Expansion of $CD4^+$ $CD25^+$ regulatory T cells from cord blood $CD4^+$ cells using the common γ-chain cytokines (IL-2 and IL-15) and rapamycin

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Abstract

Rapamycin has important roles in the modulation of regulatory T cells. We tried to expand $CD4^+CD25^+$ regulatory T cells (Treg cells) from umbilical cord blood (CB) CD4-positive cells using IL-15 or IL-2 with TGF-β and rapamycin. We were able to obtain more than 500-fold expansion of $CD4^+CD25^+$ cells from CB $CD4^+$ cells using IL-15 and TGF-β with rapamycin. These expanded $CD4^+CD25^+$ cells expressed FoxP3 mRNA at a level about 100-fold higher and could suppress allogeneic mixed lymphocyte culture by more than 50%. Early after rapamycin stimulation, CB $CD4^+$ cells showed increased expression of Foxp3 and a serine/threonine kinase Pim 2 and sustained expression of negative PI3K regulator PTEN. On the other hand, $CD4^+CD25^+$ cells expanded with rapamycin for 8 days showed much higher levels of FoxP3 mRNA expression and decreased expression of PTEN. A comparison of IL-15 stimulation and IL-2 stimulation showed slightly higher efficiency of IL-15 for expansion of $CD4^+CD25^+$ cells and for FoxP3 expression, IL-15 also showed significantly higher efficacy for inhibition of MLC. The combination of the common γ-chain cytokine IL-15, TGF-β and rapamycin may be a useful means for expanding Treg cells. Pim2 expression early after stimulation with rapamycin may be important for conferring rapamycin resistance for growth of Treg cells. IL-15 is not less useful than IL-2 for expansion of Treg cells.

Introduction

$CD4^+CD25^+$ regulatory T cells (Treg cells) contribute to the suppression of allogeneic immune responses and prevent transplant rejection and graft-versus-host disease (GVHD) [1-3]. Cord blood contains naïve T cells and Treg precursor cells that can be expanded by several cytokines [4-7]. We previously reported more than 50-fold expansion of $CD4^+CD25^+$ regulatory T cells from cord blood $CD4^+CD25^+$ cells using IL-15 and TGF-β [8]. The serine/threonine protein kinase mammalian target of rapamycin (mTOR) has important roles in the activation of effector T cells and the function and proliferation of regulatory T cells. Blockade of mTOR by rapamycin inhibits T cell proliferation, and rapamycin is therefore used for prophylaxis of GVHD and graft rejection [9]. It has been shown that rapamycin selectively expands murine naturally occurring CD4CD25FoxP3 (forkhead box P3) T cells in vitro [10] and that it induces suppressor functions in conventional CD4 T cells [11]. Selective expansion and the survival and proliferation of naturally occurring regulatory T cells can be achieved by using rapamycin [12]. FoxP3 is indispensable for the differentiation of regulatory T cells [13]. The lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) regulates the anergic response of Treg cells to IL-2 in vitro and Treg homeostasis in vivo [14]. The expression level of PTEN, a negative regulator of the

PI3K/Akt/mTOR pathway, remained high in Treg cells during stimulation [15].

Pim 2 is a serine/threonine kinase that can confer resistance to rapamycin. Pim 2 regulates late cell cycle progression induced by IL-2 [16]. FoxP3 induces Pim 2 expression in Treg cells, conferring a replicative advantage in cultures containing rapamycin [17]. Therefore, FoxP3, Pim2 and PTEN may have important roles in the expansion of Treg cells using rapamycin. IL-15 utilizes the beta and gamma chains of IL-2 receptor [18]. IL-15 has an anti-apoptotic effect and a preferential survival enhancing effect on cord blood T cells [19,20]. Therefore, IL-15 may have a positive effect to induce Treg cells with rapamycin.

In this study, we tried to expand Treg cells from cord blood $CD4^+$ cells using the common γ-chain cytokine IL-15 and TGF-β with or without rapamycin and we compared the effect of IL-15 and IL-2 in order to develop a potential strategy for cell therapy to control GVHD and graft rejection after allogeneic stem cell transplantation. Also, we analyzed FoxP3, Pim 2 and PTEN expression during expansion of Treg cells using rapamycin in order to reveal the mechanism of proliferation of Treg cells in the presence of the T cell growth inhibitor rapamycin.

