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ORIGINAL ARTICLE

# Expansion of CD45RA$^{-}$FOXP3$^{++}$ regulatory T cells is associated with immune tolerance in patients with combined kidney and bone marrow transplantation

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## Abstract

**Objectives.** Simultaneous transplantation of a solid organ and bone marrow from the same donor is a possible means of achieving transplant tolerance. Here, we attempted to identify biomarkers that indicate transplant tolerance for discontinuation of immunosuppressants in combined kidney and bone marrow transplantation (CKBMT). **Methods.** Conventional kidney transplant (KT) recipients ($n$ = 20) and CKBMT recipients ($n$ = 6) were included in this study. We examined various immunological parameters by flow cytometry using peripheral blood mononuclear cells (PBMCs), including the frequency and phenotype of regulatory T (Treg) cell subpopulations. We also examined the suppressive activity of the Treg cell population in the setting of mixed lymphocyte reaction (MLR) with or without Treg cell depletion. **Results.** Among six CKBMT recipients, three successfully discontinued immunosuppressants (tolerant group) and three could not (non-tolerant group). The CD45RA$^{-}$FOXP3$^{++}$ Treg cell subpopulation was expanded in CKBMT recipients compared to conventional kidney transplant patients, and this was more obvious in the tolerant group than the non-tolerant group. In addition, high suppressive activity of the Treg cell population was observed in the tolerant group. The ratio of CD45RA$^{-}$FOXP3$^{++}$ Treg cells to CD45RA$^{-}$FOXP3$^{+}$ cells indicated good discrimination between the tolerant and non-tolerant groups. **Conclusion.** Thus, our findings propose a biomarker that can distinguish CKBMT

patients who achieve transplant tolerance and are eligible for discontinuation of immunosuppressants and may provide insight into tolerance mechanisms in CKBMT.

**Keywords:** chimerism, combined kidney and bone marrow transplantation, regulatory T cells, tolerance

## INTRODUCTION

One of the main objectives for improving long-term outcomes in solid organ transplantation (SOT) is to reduce the usage of immunosuppressants (ISs) and ultimately discontinuing IS treatment without causing graft rejection.[1–3] Some attempts have been made in preclinical and clinical studies to achieve immune tolerance, maintaining a graft without ISs, in the setting of SOT.[4–8] One of the approaches for inducing immune tolerance is to simultaneously perform SOT and bone marrow transplantation (BMT) from the same donor.[9–14] These studies reported that SOT with BMT results in donor-specific tolerance by inducing mixed chimerism in recipients.

Currently, three major groups are actively investigating the induction of immune tolerance through combined kidney and bone marrow transplantation (CKBMT). However, these approaches do not seem to always have a clinical advantage.[6] Among patients who achieved donor hematopoietic chimerism, some were able to successfully withdraw ISs, whereas others gained no clinical benefit.

Recently, our group developed a tolerance induction strategy for kidney transplant recipients using CKBMT.[15] We have performed CKBMT with a non-myeloablative preconditioning regimen using cyclophosphamide, fludarabine, antithymocyte globulin and thymic irradiation.[15] Some patients have successfully withdrawn ISs with stable allograft function for several years after the discontinuation of ISs. However, the other patients did not have such a clinical advantage. To achieve the goals of CKBMT, it is crucial to discover biomarkers that indicate the transplant tolerance status in order to determine when to discontinue IS therapy in CKBMT recipients.

CKBMT leads to mixed chimerism, which is considered a critical step in achieving donor-specific tolerance.[16] Assays for mixed lymphocyte reaction (MLR) can be used to assess the status of donor-specific tolerance or hypo-responsiveness.[17,18] Therefore, an expectation exists that the MLR assay would be available as an indicator of IS discontinuation, but MLR assays after CKBMT exhibit a weak correlation with each patient's clinical course, including allograft rejection.[10,19]

$CD4^+CD25^+CD127^{lo}FOXP3^+$ regulatory T (Treg) cells play a major role in inducing immune homeostasis and self-tolerance.[20,21] In addition, Treg cells prevent allograft rejection by regulating alloreactive immune responses.[21–23] In humans, the $CD4^+$ T cells expressing FOXP3 can be classified into three distinct subpopulations based on the expression of CD45RA and FOXP3: $CD45RA^+FOXP3^{lo}$ Treg cells, $CD45RA^-FOXP3^{hi}$ Treg cells and $CD45RA^-FOXP3^{lo}$ cytokine-secreting cells.[24] Interestingly, an increased frequency of $CD45RA^-FOXP3^{hi}$ Treg cells has been observed in spontaneously operational tolerant recipients following conventional kidney transplantation (KT).[25]

In the present study, we tried to identify immunological parameters that correlate with transplant tolerance in a cohort of six CKBMT patients. First, we compared various T cell-related factors between conventional KT and CKBMT recipients. We also examined the Treg cells as $CD4^+CD25^+CD127^{lo}FOXP3^+$ and their subpopulations by their surface phenotype based on the expression of CD45RA and FOXP3: $CD45RA^+FOXP3^+$ Treg cells, $CD45RA^-FOXP3^{++}$Treg cells and $CD45RA^-FOXP3^+$ Treg cells. We found that the $CD45RA^-FOXP3^{++}$ Treg cell subpopulation was expanded in CKBMT recipients. The expansion of $CD45RA^-FOXP3^{++}$ Treg cells was more striking in tolerant recipients who successfully discontinued ISs. Our findings demonstrate that the expansion of $CD45RA^-FOXP3^{++}$ Treg cells distinguishes CKBMT patients who achieve transplant tolerance and are eligible for discontinuation of ISs.

## RESULTS

### Characteristics of patients with KT or CKBMT

The clinical characteristics of the KT ($n$ = 20) and CKBMT ($n$ = 6) patients are described in Table 1. Our preconditioning schedule for conventional KT

**Table 1.** Characteristics of KT and CKBMT recipients

| | KT ($n$ = 20) | CKBMT ($n$ = 6) |
|---|---|---|
| General characteristics | | |
| Gender (males/females) | 18/2 | 6/0 |
| Age, years (range) | 53 (36–65) | 34.5 (31–47) |
| Deceased donor | 5 (25.0) | 0 (0) |
| Post-transplantation period, months (range) | 24 (13–24) | 50.5 (52–74) |
| Induction drug | | |
| rATG | 1.5 mg $kg^{-1}$ × 3 | 1.5 mg $kg^{-1}$ × 3–4 |
| Rituximab | – | 375 mg $m^{-2}$ × 2 |
| Cyclophosphamide | – | 22.5–60 mg $kg^{-1}$× 2 |
| Fludarabine monophosphate | – | 10–15 mg $m^{-2}$ × 4 |
| Cause of renal failure, $n$ (%) | | |
| Diabetes | 10 (50.0) | 1 (16.7) |
| Hypertension | 4 (20.0) | 0 (0) |
| IgA nephropathy | 3 (15.0) | 1 (16.7) |
| Glomerulonephritis | 1 (5.0) | 4 (66.7) |
| Unknown | 1(5.0) | 0 (0) |
| Acute cellular rejection[a] | 6 (30.0) | 3 (50.0) |
| Antibody-mediated rejection[a] | 1 (5.0) | 0 (0) |
| de novo DSA[a] | 0 (0) | 0 (0) |
| Infection, $n$ (%) | | |
| BK viruria (> 7 log unit) | 6 (30.0) | 4 (66.7) |
| BK viraemia (> 4 log unit) | 2 (10.0) | 4 (66.7) |
| BK nephritis | 0 (0) | 2 (33.3) |
| CMV antigenemia (> 50/200 000 WBCs) | 2 (10.0) | 5 (83.3) |
| Graft failure[a] | 2 (10.0) | 0 (0) |

CMV, cytomegalovirus; DSA, donor-specific antibody.
[a]Monitored for 2 years after transplantation.

