* treated patients (Fig. 1) there was a significant drop in $CD4^+CD25^{high}$ cells in cultures containing the antibody (Fig. 5, upper panels). Tracking of the dye-labeled $CD4^+CD25^{high}$ subset revealed a shift in CD25 density towards the $CD25^{low}$ compartment (Fig. 5, lower panels; MFI: 320–44). Furthermore, an increase in the frequency of $CD25^-$ cells by 50% was observed. However, the proportion of dye-labelled cells among the lymphocyte population did not differ between cultures containing *Basiliximab* or medium alone (6.6% vs. 6.4% of $CD4^+$ cells, respectively) supporting the assumption that the antibody does not eliminate $CD25^{high}$ cells but down-regulates their CD25 expression.

Suppression assays were performed to evaluate the impact of *Basiliximab* on the function of regulatory T-cells. Sorted T_{regs} treated with *Basiliximab* showed modulation of CD25 expression meanwhile the density of CD127 was unaffected (Fig. 6a) similarly to the observations made in patients (Fig. 4a). The proliferative response of $CD4^+CD25^-$ T-cells upon stimulation was effectively suppressed by addition of nonmodulated $CD4^+CD25^{high}CD127^{low}$ T_{regs}. Nevertheless, a comparable suppressive effect was observed applying *Basiliximab* treated T_{regs} indicating that CD25-modulation does not interfere with the suppressive function (Fig. 6b). Furthermore, in an attempt to analyze the effect of *Basiliximab* on CD25 mRNA expression real-time-PCR was performed. No differences between the two T_{reg}-groups were observed (data not shown).

Discussion

Prevention of acute rejection episodes is an important goal early after solid organ transplantation and establishment of efficient immunosuppressive protocols with low drug toxicity is highly required. Thus, recent regimens are

based on a combination of drugs such as calcineurin inhibitors, anti-proliferative agents and steroids and often are supplemented by induction therapy with non-/depleting antibodies. The impact of immunosuppression on T_{regs} is still insufficiently understood [20], however it is essential to ensure that novel immunosuppressive strategies do not interfere with the development or function of regulatory T-cells but rather promote transplantation tolerance. In this study, we have focused on the influence of anti-CD25-mAb *Basiliximab* on the frequency of T_{regs} in patients early after kidney transplantation.

In animal models, anti-CD25-antibodies have been shown to directly affect T_{regs}. Following antibody application, regulatory T-cells in the peripheral blood were eliminated and/or their function impaired [21,22]. Previous studies on the use of these antibodies in humans have shown inconsistent results. *Daclizumab* was seen to be associated with a transient elimination of T_{regs} [23] and inhibition of FoxP3 mRNA induction [24]. Similar findings are reported for the application of *Basiliximab* [16,17] whereas a recent publication also promotes the down-regulation of CD25 rather than elimination of regulatory T-cells [25].

To exclude in advance that a reduction of $CD25^+$ cells observed would be due to the generalized effects of organ Tx with basic immunosuppression such as CNI, we compared *Basiliximab* treated patients to an appropriate control group of transplanted individuals not receiving induction therapy as part of the immunosuppressive regimen as the single difference.

The most prominent observation of this study was the distinct reduction of CD25 particularly on regulatory T-cells. Due to fading of CD25 on T_{regs}, monitoring of these cells was impeded and only feasible by staining for a combination of several markers. By this means, we have shown that $CD4^+CD25^+FoxP3^+$ T_{regs} were not entirely eliminated as might have been suggested regarding the disappearance of the $CD4^+CD25^{high}$ subpopulation following *Basiliximab* injection. In parallel to the pharmacokinetics of *Basiliximab* the reduction of CD25 expression lasted for at least 4–6 weeks with continuous recovery thereafter. The serum concentration of *Basiliximab* is known to drop below saturation levels of IL-2R after approximately 30 days [26]. In patients treated with triple immunosuppression, as were the patients of this study, the clearance of *Basiliximab* might be even lower resulting in a prolonged duration of CD25 saturation [27]. In accordance with this, reconstitution of the T_{reg} subset close to pre-Tx level in our patients was observed by 3 months following anti-CD25-mAb administration. Nevertheless, all patients included in this study presented with a reduced MFI of CD25 on T_{regs} by this time compared to baseline. But this effect can be addressed to the basic immunosuppressive medication given as it is well known that calcineurin inhibitors block IL-2 synthesis in T lymphocytes and thus interfere with the proliferation/activation of T-cells [28].