Materials and methods

Isolation of $CD4^+$ cells from cord blood and culture

CD4-positive cells were isolated from umbilical cord blood cells (Hokkaido Cord Blood Bank, Sapporo, Japan) by positive selection using magnetic cell sorting (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). FACS analysis showed that cells were routinely more than 90% CD4-positive for the CD4-positive fraction.

$CD4^+$ cells (0.2 X 10^6/mL) were cultured with 10 μl of anti-CD3/CD28 mAb-coated dynabeads (Dynal Biotech, Lake Success, NY, USA) and IL-2 (5 ng/mL) or IL-15 (5 ng/mL) with or without TGF-β (1 ng/mL) (R & D Systems, Minneapolis, MN, USA) in RPMI-1640 with 10% fetal calf serum (FCS) in 24-well plates in the presence or absence of rapamycin (Sigma-Aldrich; 0.5-1.0 nM). Cell cultures were split approximately one-fourth after 3 days of culture and fresh medium, cytokines and anti-CD3/CD28 dynabeads were added.

Immunofluorescent staining for flow cytometric analysis and monoclonal antibodies

The phycoerythrin (PE)-conjugated monoclonal antibody (mAb) Mik-b3 (anti-CD122) was obtained from Becton Dickinson (BD, San

Jose, CA, USA) and 4E3 (anti CD25) was obtained from Miltenyi Biotec GmbH. FITC-conjugated mAbs 110416 (anti-GITR) and ACT35 (anti-OX40) were obtained from R&D and BD, respectively. Intracellular FoxP3 was stained using FoxP3 fixation/permeabilization reagent (eBioscience, San Diego, CA, USA). The fluorescence intensity of the cells was analyzed using a FACS Calibur (BD). Statistical analysis was performed using Student's *t*-test.

Mixed lymphocyte culture (MLC)

Responder cells (50×10^3) were cultured with 50×10^3 irradiated (30 Gy) allogeneic, third-party PBMC stimulators in 200 μl of RPMI 1640 supplemented with 10% fetal calf serum in round-bottom 96-well plates (Corning, New York). After 7 days of incubation at 37 °C in 5% CO_2, cultures were pulsed with ^{3}H-thymidine (1.0 μCi/well) for the final 16 hours. The cells were then harvested, and ^{3}H-thymidine incorporation was measured in triplicate using a 196 gas flow counter (Packard). $CD4^+CD25^+$ cells (>95%) expanded from cord blood $CD4^+$ cells for 8 days were added to the MLC on day 0 (responder cells : Treg cells, 10:1 and 10:3).

Real-time PCR

Total RNA was prepared using a QIAamp RNA Blood Mini Kit (QIAGEN), and cDNA was synthesized from 1.2 μg total RNA using a TaKaRa RNA PCR Kit(AMV) Ver.3.0 following the instructions of the manufacturer (TAKARA, Shiga, Japan). Assays-on-Demand™Gene Expression products for FoxP3, Pim2 and PTEN were obtained from Applied Biosystems. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Relative quantification assays for gene expression were performed using an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems)

Results

Expansion of $CD4^+CD25^+$ cells from $CD4^+$ cells derived from cord blood

There were 3.1 ± 1.5% of $CD4^+CD25^+$ cells with only 0.2 ± 0.1% of $CD25^+FoxP3^+$ cells in lymphocytes of cord blood before stimulation. We could get an 893 ± 475-fold expansion of $CD4^+CD25^+$ cells (n=5, $CD4^+CD25^+$ > 95%) from cord blood $CD4^+$ cells after 8 days of culture using IL-15 (Table 1). Addition of TGF-β enhanced FoxP3 expression in IL-15-expanded $CD25^+$ cells (19.1 ± 12.0% vs 51.6 ± 21.4%, n=5, $p<0.05$). Furthermore, addition of rapamycin (0.5 nM) significantly enhanced FoxP3 expression in $CD4^+$ cells expanded by IL-15 with TGF-β (87.4 ± 7.9 %, n=5, $p<0.01$)(Fig.1). Also, these $CD4^+CD25^+$ cells coexpressed glucocorticoid-induced tumor necrosis factor receptor (GITR), OX40 and IL-15 receptor α chain (CD122). The calculated absolute number of $CD4^+CD25^+$ cells after 8 days of culture with IL-15 together with TGF-β and rapamycin tended to be small (5 x 10^6 cells from 0.2 x 10^6 cord blood $CD4^+$ cells) compared with the number of cells obtained in other conditions (about 6 x 10^6 cells)(Table 2). However, IL-15 stimulation with TGF-β and rapamycin induced much more expansion of $CD25^+FoxP3^+$ cells (3.856 ± 1.035 x 10^6). Also, the calculated FoxP3 : CD4 ratio