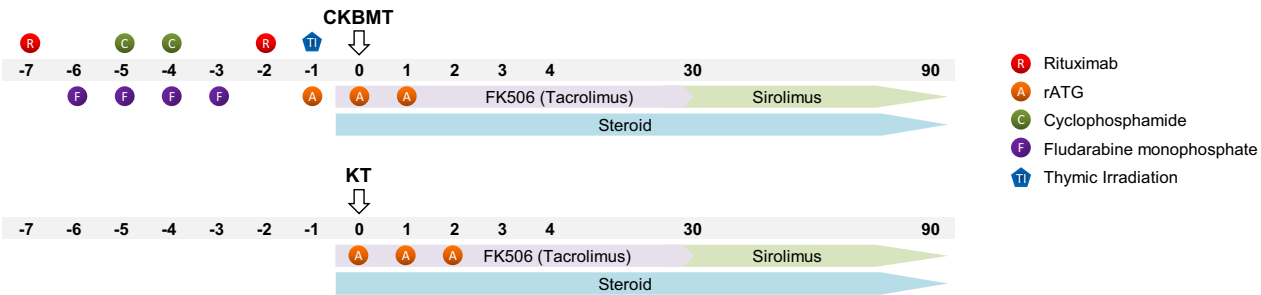



**Figure 1.** Preconditioning regimens in CKBMT and conventional KT. The preconditioning regimen for CKBMT (top) consisted of rituximab on days −7 and −2, fludarabine monophosphate on day −6 and given consecutively for 4 days, cyclophosphamide on days −5 and −4, rATG on day −1 and given consecutively for 3 days, and thymic irradiation on day −1. On day 0, transplantation of donor bone marrow cells was followed by kidney transplantation. In CKBMT recipients, tacrolimus and steroid were administered as the maintenance drug. One-month post-transplantation, the immunosuppressive regimen in CKBMT recipients was switched from tacrolimus to sirolimus. Sirolimus and steroids were tapered to withdrawal after a protocol biopsy showing the absence of any clinical rejection. Bottom, KT recipients received rATG as an induction drug on days 0, +1 and +2, and then treated with steroid, tacrolimus and mycophenolate mofetil.

and CKBMT is presented in Figure 1. Table 2 provides more detailed clinical information on the CKBMT patients, including preconditioning drugs and the duration of chimerism. The preconditioning regimen for CKBMT included fludarabine monophosphate, cyclophosphamide, rituximab, rabbit-antithymocyte globulin (rATG) and thymic irradiation. Tacrolimus, sirolimus and steroid were used as maintenance drugs. Conventional KT patients received rATG-based induction therapy, and tacrolimus, sirolimus and steroid were used for maintenance as in the

**Table 2.** Characteristics of the tolerant and non-tolerant groups of CKBMT recipients

| | Tolerant group | | | Non-tolerant group | | |
|---|---|---|---|---|---|---|
| | Subject 4 | Subject 6 | Subject 7 | Subject 1 | Subject 2 | Subject 5 |
| Age/Sex | 31/M | 28/M | 42/M | 47/M | 33/M | 36/M |
| Cause of ESRD | MPGN | IgA nephropathy | MPGN | Diabetes | GN (Clinically) | GN (Clinically) |
| HLA mismatch | 3/6 | 3/6 | 3/6 | 1/6 | 3/6 | 4/6 |
| $CD34^+$ cells ($\times 10^6$ $kg^{-1}$) | 1.38 | 3.24 | 2.93 | 1.88 | 1.26 | 2.13 |
| CP (mg $kg^{-1}$) | 22.5 × 2 | 22.5 × 2 | 22.5 × 2 | 60 × 2 | 60 × 2 | 22.5 × 2 |
| FDR (mg $m^{-2}$) | 15 × 4 | 10 × 4 | 10 × 4 | – | – | 15 × 4 |
| rATG (mg $kg^{-1}$) | 1.5 × 4 | 1.5 × 3 | 1.5 × 3 | 1.5 × 3 | 1.5 × 3 | 1.5 × 4 |
| Peak chimerism percentage | 45.5% | 5.60% | 17.40% | 95.30% | 64.20% | 16.3% |
| Chimerism duration (weeks) | 8 | 3 | 2 | 3 | 3 | 8 |

CP, cyclophosphamide; ESRD, end-stage renal disease; FDR, fludarabine; GN, glomerulonephritis; MPGN, membranoproliferative glomerulonephritis; rATG, thymoglobulin.

CKBMT regimen. Biopsy for determining acute cellular rejection and antibody-mediated rejection was performed within 2 years after transplantation. Six of the 20 KT recipients experienced allograft rejection. Among KT patients with cellular and antibody rejection, two recipients developed graft failure within 2 years after transplantation.

Among the six CKBMT patients, five who underwent early graft rejection were tapered in their immunosuppression and eventually stopped using ISs; the exception was patient 2. Three patients (4, 6 and 7) maintained stable allograft function for several years after IS discontinuation, whereas two patients (1 and 5) experienced graft rejection after IS discontinuation. On the basis of these clinical results, the six CKBMT recipients were divided into two groups (Supplementary figure 1): the tolerant group (patients 4, 6 and 7) and the non-tolerant group (patients 1, 2 and 5). Graft rejection was proven by biopsy in the non-tolerant recipients. The tolerant recipients continued to maintain stable allograft function with no evidence of acute cellular rejection or antibody-mediated rejection. More detailed information on the CKBMT patients and clinical outcomes was described in a previous study.[15]

## The relative frequency of memory T cells is increased by CKBMT

Next, we examined the frequency of lymphocytes, T cells and their subpopulations in the peripheral blood by flow cytometry (Figure 2a). The absolute number of lymphocytes was decreased at 0.5–1 month after CKBMT, followed by a robust increase, but was relatively stable after KT (Figure 2b). The absolute number of $CD3^+$ T cells in CKBMT recipients was significantly increased during the course of follow-up after CKBMT (Figure 2c). Among the CKBMT recipients and KT recipients, the percentage of $CD8^+$ T cells was higher than the percentage of $CD4^+$ among T cells in all recipients (Figure 2d).