Due to down-regulation of CD25 induced by *Basiliximab*, a shift within the $CD4^+FoxP3^+$ subpopulation was observed: the frequency of $CD4^+CD25^+FoxP3^+$ T_{regs} was transiently reduced, whereas the number of $CD4^+CD25^-FoxP3^+$ cells was increased. In accordance to previous reports [16,25], the course of $CD4^+FoxP3^+$ cells following *Basiliximab* treatment remained almost stable suggesting that the drop of $CD4^+CD25^+FoxP3^+$ T_{regs} may result from blocking of the diagnostic anti-CD25-mAb by *Basiliximab* or down-regulation of CD25 molecules rather than from elimination of the cells. We performed *in vitro* experiments to distinguish between these possibilities. Preincubation with *Basiliximab* did not prevent the anti-CD25-mAb used for our flow cytometric analyses from binding to the IL-2Rα (clone M-A251, epitope B, BD Bioscience) and is in accordance with previous findings by others [17,29]. Blockade by *Basiliximab* pretreatment was observed using different antibody clones recognizing other epitopes on the α-chain of the IL-2R (data not shown, e.g. clone 2A3 for epitope A, BD Bioscience). Furthermore, incubation of PBMCs in the presence of *Basiliximab* at 37 °C markedly reduced CD25 expression on T_{regs} without eliminating the cells as seen by our cell-tracking experiments. These findings are supported by Wang *et al.* [25] who despite the disappearance of $CD4^+CD25^+FoxP3^+$ T_{regs} did not find an increased rate of apoptosis in patients treated with *Basiliximab*. Game *et al.* [30] even observed a lower occurrence of apoptosis for *Basiliximab* treated $CD4^+CD25^+$ cells *in vitro* compared to untreated cells. Thus, these *in vitro* results strongly suggest that *Basiliximab* directly affects T_{regs} by modulation of their CD25 expression.

A decrease in the concentration of CD25 molecules on the cell surface could be due to internalization and/or shedding. Decrease of CD25 expression after anti-CD25-therapy has not been associated with an appropriate alteration of intracellular CD25 protein nor mRNA expression [22]. The latter is in accordance with our findings that modulated T_{regs} did not show altered CD25 mRNA expression. In addition, Warlé *et al.* [31] measured an increased level of soluble CD25 (sIL-2R) in the blood of *Basiliximab* treated liver transplant patients compared to untreated controls. The latter phenomenon was also explained by increased shedding of CD25 from T lymphocytes as a consequence of saturating levels of *Basiliximab* during the first months after Tx and is based on the knowledge that binding of IL-2 to the IL-2R induces shedding of the α-chain of the receptor [32]. In combination, these observations thus favour shedding

as the underlying mechanism for the down-regulation of CD25 upon binding of *Basiliximab*. This is further supported by our finding that the transiently increasing $CD4^+CD25^-FoxP3^+$ subset is of the $CD127^{low}$ phenotype which has not been reported so far. As previously shown by others, the expression of CD127 on human T_{regs} is down-regulated in contrast to activated effector T-cells [11,12]. The commonly referred to T_{regs} thus are of the $CD4^+CD25^+CD127^{low}FoxP3^+$ phenotype. Due to CD25 receptor modulation by shedding of the CD25 molecule T_{regs} might be shifted from the $CD25^+$ to the $CD25^-$ subset following *Basiliximab* induction therapy. The sudden and short lived nature of this phenomenon – the frequency of $CD4^+CD25^-CD127^{low}FoxP3^+$ cells had returned to pre-Tx level 3 months following Tx – argues against a newly developing $CD4^+FoxP3^+$ T_{reg} subpopulation from the $CD25^-$ compartment. The survival of T_{regs} transiently lacking the IL-2Rα or during the blockade of this receptor by *Basiliximab* might be enabled by compassing the IL-2 pathway via other common γ-chain dependent cytokines such as IL-4, IL-7 or IL-15 [33,34]. In this context it should be noted that in certain cytokine milieus T_{regs} might convert into Th17 cells [35].

There is no indication that the suppressive function of T_{regs} is impaired following *Basiliximab* treatment and this is supported by the *in vitro* experiments of this study. Even in the presence of *Basiliximab* suppression by $CD4^+CD25^+$ cells *in vitro* was reported to be possible [30]. Other recent investigations also have shown that the regulatory function of $CD4^+CD25^{high}FoxP3^+$ T-cells in the course of Tx is not negatively influenced by anti-CD25-antibody application [17,36]. In combination with the net effect of this antibody-treatment to significantly reduce the incidence of AR following solid organ transplantation these are promising results.

In summary, this study has shown that the use of *Basiliximab* directly affects peripheral T_{regs} in transplanted individuals. *In vivo* and *in vitro* results demonstrate that *Basiliximab* transiently reduces the frequency of $CD4^+CD25^+CD127^{low}FoxP3^+$ T-cells by modulation of their CD25 expression but without impairment of their suppressive function. Long-term follow up of these patients will provide further evidence whether the application of this anti-CD25-mAb indeed is unobjectionable for the use in tolerance promoting protocols.

Authorship

FWRV: designed study, performed research, collected/analysed data and wrote the paper. KT: performed research, collected data, revised manuscript. JT: performed research, collected data. SK: performed research, collected data. AS: contributed patient material, revised manuscript. FL: contributed patient material, provided clinical data, revised manuscript. JK: contributed patient material, revised manuscript. TB: contributed patient material, provided clinical data, revised manuscript. RS: designed study, analyzed data, revised manuscript.

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