was highest after IL-15 stimulation with TGF-β and rapamycin (0.771). Therefore, rapamycin selectively helped to expand Treg cells.

We used IL-2 instead of IL-15 as a control study. Calculated absolute numbers of $CD4^+CD25^+$ cells and $CD25^+FoxP3^+$ cells after 8 days of culture with IL-2 together with TGF-β and rapamycin were $4.631 \pm 0.794 \times 10^6$ and $3.182 \pm 1.272 \times 10^6$, respectively, and the FoxP3: CD4 ratio was 0.687. IL-2 stimulation showed slightly lower efficiency for expansion of Treg cells, but there were no statistical differences (Tables 3,4).

Inhibition of allogeneic MLC by $CD4^+CD25^+$ cells expanded from cord blood

$CD4^+CD25^+$ cells (>95%) expanded from cord blood $CD4^+$ cells with anti-CD3/CD28 mAb-coated dynabeads and IL-15 plus TGF-β using rapamycin inhibited allogeneic MLC by about 80% (responder cells : Treg cells, 10:1 and 10:3)(Table 5). Also, $CD4^+CD25^+$ cells expanded with rapamycin inhibited MLR much more significantly than did cells expanded without rapamycin. IL-2 stimulation showed slightly lower efficiency for inhibition of MLC. Also, a significantly lower inhibition rate was found when IL-2 plus TGF-β with rapamycin was used than when IL-15 plus TGF-β with rapamycin was used (responder cells : Treg cells, 10:1, Table 6).

Significantly lower absolute counts (CPM) of ^{3}H-thymidine incorporation in allogeneic third party MLC were always found when cultured with expanded $CD25^{+}CD4^{+}$ cells than when cultured without expanded $CD25^{+}CD4^{+}$ cells ($p<0.01$, Fig.2). This result indicated that expanded $CD25^{+}CD4^{+}$ cells were anergic against allogeneic antigens and could not response allogeneic third party stimulators.

Increased expression of FoxP3 and Pim2 in rapamycin-induced $CD4^{+}CD25^{+}$ cells expanded from cord blood

Early after rapamycin stimulation (6 hours), cord blood $CD4^{+}$ cells showed increased expression of Foxp3 and Pim 2, which share common downstream targets of Akt (protein kinase B) correlated with cell cycle progression of Treg cells. Also, expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of the PI3K/Akt/mTOR pathway, was sustained at a relatively high level in rapamycin-induced cells compared with that in cells induced by only IL-15 and TGF-β (Table 7). FoxP3 expression level was upregulated after 6 hours of stimulation with rapamycin and was sustained at a high level on day 8. On the other hand, Pim 2 expression level was upregulated only early after rapamycin stimulation. $CD4^{+}CD25^{+}$ cells expanded from cord blood $CD4^{+}$ cells with rapamycin for 8 days expressed a much higher level

of FoxP3 mRNA than that in $CD4^+CD25^+$ cells expanded without rapamycin. However, the expression level of Pim2 was not so elevated and the expression level of PTEN was downregulated after 8 days of stimulation even using rapamycin. IL-2 stimulation showed almost the same tendency for induction of these genes, though slightly lower efficiency for induction of FoxP3 on day 8 by stimulation with rapamycin was noted (93.3 ± 15.1 vs 60.2 ± 47.5, p=0.068)(Table 8).