We evaluated the proportions of naïve ($CCR7^+$ $CD45RA^+$) and memory (central memory, $T_{CM}$: $CCR7^+CD45RA^-$, effector memory, $T_{EM}$: $CCR7^-CD45RA^-$, and effector memory re-expressing CD45RA, $T_{EMRA}$: $CCR7^-CD45RA^+$) T cell subsets among the $CD4^+$ and $CD8^+$ T cell populations by flow cytometry (Figure 2a and e). Three months after transplantation, nearly 100% of $CD4^+$ or $CD8^+$ T cells in CKBMT recipients were memory cells, whereas both memory and naïve cells were observed in KT recipients (Figure 2e, f and Supplementary figure 2a). The dominance of the memory cell population among the $CD4^+$ and $CD8^+$ T cell populations was observed at the 1- and 2-year follow-up in CKBMT recipients (Figure 2f). However, the frequency of memory T cells was not different between the tolerant and non-tolerant groups (Figure 2g, h and Supplementary figure 2b).

We performed MLR assays with peripheral blood mononuclear cells (PBMCs) from CKBMT recipients using autologous, donor and third party pooled PBMCs as stimulants. However, the proliferation of $CD4^+$ or $CD8^+$ T cells in response to donor PBMCs was not different between the tolerant and non-tolerant groups (Supplementary figure 3).

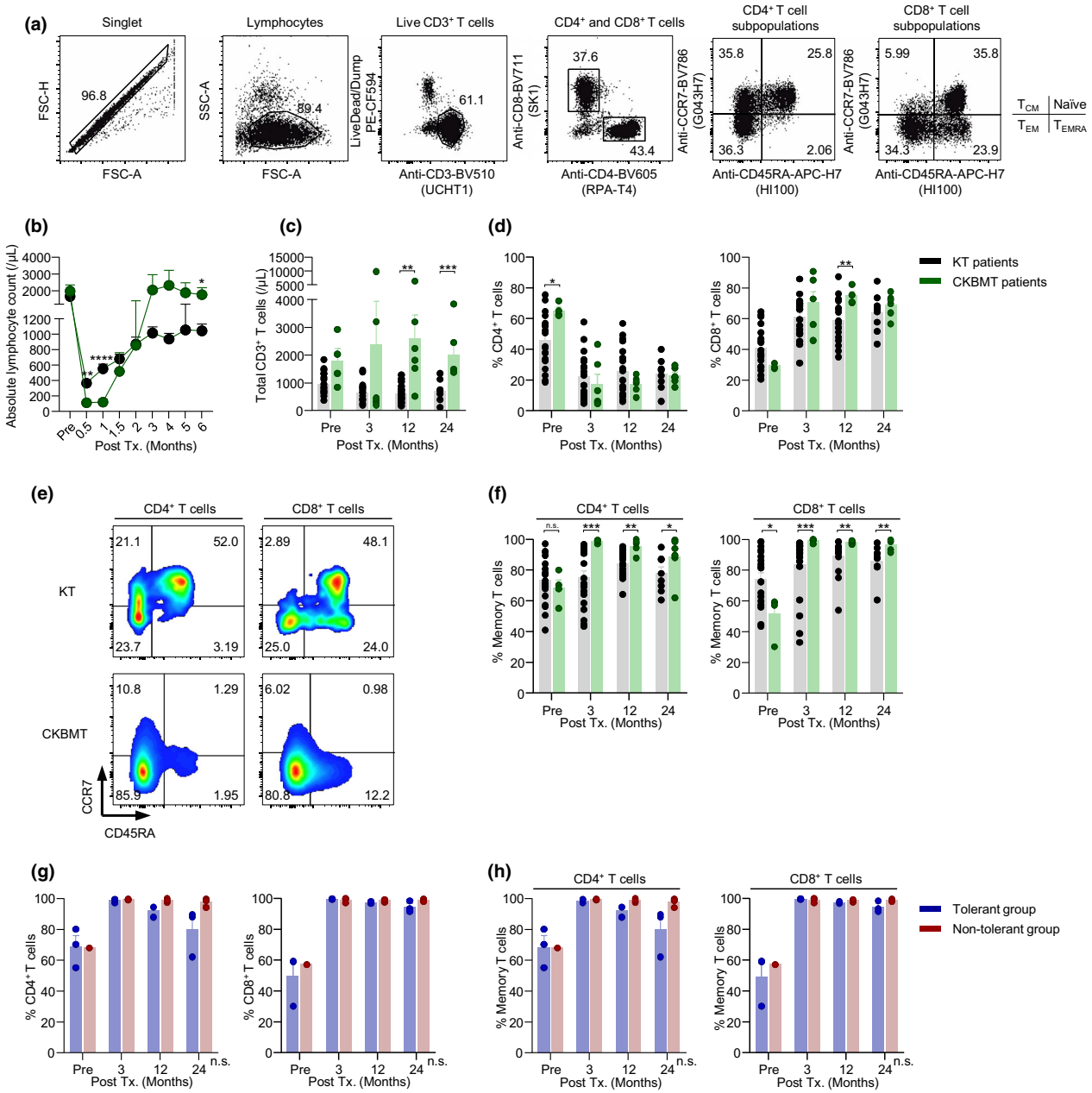



**Figure 2.** Characterisation of recovering T cells after KT and CKBMT. (**a**) Gating strategy for naïve and memory T-cell populations in $CD4^+$ and $CD8^+$ T cells. T cells were identified by lymphogating and live cell gating (excluding $CD14^+$ myeloid cells and $CD19^+$ B cells). After gating $CD4^+$ and $CD8^+$ cells, naïve and memory T cells were assessed based on CCR7 and CD45RA expression: naïve ($CCR7^+CD45RA^+$), central memory ($T_{CM}$, $CCR7^+CD45RA^-$), effector memory ($T_{EM}$, $CCR7^-CD45RA^-$) and effector memory re-expressing CD45RA ($T_{EMRA}$, $CCR7^-CD45RA^+$). **(b)** Absolute lymphocyte counts (ALCs) in conventional KT recipients (black circles and bars, $n = 20$) and CKBMT recipients (green circles and bars, $n = 6$) were assessed from the pre-transplant period to 6 months after transplantation. The recovering lymphocytes in each group were compared. **(c)** The frequency of $CD3^+$ T cells following lymphopenia was monitored in KT and CKBMT recipients. **(d)** The relative frequency of $CD4^+$ and $CD8^+$ T cells among total T cells was monitored in KT and CKBMT recipients. **(e)** A representative figure for the expression of CCR7 and CD45RA in the $CD4^+$ T cell and $CD8^+$ T cell gates 3 months after transplantation. **(f)** The relative frequency of memory T cells among $CD4^+$ T cells and $CD8^+$ T cells was analysed by flow cytometry at the indicated time points. Memory T cells exclude $CCR7^+CD45RA^+$ naïve T cells. **(g)** The relative frequency of $CD4^+$ and $CD8^+$ T cells among total T cells was monitored in the tolerant (blue circles and bars) and non-tolerant (red circles and bars) groups at the indicated time points. (**h**) The percentage of memory T cells in $CD4^+$ and $CD8^+$ T cells was assessed in the tolerant and non-tolerant groups. Data are presented as mean ± standard deviation. n.s. = not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$.