Discussion

Treg cells have an important role in the regulation of allogeneic immune responses after allogeneic stem cell transplantation. Rapamycin inhibits the mammalian target of rapamycin (mTOR) pathway and has an impact on expansion and activity of Treg cells. Treg cells are resistant to rapamycin-induced apoptosis and thus selectively expand in the presence of rapamycin [9].

IL-2 has been used for the expansion of cytotoxic CD8 cells and also Treg cells. IL-15 enhances T cell and NK cell proliferation by using the β and γ chains of the IL-2 receptor [21]. It has been reported that IL-15 enhanced in vitro T cell survival and effector function after autologous stem cell transplantation [22]. Also, IL-15 enhanced TNF-α and IL-10 production during cord blood alloreactivity but failed to enhance allospecific proliferation [23]. It has been reported that peripheral $CD4^+CD25^-$ naive T cells are converted to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3 and that they develop suppressive activity [24, 25]. We previously reported expansion of $CD4^+CD25^+$ regulatory T cells from cord blood $CD4^+CD25^+$ cells using IL-15 [8]. Therefore, there is a possibility that IL-15, TGF-β and rapamycin can be used to induce the expansion of Treg cells derived from cord blood. In this study, $CD4^+CD25^+$ cells could

be expanded efficiently by more than 500 fold from cord blood $CD4^+$ cells using the common γ-chain cytokine IL-15, TGF-β and rapamycin. Addition of rapamycin to the stimulation of IL-15 and TGF-β helped to induce selective expansion of $CD25^+FoxP3^+$ cells (FoxP3 : CD4 ratio was 0.371 without rapamycin vs 0.771 with rapamycin), though the number of $CD4^+CD25^+$cells was relatively small(6.368 ± 1.071 without rapamycin vs 5.001 ± 1.207 with rapamycin). Also, these expanded $CD4^+CD25^+$ cells could suppress third-party MLR and these $CD25^+CD4^+$ cells were anergic against allogeneic antigens and could not response allogeneic third party stimulators. These results indicate that stimulation with anti-CD3/CD28 mAb-coated dynabeads and IL-15 plus TGF-β with rapamycin may be a powerful inducer of selective expansion of Treg cells with suppressive activity and FoxP3 expression from cord blood $CD4^+$ cells. A comparison of IL-15 stimulation and IL-2 stimulation showed a slightly higher efficiency of IL-15 for expansion of the proportion and absolute number of $CD4^+CD25^+$ cells and for FoxP3 expression, and IL-15 also showed significantly higher efficacy for inhibition of MLC.

It has been reported that rapamycin promoted the outgrowth of Treg cells [10-12]; however, Hippen et al. [26] reported that rapamycin had no advantage for increasing the overall number of Treg cells from cord blood $CD4^+CD25^+$ cells in expansion culture using IL-2 and anti-CD3/CD28 mAb-coated dynabeads.

Therefore, rapamycin-mediated enrichment of Treg cells may be due to the selective expansion of conventional $CD4^+$ cells in cord blood.

However, the mechanism of Treg cell-specific proliferation in the presence of rapamycin, which has a potent immunosuppressive action to inhibit growth factor-induced T cell proliferation, is not clear. The IL-2 receptor and STAT5 pathway is essential for activity of Treg cells [27-29]. Pim2 kinase is involved in conferring resistance of Treg cells to rapamycin-mediated inhibition when STAT5-signalling cytokines are present [17]. IL-15 receptor contains IL-2 receptor β and uses the STAT5 signal pathway [30]. In this study, we investigated the expression levels of FoxP3, Pim2 and PTEN during cord blood $CD4^+$ cell culture with rapamycin to clarify the mechanism of Treg cell proliferation in the presence of the T cell growth inhibitor rapamycin. Cord blood $CD4^+$ cells cultured with rapamycin showed an increased expression level of serine/threonine protein kinase Pim2 early after stimulation and a decreased mRNA expression level of the negative PI3K regulator PTEN at late phase of culture. Increased expression of Pim2 by IL-15 and TGF-β with rapamycin may be able to confer rapamycin resistance for growth of Treg cells. Also, decreased expression of PTEN may give an advantage to Treg cells for proliferation. Taken together, the results suggest that increased expression of Pim2 at the early phase and decreased

expression of PTEN at the late phase compensate for AKT inactivity and promote cell cycle progression of Treg cells derived from cord blood. Therefore, rapamycin may be useful for expansion of Treg cells. IL-2 stimulation showed almost the same tendency for induction of these genes; however, IL-15 had a little higher efficiency for induction of FoxP3 on day 8. Therefore, IL-15 is not less useful than IL-2 for expansion of Treg cells.