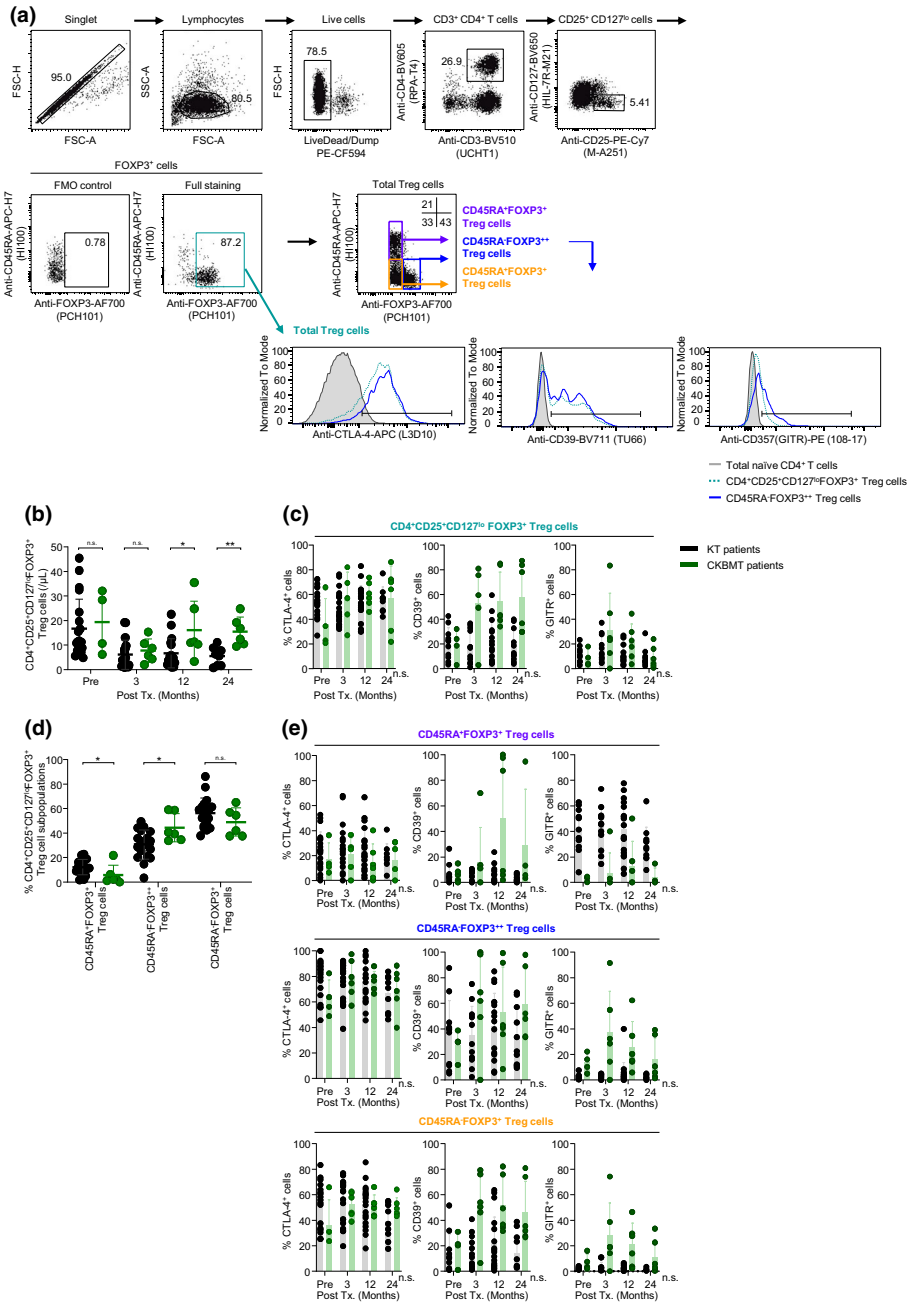



**Figure 3.** Functionally different phenotypes of regulatory T (Treg) cells in KT ($n$ = 20) and CKBMT recipients ($n$ = 6). **(a)** Gating strategy for the total Treg cell population. In flow cytometric analysis, Treg cells were defined as CD25$^{+}$ CD127$^{lo}$ FOXP3$^{+}$ cells among live CD3$^{+}$ CD4$^{+}$ T cells. Gating of FOXP3$^{+}$ cells among CD4$^{+}$CD25$^{+}$CD127lo cells was based on fluorescence-minus on (FMO) control. Three Treg cell subpopulations were defined by the surface expression of CD45RA and FOXP3 in the total Treg cell gating: CD45RA$^{+}$FOXP3$^{+}$ Treg cells (purple line), CD45RA-FOXP3$^{++}$ Treg cells (blue line) and CD45RA-FOXP3$^{+}$ Treg cells (orange line). Histograms show the expression of receptors related to suppressive functions, including CTLA-4, CD39 and CD357 (GITR), among CD4$^{+}$CD25$^{+}$CD127$^{lo}$FOXP3$^{+}$ Treg cells (turquoise line) and CD45RA$^{+}$FOXP3$^{++}$ Treg cells (blue line). PBMCs were stained with a combination of several antibodies as described in the Methods. **(b)** The absolute counts of Treg cells were analysed in conventional KT recipients (black circles and bars) and CKBMT recipients (green circles and bars) at the indicated post-transplant time points. **(c)** The relative frequencies of CTLA-4$^{+}$, CD39$^{+}$ and GITR$^{+}$ cells among total Treg cells were assessed by flow cytometry. **(d)** The relative frequency of three distinct subpopulations of Treg cells was examined and compared for KT and CKBMT recipients 1 year after transplantation. **(e)** The relative frequencies of CTLA-4$^{+}$, CD39$^{+}$ and GITR$^{+}$ cells among three Treg cell subpopulations were compared for KT and CKBMT recipients at the 3-, 12- and 24-month follow-up. KT and CKBMT recipients are represented by black circles and green circles, respectively. Data are presented as mean ± standard deviation. n.s.: not significant, *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001 and ****$P$ < 0.0001.

## The relative frequency of Treg cells is increased by CKBMT

Next, we examined the frequency of Treg cells in the peripheral blood by flow cytometry (Figure 3a and Supplementary figure 4). Treg cells were defined as $CD4^+CD25^+CD127^{lo}FOXP3^+$ T cells. Three months after transplantation, the absolute number of Treg cells in CKBMT patients did not differ from the number in KT patients (Figure 3b). However, at the 1- and 2-year follow-up, the absolute number of Treg cells was significantly higher in CKBMT patients than in KT patients. We also examined the expression of receptors related to suppressive functions, including CTLA-4, GITR and CD39 (Figure 3c). We found no difference in the expression of CTLA-4 and GITR between CKBMT and KT recipients, and the expression of CD39 tended to be higher in CKBMT recipients than KT recipients, but was not significant.

We further assessed three distinct subpopulations of $CD4^+$ T cells expressing FOXP3, $CD45RA^+FOXP3^+$ Treg cells, $CD45RA^-FOXP3^{++}$ Treg cells and $CD45RA^-FOXP3^+$ Treg cells (Figure 3a). Previous studies have shown $CD45RA^-FOXP3^{++}$ Treg cells exert the highest suppressive activity among the three subpopulations.[24] One year after transplantation, CKBMT recipients had a significantly higher frequency of $CD45RA^-FOXP3^{++}$ Treg cells than KT recipients, but they had a lower frequency of $CD45RA^+FOXP3^+$ Treg cells (Figure 3d). We found no difference in the frequency of $CD45RA^-FOXP3^+$ cells, or in the expression of CTLA-4 among the $CD45RA^-FOXP3^{++}$ Treg subpopulation between the CKBMT and KT recipients (Figure 3e). The expression of GITR and CD39 on the Treg subpopulations tended to be higher in CKBMT recipients than KT recipients but was not significant.

## The ratio of two $CD45RA^-FOXP3^+$ T-cell subpopulations is associated with graft tolerance

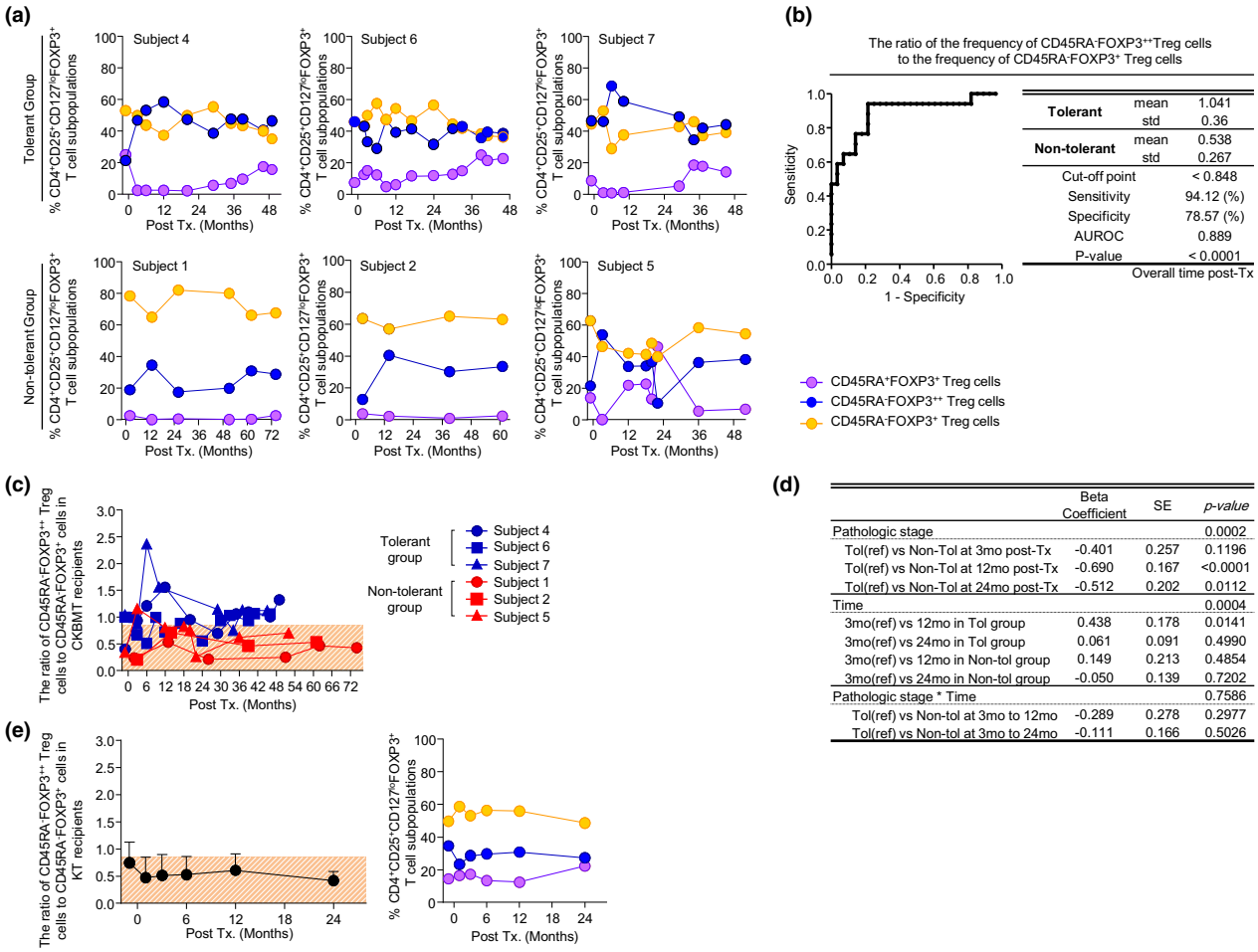
We also examined the changes in the frequency of two $CD45RA^-FOXP3^{+/++}$ T-cell subpopulations in the tolerant and the non-tolerant groups. In the non-tolerant group, $CD45RA^-FOXP3^+$ cells dominated over $CD45RA^-FOXP3^{++}$ Treg cells during the course of follow-up (Figure 4a). However, the frequency of $CD45RA^-FOXP3^{++}$ Treg cells was similar to the frequency of $CD45RA^-FOXP3^+$ cells during the course of follow-up in the tolerant group. Next, we calculated the ratio of the frequency of $CD45RA^-FOXP3^{++}$ Treg cells to the frequency of $CD45RA^-FOXP3^+$ cells at all available time points and analysed whether the ratio can distinguish between the tolerant group and the non-tolerant group. Receiver operating curve (ROC) analysis of the ratio revealed a significant area under the curve of 88.9% ($P < 0.0001$), with 94.12% sensitivity and 78.57% specificity (Figure 4b). The cut-off point (ratio $< 0.848$) was determined by ROC analysis. The ratio showed good discrimination of tolerance and non-tolerance with the cut-off points under 0.848.

The ratio of the frequency of $CD45RA^-FOXP3^{++}$ Treg cells to the frequency of $CD45RA^-FOXP3^+$ cells was plotted for six patients during the course of follow-up (Figure 4c). Tolerant recipients had a ratio higher than the cut-off value at many time points, particularly after 24 months post-CKBMT. However, the non-tolerant recipients exhibited a ratio lower than the cut-off value at almost all time points. The longitudinal relationship of the ratio to pathological status was evaluated utilising the Generalized Estimating Equation (GEE)[26,27] (Figure 4d). GEE analysis indicated that maintaining a high ratio was associated with tolerant status over time. Interestingly, in KT patients without any procedure for inducing graft tolerance, the kinetics of the $CD4^+CD25^+CD127^{lo}FOXP3^+$ subpopulations and ratio of two different $CD45RA^-FOXP3^+$ T-cell subpopulations exhibited similar patterns as in the non-tolerant CKBMT recipients (Figure 4e). These results suggest that the balance of $CD45RA^-FOXP3^{++}$ Treg cells and $CD45RA^-FOXP3^+$ cells is crucial for inducing transplant immune tolerance.

## Suppressive activity of Treg cells in CKBMT recipients

Finally, we evaluated the suppressive activity of Treg cells from recipients after CKBMT. The Treg cells were isolated and co-cultured with autologous $CD8^+$ or $CD4^+CD25^-$ T cells in the presence of anti-CD3/CD28. Treg cells from the tolerant group highly suppressed $CD8^+$ or $CD4^+CD25^-$ responder cells as well as those from healthy donors, but Treg cells from the non-tolerant group did not (Figure 5a).