Regulatory T cell play a vital role in down-regulating GVHD and are critically important for the establishment of active dominant tolerance to both allo- and self-major histocompatibility complex antigens [31]. Also, one of the mechanisms involved in tolerance is the suppression of graft-specific alloreactive T cells, which largely mediate graft rejection, by Treg cells. The ability to isolate and expand Treg cells with immune suppressive activity will enable new forms of adoptive immunotherapy for GVHD after allogeneic stem cell transplantation, rejection after organ transplantation and autoimmune disorders [32-35]. Trzonkowski et al reported first-in-man clinical results of the treatment of patients with acute and chronic GVHD with human ex vivo expanded $CD4^+CD25^+CD127^-$ Treg cells [36]. Recently, Burnstein et al reported a method of $CD4^+CD25^+FoxP3^+$ Treg cells enrichment from cryopreserved third cord blood unit followed by expansion culture including anti-CD3/anti-CD28 antibody

coated beads and IL-2 [37]. At first, enrichment of $CD25^+$ cells was accomplished by positive selection with anti-CD25 magnetic beads and then expanded Treg cells using anti-CD3/anti-CD28 antibody coated beads and IL-2 for 18 days. They obtained median 211 (range 13-1796) folds expansion of Treg cells with median 86 % (range 62-97) purity of $CD4^+CD25^+$ positive cells. 23 patients received a dose of 0.1 to 30 x 10^5 third cord blood unit derived Treg cells /kg after double cord blood transplantation. Compared to identically treated 108 historical control patients without Treg cells, there was a reduced incidence of grade II-IV acute GVHD without deleterious affect on risks of infection, relapse or early mortality in Treg cells treated patients. Therefore, allogeneic Treg cells expanded from cord blood may have clinical effect for preventing GVHD. In this study, we showed the combination of the common γ-chain cytokine IL-15, TGF-β and rapamycin with anti-CD3/anti-CD28 antibody coated beads may be an effective means for expanding Treg cells from cord blood $CD4^+$ cells with high expansion efficacy (mean 706 folds) and high purity (mean 98.4% $CD4^+CD25^+$ positive) for 8 days culture. Therefore, ex vivo expanded Treg cells under GMP standard condition from allogeneic cord blood by our method may enable adaptive Treg cell therapy for the prevention and treatment of GVHD after allogeneic stem cell transplantation. Also, there is a possibility to use these expanded Treg cells for the treatment

of rejection after organ transplantation.

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Figure legend

Fig.1. Surface expression of CD4 and CD25 (a,b) and intracellular FoxP3 expression (c, d, e) in lymphocytes of cord blood before stimulation (A) and after 8 days of stimulation by IL-15 and TGF-β with rapamycin (B).

Fig.2. Absolute count (CPM) of ^{3}H-thymidine incorporation in allogeneic third party MLC by $CD25^{+}CD4^{+}$ cells expanded from CD4-positive cord blood with rapamycin.

Values indicate absolute count (CPM) of ^{3}H-thymidine incorporation in allogeneic third party MLC (means ± SDs, n=6). Significant differences were found in the values after stimulation without $CD25^{+}CD4^{+}$ cells (control) and with $CD25^{+}CD4^{+}$ cells ($P<0.01$). Responder cells and Treg cells ratio 10:0 as control, 10:1 and 10:3.