We also assessed the suppressive activity of Treg cells after CKBMT in the setting of MLR assays.




| | | Beta Coefficient | SE | *p-value* |
|---|---|---|---|---|
| Pathologic stage | | | | 0.0002 |
| Tol(ref) vs Non-Tol at 3mo post-Tx | | -0.401 | 0.257 | 0.1196 |
| Tol(ref) vs Non-Tol at 12mo post-Tx | | -0.690 | 0.167 | <0.0001 |
| Tol(ref) vs Non-Tol at 24mo post-Tx | | -0.512 | 0.202 | 0.0112 |
| Time | | | | 0.0004 |
| 3mo(ref) vs 12mo in Tol group | | 0.438 | 0.178 | 0.0141 |
| 3mo(ref) vs 24mo in Tol group | | 0.061 | 0.091 | 0.4990 |
| 3mo(ref) vs 12mo in Non-tol group | | 0.149 | 0.213 | 0.4854 |
| 3mo(ref) vs 24mo in Non-tol group | | -0.050 | 0.139 | 0.7202 |
| Pathologic stage * Time | | | | 0.7586 |
| Tol(ref) vs Non-tol at 3mo to 12mo | | -0.289 | 0.278 | 0.2977 |
| Tol(ref) vs Non-tol at 3mo to 24mo | | -0.111 | 0.166 | 0.5026 |

**Figure 4.** Ratio of Treg subpopulations in the tolerant group and non-tolerant group. **(a)** The kinetics of three $CD4^+CD25^+CD127^{lo}FOXP3^+$ Treg cells subpopulations (orange lines and circles: the kinetics of $CD45RA^+FOXP3^+$ Treg cells, blue lines and circles: the kinetics of $CD45RA^-FOXP3^{++}$ Treg cells, and purple lines and circles: the kinetics of $CD45RA^-FOXP3^+$ cells) in the tolerant group and non-tolerant group were monitored at various post-transplant time points. **(b)** ROC curve estimation based on the ratio of the frequency of $CD45RA^-FOXP3^{++}$ Treg cells to the frequency of $CD45RA^-FOXP3^+$ cells in CKBMT recipients ($n$ = 6). **(c)** Kinetics of the ratio of two $CD4^+CD25^+CD127^{lo}CD45RA^-FOXP3^+$ T-cell subpopulations in each CKBMT recipient (frequency of $CD45RA^-FOXP3^{++}$ Treg cells to $CD45RA^-FOXP3^+$ cells). The area under the cut-off value (0.848) of the ratio of Treg subpopulations is indicated by the shading and red diagonal line. **(d)** GEE analysis was performed to evaluate the association with time-lag and pathological stage in the tolerant group and non-tolerant group. **(e)** Kinetics of the relative frequencies of Tregs (right panel) and the ratio of $CD45RA^-FOXP3^{++}$ Treg cells to $CD45RA^-FOXP3^+$ cells (left panel) in conventional KT recipients ($n$ = 20). Data are presented as mean ± standard deviation.

PBMCs from the tolerant ($n$ = 3) and non-tolerant ($n$ = 2) groups with or without Treg cell depletion were co-cultured with irradiated donor PBMCs for 6 days. The proliferation of $CD4^+$ and $CD8^+$ responder cells was increased by depleting Treg cells in the tolerant group, demonstrating the suppressive activity of Treg cells on the allo-response (Figure 5b and c). However, the proliferation of $CD4^+$ and $CD8^+$ responder cells was decreased by the depletion of Treg cells in the non-tolerant group, indicating an absence of the suppressive activity of Treg cells (Figure 5b and c). In the non-tolerant group, high levels of $CD4^+$ T cell proliferation in Treg non-depleted PBMCs may be explained by the proliferation of Treg cells.

These data indicate that graft tolerance after CKBMT is associated with not only a high ratio of $CD45RA^-FOXP3^{++}$ Treg cells to $CD45RA^-FOXP3^+$ cells, but also high suppressive activity of the Treg cell population, which may play a crucial role in the maintenance of graft tolerance.

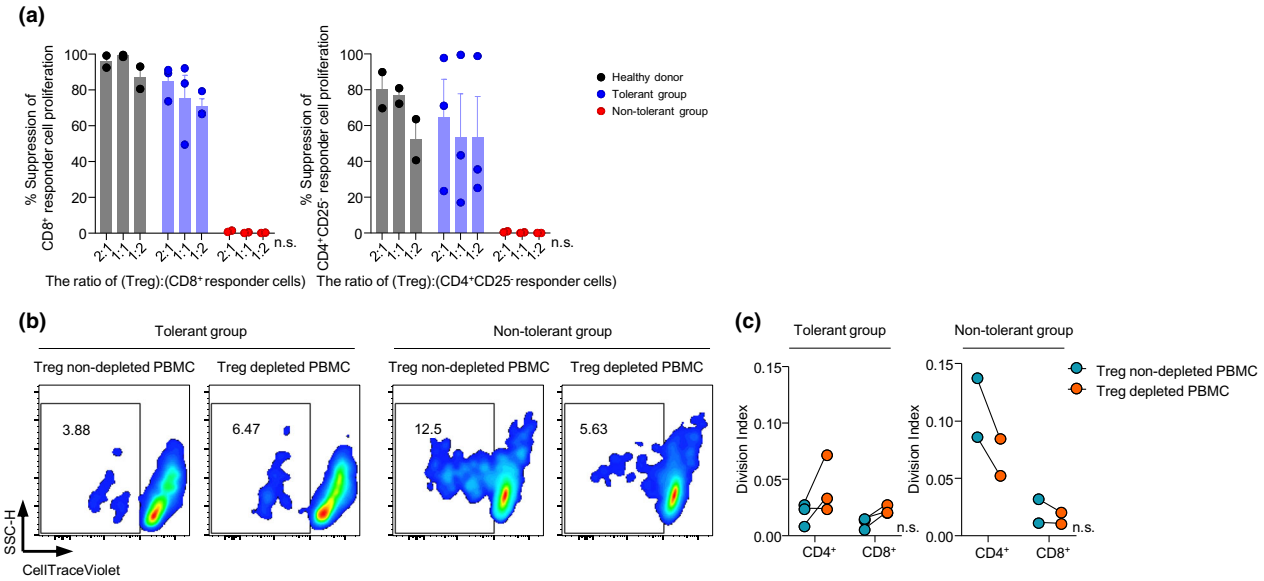



**Figure 5.** Suppressive activity of the Treg cell population in the tolerant and non-tolerant groups. **(a)** The suppressive activity of the Treg cell population in CKBMT recipients ($n$ = 5) was measured as T-cell receptor-stimulated (anti-CD3 and anti-CD28) proliferation of $CD8^+$ or $CD4^+CD25^-$ responder T cells co-cultured with $CD4^+CD25^+CD127^{lo}$ total Treg cells. The per cent suppression of responder T-cell proliferation was calculated as [% Suppression = 100 − {(division index of responder T cells only)/(division index of responder T cells in co-culture with Treg cells)} × 100] and compared between the tolerant (blue bars), non-tolerant (red bars) and healthy control (black bars) groups. **(b)** Representative flow cytometry plots for proliferating $CTV^{lo}$ T cells in the gate of responder $CD4^+$ T cells during MLR using Treg-depleted/non-depleted recipient PBMCs. **(c)** The division index among responder $CD4^+$ and $CD8^+$ T cells was calculated. The experiment was performed with Treg-depleted (orange circles) or non-depleted PBMCs (turquoise circles) in the tolerant group (left panel) and non-tolerant group (right panel). These PBMCs were co-cultured with each donor PBMC. Data are presented as mean ± standard deviation. n.s. = not significant.