Table 1. Expansion of $CD25^+CD4^+$cells from CD4-positive cord blood cells with IL-15 and rapamycin
-Proportion-

	Fold expansion	$CD25^+/CD4^+$	$CD25^+/GITR^+$	$CD25^+/OX40^+$	$CD122^+/CD4^+$	$CD25^+/FoxP3^+$
IL-15	893± 475	98.4 ± 1.0	45.3± 19.0	36.3 ± 11.3	35.9 ± 18.1	19.1 ± 12.0 [a]
IL-15+TGF-β	944 ± 591	98.5 ± 1.3	53.5 ± 18.1	41.3 ± 11.0	52.9 ± 28.6	51.6 ± 21.4 [a]
Rp+IL-15	922 ± 502	99.7 ± 0.3	65.4 ± 18.0	41.1 ± 14.3	23.2 ± 13.5	42.8 ± 20.0 [a]
Rp+IL-15+TGF-β	706 ± 522	98.4 ± 2.7	63.9 ± 19.5	38.7 ± 22.7	36.0 ± 23.9	87.4 ± 7.9

Values indicate fold expansion of $CD25^+CD4^+$ cells and percentage of indicated marker-expressing cells (means ± SDs, n=5). Significant differences were found in the values after stimulation with rapamycin (Rp) and IL-15/TGF-β and other conditions ($P<0.01^a$).

Table 2. Expansion of $CD25^+CD4^+$cells from CD4-positive cord blood with IL-15 and rapamycin - Absolute number-

	$CD25^+/CD4^+$	$CD25^+/FoxP3^+$	$FoxP3^+ : CD4^+$ ratio
IL-15	6.214± 0.631 [c]	1.116 ± 0.702 [a]	0.178
IL-15+TGF-β	6.368 ± 1.071 [c]	2.365 ± 1.282 [c]	0.371
Rp+IL-15	6.222 ± 0.668 [c]	1.853 ± 0.927 [b]	0.298
Rp+IL-15+TGF-β	5.001 ± 1.207	3.856 ± 1.035	0.771

Values indicate absolute numbers of $CD25^+CD4^+$ cells and $CD25^+FoxP3^+$ cells ($x10^6$, means ± SDs, n=5) expanded from $0.2x10^6$ CD4-positive cord blood cells. Significant differences were found in the values after stimulation with rapamycin (Rp) and IL-15/TGF-β and other conditions ($P<0.01^a$, $P<0.05^b$, $P<0.01^c$).

Table 3. Expansion of $CD25^+CD4^+$cells from CD4-positive cord blood cells with IL-2 and rapamycin -Proportion-

	Fold expansion	$CD25^+/CD4^+$	$CD25^+/GITR^+$	$CD25^+/OX40^+$	$CD122^+/CD4^+$	$CD25^+/FoxP3^+$
IL-2	999± 600	99.1 ± 0.5	42.8± 16.5	36.0 ± 10.8	41.5 ± 21.5	16.6 ± 10.0 [a]
IL-2+TGF-β	972 ± 470	99.4 ± 0.2	56.0 ± 23.2	42.8 ± 15.7	55.8 ± 30.1	53.0 ± 24.8
Rp+IL-2	961 ± 594	99.7 ± 0.1	63.5 ± 18.0	40.2 ± 15.0	25.2 ± 16.1	42.4 ± 20.1
Rp+IL-2+TGF-β	668 ± 412	99.4 ± 0.7	64.3 ± 21.5	34.6 ± 26.5	69.6 ± 13.4	68.0 ± 22.7

Values indicate fold expansion of $CD25^+CD4^+$ cells and percentage of indicated marker-expressing cells (means ± SDs, n=5). Significant differences were found in the values after stimulation with rapamycin (Rp) and IL-2/TGF-β and other conditions ($P<0.01^a$).

Table 4. Expansion of $CD25^+CD4^+$cells from CD4-positive cord blood cells with IL-2 and rapamycin - Absolute number-

	$CD25^+/CD4^+$	$CD25^+/FoxP3^+$	$FoxP3^+ : CD4^+$ ratio
IL-2	6.665± 1.63 [b]	0.89 ± 0.458 [a]	0.134
IL-2+TGF-β	6.591 ± 0.589 [a]	2.905 ± 1.816	0.44
Rp+IL-2	6.699 ± 1.765 [b]	2.119 ± 0.756	0.316
Rp+IL-2+TGF-β	4.631 ± 0.794	3.182 ± 1.272	0.687

Values indicate absolute number of $CD25^+CD4^+$ cells and $CD25^+FoxP3^+$ cells ($x10^6$, means ± SDs, n=5) expanded from $0.2x10^6$ CD4-positive cord blood cells. Significant differences were found in the values after stimulation with rapamycin (Rp) and IL-2/TGF-β and other conditions ($P<0.01^a$, $P<0.05^b$, $P<0.01^c$).