## DISCUSSION

In the present study, we demonstrated that the expansion of $CD45RA^-FOXP3^{++}$ Treg cells is likely associated with immune tolerance in patients with combined kidney and bone marrow transplantation. We first found that the $CD45RA^-FOXP3^{++}$ Treg cell subpopulation was expanded in CKBMT recipients compared to conventional KT patients. Importantly, the expansion of $CD45RA^-FOXP3^{++}$ Treg cells was more striking observed in the tolerant group than the non-tolerant group, and the ratio of $CD45RA^-FOXP3^{++}$ Treg cells to $CD45RA^-FOXP3^+$ cells showed good discrimination for the tolerant and non-tolerant groups. In addition, the suppressive activity of the Treg cell population was high after CKBMT in the tolerant group but absent in the non-tolerant group, although the difference was not significant.

$CD25^+CD127^{lo}FOXP3^+$ Treg cells have been considered to contribute to the induction of transplant tolerance.[25,28] In CKBMT recipients with allograft tolerance, early expansion of peripheral Treg cells has been implicated in the induction of tolerance.[29–33] In addition, $FOXP3^+$ Treg cells were enriched in the graft tissues of tolerant recipients.[34] $CD45RA^-FOXP3^{++}$ Treg cells have been reported to exhibit the highest suppressive ability among Treg subpopulations.[24] In conventional KT recipients with operational tolerance, the relative frequency of $CD45RA^-FOXP3^{++}$ Treg cells was high in peripheral blood.[25] In addition, an increased frequency of $CD45RA^-FOXP3^{++}$ Treg cells has been observed in CKBMT recipients compared to pre-transplant status, although that previous study used a different marker to define activated Treg cells, HLA-DR.[33]

$CD45RA^-FOXP3^+$ cells exert inflammatory cytokine-secreting functions rather than suppressive functions.[24] In the current study, the ratio of $CD45RA^-FOXP3^{++}$ Treg cells and $CD45RA^-FOXP3^+$ cells was maintained at relatively high levels in the tolerant group. In contrast, the ratio was maintained at low levels in the non-tolerant group and the conventional KT group. $CD45RA^-FOXP3^+$ cells may contribute to

exaggerated allo-responses by secreting inflammatory cytokines, including IL-17A,[24,35] IFN-$\gamma$[24] and TNF,[36] disrupting transplant tolerance after CKBMT. In the balance between CD45RA$^-$FOXP3$^{++}$ Treg cells and CD45RA$^-$FOXP3$^+$ cells, CD45RA$^-$FOXP3$^{++}$ Treg cells become dominant after CKBMT, and the inflammatory action of CD45RA$^-$FOXP3$^+$ cells may be efficiently checked by CD45RA$^-$FOXP3$^{++}$ Treg cells, successfully maintaining transplant tolerance.

We also examined the frequency of T cells and their subpopulations in the peripheral blood after transplantation. We found that the memory T-cell subpopulation among CD4$^+$ and CD8$^+$ T cells in CKBMT recipients is expanded following lymphopenia. Severe lymphopenia results in the predominant reconstitution of memory T cells, especially in the CD8$^+$ T-cell population.[9,14,29] Rapid emergence of memory T cells after lymphodepletion is likely caused by homeostatic proliferation.[37,38] Although reconstituted T cells are preferentially memory cells, a high frequency of memory T cells is not associated with an increased risk of rejection.[38] In cynomolgus monkeys with CKBMT, the frequency of total memory T cells has been shown to be robustly increased in all recipient monkeys, regardless of the outcome of CKBMT,[39] although donor MHC-specific memory T cells were found to be expanded only in recipients with acute cellular rejection.[38,39] Therefore, the increase in the frequency of total memory T cells seems to be related more to homeostatic proliferation following severe lymphopenia than the rejection/tolerance status.

Whether donor chimerism *(i.e. full or mixed, and durable or transient)* is helpful for inducing graft tolerance following CKBMT is still unclear.[6,16,40,41] Moreover, underlying mechanisms of chimerism-induced transplant tolerance are not completely understood. Our group aimed to achieve transient mixed chimerism, which requires less toxic preconditioning. We optimised the protocol for non-myeloablative preconditioning for CKBMT[15] and successfully induced transplant tolerance in half of recipients, with the expansion of the CD45RA$^-$FOXP3$^{++}$ Treg cell subpopulation. However, we do not know yet how transient mixed chimerism results in the expansion of the CD45RA$^-$FOXP3$^{++}$ Treg cell subpopulation in CKBMT recipients, and it needs to be elucidated in further studies.

The current study has some limitations. First, the number of CKBMT patients is too small to come to a solid conclusion, although we suggest that the ratio of CD45RA$^-$FOXP3$^{++}$ Treg cells to CD45RA$^-$FOXP3$^+$ cells reflects the tolerant/non-tolerant status. Our center is currently performing 4–5 CKBMT procedures per year and has experience with 16 cumulative cases thus far. We have a plan to validate the current preliminary results by analysing a larger CKBMT cohort in the near future. Second, we could not show a significant difference in the suppressive activity of Treg cells between the tolerant and non-tolerant groups due to the small number of CKBMT patients, although only the tolerant group had high levels of Treg suppressive activity. A difference in the Treg suppressive activity between the tolerant and non-tolerant groups needs to be investigated further in a larger cohort because it can explain a tolerance mechanism in CKBMT recipients.

In summary, we found that the relative frequency of CD4$^+$CD25$^+$CD127$^{lo}$FOXP3$^+$ Treg cells significantly increased in the peripheral blood of CKBMT recipients. Moreover, the ratio of CD45RA$^-$FOXP3$^{++}$ Treg cells to CD45RA$^-$FOXP3$^+$ cells reflects the tolerant/non-tolerant status and may serve as a reliable biomarker. The value of the ratio of two different CD45RA$^-$FOXP3$^+$ T-cell subpopulations needs to be further validated in a larger cohort, and the underlying mechanisms of activated Treg cell expansion after CKBMT in our preliminary study need to be clarified.

## METHODS

### Patients

Seven patients underwent CKBMT at Samsung Medical Center between November 2011 and May 2014. One patient was excluded from this analysis because he lost his graft from severe BK virus-associated nephropathy 8 months after CKBMT.[15] To compare CKBMT to conventional KT, 20 patients who received ABO blood type-compatible kidney transplants at Samsung Medical Center from October 2015 to June 2017 were enrolled in the study. KT recipient and CKBMT recipient characteristics are compared in Table 1. The characteristics of the CKBMT recipients were described previously[15] and are presented in Table 2. All protocols were approved by the Institutional Review Board of Samsung Medical Center, Sungkyunkwan University School of Medicine (IRB No. SMC 2012-09-019 and SMC 2010-07-210).