Table 5. Inhibition of allogeneic third party MLC by $CD25^+CD4^+$ cells expanded from CD4-positive cord blood with IL-15 and rapamycin.

Responder: $CD25^+CD4^+$ ratio	10:1	10:3
IL-15	15.7±17.6[a]	25.3±20.6[a]
IL-15+TGF-β	52.2±18.7[a]	67.6±15.8[b]
Rp+IL-15	22.6±22.8[a]	32.8±20.9[a]
Rp+IL-15+TGF-β	80.0±8.7 [c]	81.0±8.2

Values indicate percentages of inhibition of allogeneic third-party MLC reaction estimated by ^{3}H-thymidine incorporation (means ± SDs, n=9). Significant differences were found in the values after stimulation with Rp+IL-15+TGF-β and other cytokines ($P<0.01^a$, $P<0.05^b$) and also in comparison with IL-2 ($P<0.05^c$).

Table 6. Inhibition of allogeneic third party MLC by $CD25^+CD4^+$ cells expanded from CD4-positive cord blood with IL-2 and rapamycin.

Responder: $CD25^+CD4^+$ ratio	10:1	10:3
IL-2	5.9±7.6[a]	15.2±18.2[a]
IL-2+TGF-β	40.6±23.58[b]	65.7±15.6
Rp+IL-2	20.1±18.2[a]	25.7±20.0[a]
Rp+IL-2+TGF-β	63.8±21.0[c]	73.0±16.7

Values indicate percentage of inhibition of allogeneic third party MLC reaction estimated by ^3H-thymidine incorporation (means ± SDs, n=9). Significant differences were found in the values after stimulation with Rp+IL-2+TGF-β and other cytokines ($P<0.01^a$, $P<0.05^b$), also in comparison with IL-15 ($P<0.05^c$).

Table 7. Quantitative analysis of FoxP3, Pim2 and PTEN mRNA expression in cord blood $CD4^+$ cells expanded with IL-15 and rapmycin

Cytokine	FoxP3	Pim2	PTEN
6hrs			
IL-15+TGF-β	25.6±13.9	3.0±1.1	1.0±0.7
Rp+IL-15+TGF-β	38.7±9.3[a]	4.9±1.7[a]	1.4±1.2
8days			
IL-15+TGF-β	64.2±30.9[d]	1.2±0.8[d]	0.4±0.4[d]
Rp+IL-15+TGF-β	93.3±15.1[b,c]	1.6±0.8[c]	0.1±0.1[d]