## Preconditioning regimen and immunosuppression tapering

The medication protocols for KT and CKBMT are summarised in Figure 1. The preconditioning regimen for CKBMT was reported previously.[15] Briefly, the preconditioning regimen for CKBMT consisted of rituximab (Mabthera, Roche Pharma AG, Basel, Switzerland) twice, cyclophosphamide (Endoxan Inj., Baxter Oncology GmbH, Westfalen, Germany) twice, fludarabine monophosphate (Fludara Inj., Bayer AG, Wuppertal, Germany) four times, rATG (Thymoglobulin, Sanofi Genzyme Co., Boston, Massachusetts, USA) for 3–4 days and thymic irradiation on day −1. The preconditioning regimen was modified two times due to engraftment syndrome, cyclophosphamide toxicity and uncontrolled BK virus reactivation. Tacrolimus (Prograf, Astellas Pharma, Tokyo, Japan), sirolimus (Rapamune, Pfizer, New York USA) and steroid were used as maintenance drugs. Steroids were tapered down and stopped 3–6 months after CKBMT. Tacrolimus or sirolimus tapering was performed after a 1-year biopsy if the result was not suggestive of pathological acute rejection. In the case of KT, all patients received a total dose of 4.5 mg/kg rATG as an induction drug and were administered tacrolimus, mycophenolate and steroid as maintenance drugs.

## Blood sampling

Whole blood was obtained from patients who underwent CKBMT and KT. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque (GE Healthcare, Chicago, Illinois, USA) density gradient centrifugation and cryopreserved in liquid nitrogen until use.

## Antibodies

The following fluorochrome-conjugated monoclonal antibodies were used for multicolour flow cytometry: anti-CD45-BUV395 (HI30), anti-CD3-BV510, –FITC, or -PerCP-Cy5.5 (UCHT1), anti-CD4-BV605 (RPA-T4), anti-CD8-BV711 or –APC-R700 (SK1), anti-CD45RA-APC-H7 (HI100), anti-CD127-BV650 or –FITC (HIL-7R-M21), anti-CD25-BV650, -BB515, or -PE-Cy7 (M-A251), anti-CD272-BV650 or -APC (J168-540.90.22), anti-CD39-BV711 (TU66), anti-CD19-PE-CF594 (HIB19) and anti-CD14-PE-CF594 (MQP9) (all from BD Bioscience, San Jose, California, USA); anti-FOXP3-PE or –Alexa Fluor 700 (PCH101) (all from eBioscience, San Diego, California, USA); anti-CD197-BV786 or –BV510 (G043H7), anti-PD-1-BV421 or –PerCP-Cy5.5 (EH12.2H7), anti-CD357 (GITR)-PE (108-17) and anti-CTLA-4-APC or –PE-cy7 (L3D10) (all from Biolegend, San Diego, California, USA).

## Flow cytometric analysis

PBMCs were stained with fluorochrome-conjugated antibodies. Dead cells were excluded by staining with Live/Dead red fluorescent reactive dye (Invitrogen, Carlsbad, California, USA). For intracellular staining, surface-stained cells were permeabilised using the FOXP3/Transcription Factor Staining Buffer Set (Invitrogen, Waltham, Massachusetts, USA, catalog number: 00-5523-00) and further stained for intracellular proteins. Anti-FOXP3 (PE, or Alexa Fluor 700) and anti-CTLA-4 (APC or PE-cy7) were used for intracellular staining. The stained cells were analysed using an LSR II and LSRFortessa instrument and FACSDiva (BD Bioscience) or FlowJo software (BD Bioscience).[36]

## *In vitro* Treg suppression assays

Treg cells were isolated from recipient PBMCs and commercial normal PBMCs (ePBMC®, Cellular Technology Limited, Shaker Heights, Cleveland, USA; as a healthy control) using the $CD4^+CD25^+CD127^{dim/-}$ Treg Isolation Kit α (Miltenyi Biotec, Bergisch Gladbach, Germany) and an AutoMACS separator (Miltenyi Biotec). Isolated Treg cells labelled with CellTraceRed (CTR, Invitrogen) were co-cultured with autologous sorted $CD8^+CD25^-$ and $CD4^+CD25^-$ T cells (as responder cells) labelled with CellTraceViolet (CTV, Invitrogen) in the presence of anti-CD3/CD28. A total of $2 \times 10^4$ responder T cells were co-cultured at different ratios with isolated autologous Treg cells. After 5 days of co-culture, the proliferative activity of the $CD8^+$ responder T cells was measured by calculating the percentage of dividing $CTV^{lo}$ cells. The percentage of suppression was calculated as [% Suppression = 100 − {(division index of responder T cells only)/(division index of responder T cells in co-culture with Treg cells)} × 100].

## Mixed lymphocyte reaction

MLR assay was performed as described previously.[15] Stimulator cells, including donor PBMCs and third party pooled PBMCs, were γ-irradiated at 30 Gy and labelled with CTR dye. Responder PBMCs, from each CKBMT patient, were labelled with CTV dye and co-cultured with stimulator cells in a 37°C $CO_2$ incubator for 6 days. The proliferative activity of responder cells was assessed as the stimulation index.[42]

## Mixed lymphocyte reaction using Treg-depleted PBMCs

To determine how the proliferative response of responder PBMCs to each donor PBMC is affected by Treg cells, we performed modified mixed lymphocyte reactions. Treg cells were depleted from PBMCs using the Treg Isolation Kit α (Miltenyi Biotec) with an AutoMACS separator (Miltenyi Biotec). The absence of Treg cells was confirmed by flow cytometry. Approximately $1 \times 10^5$ Treg-depleted or non-depleted recipient PBMCs were labelled with CTV dye and co-cultured with $1 \times 10^5$ γ-irradiated donor PBMCs labelled with CTR dye. After 6 days of co-culture, the proliferative activity of Treg-depleted/non-depleted PBMCs was measured by gating the percentage of proliferating $CTV^{lo}$ cells. The proliferative activity of responder cells was assessed as the division index.[42,43]

### Statistical analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Differences between two groups were compared by the exact Wilcoxon rank sum test and Mann–Whitney *U*-test. The Generalized Estimating Equation was applied to repeat measurements of parameters. The diagnostic cut-off point was determined by receiver operating characteristic (ROC) analysis of the patient's percentages of regulatory T-cell subpopulations. The ROC analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, California, USA). Significance was set at $P < 0.05$.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

**Yeongbeen Kwon**: Study design, methodology, data acquisition, investigation, writing–original draft and writing–review and editing. **Kyo Won Lee**: Study design, investigation, patient recruitment and writing-review and editing. **You Min Kim**: Study design, methodology, data acquisition, investigation and writing–review and editing. **Hyojun Park**: Investigation and patient recruitment. **Min Kyung Jung**: Providing technical expertise. **Young Joon Choi**: Investigation and data analysis. **Jin Kyung Son**: Data acquisition. **JuHee Hong**: Data acquisition. **Su-Hyung Park**: Providing technical expertise. **Ghee Young Kwon**: Pathologic analysis. **Heejin Yoo**: Statistical analysis. **Kyunga Kim**: Statistical analysis. **Sung Joo Kim**: Study design, patient recruitment and manuscript revision. **Jae Berm Park**: Study design, project administration, manuscript revision and supervision. **Eui-Cheol Shin**: Study design, project administration, writing-review and editing and supervision.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.