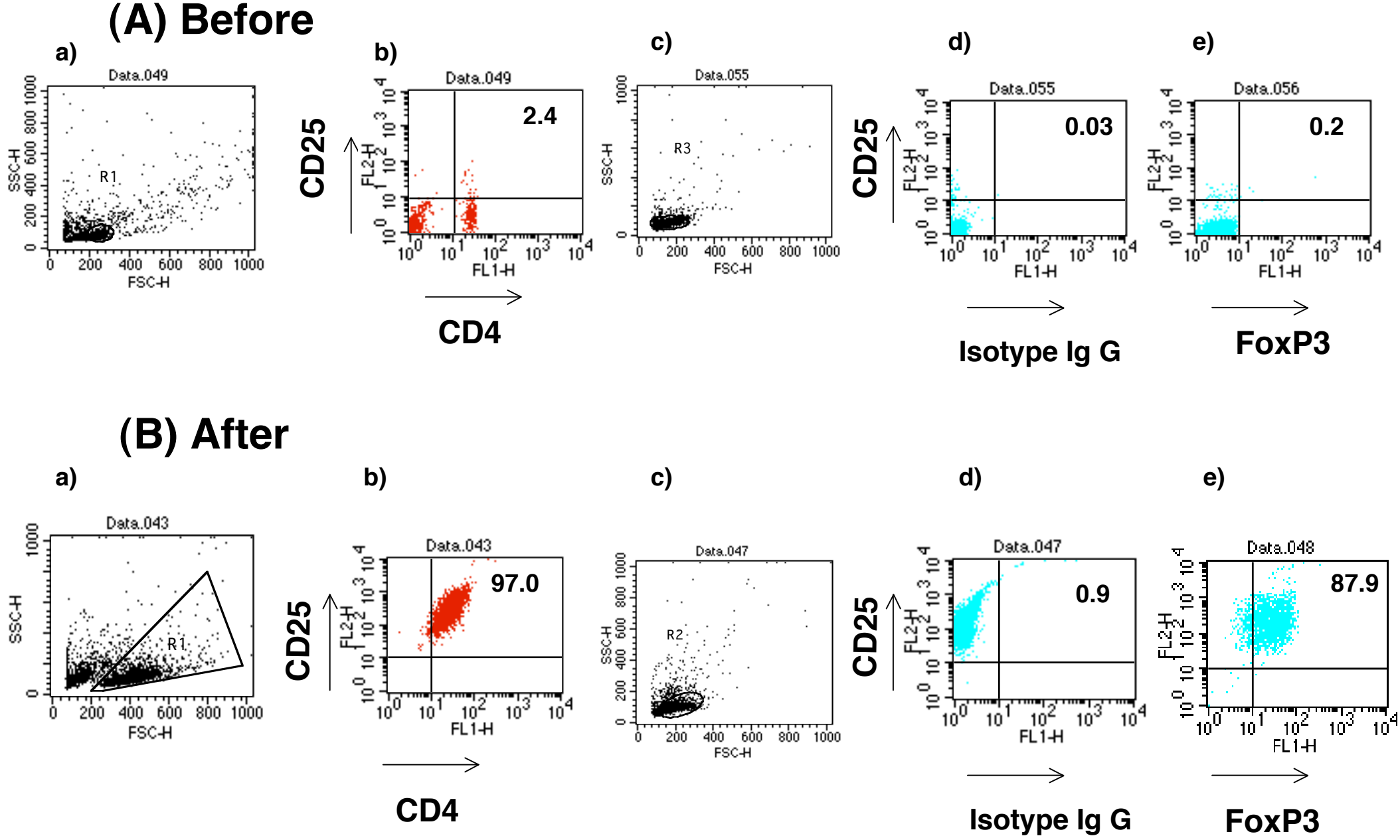
Fold expression level of FoxP3 compared with expression level in cord blood cells before stimulation (means ± SDs, n=5). Significant differences were found in the values after stimulation with or without rapamycin ($p<0.01^a$, $P<0.05^b$) and at 6 hours and 8 days after stimulation ($p<0.01^c$, $P<0.05^d$).

Table 8. Quantitative analysis of FoxP3, Pim2 and PTEN mRNA expression in cord blood $CD4^+$ cells expanded with IL-2 and rapmycin

Cytokine	FoxP3	Pim2	PTEN
6hrs			
IL-2+TGF-β	31.1 ±17.8	3.2±1.4	1.0±0.7
Rp+IL-2+TGF-β	39.4±21.4	4.1±1.3[a]	1.2±0.1
8days			
IL-2+TGF-β	56.7±28.0[d]	1.0±0.7[d]	0.2±0.2[d]
Rp+IL-2+TGF-β	60.2±47.5	1.2±0.9[c]	0.4±0.1

Fold expression level of FoxP3 compared with expression level in cord blood cells before stimulation (means ± SDs, n=5). Significant differences were found in the values after stimulation with or without rapamycin ($p<0.01^a$, $P<0.05^b$) and at 6 hours and 8 days after stimulation ($p<0.01^c$, $P<0.05^d$).

Fig.1.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FSC-H
b)
Data.049
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CD25
FL2-H
FL1-H
CD4
c)
Data.055
R3
SSC-H
FSC-H
d)
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CD25
FL2-H
FL1-H
Isotype Ig G
e)
Data.056
0.2
FL2-H
FL1-H
FoxP3
(B) After
a)
Data.043
R1
SSC-H
FSC-H
b)
Data.043
97.0
CD25
FL2-H
FL1-H
CD4
c)
Data.047
R2
SSC-H
FSC-H
d)
Data.047
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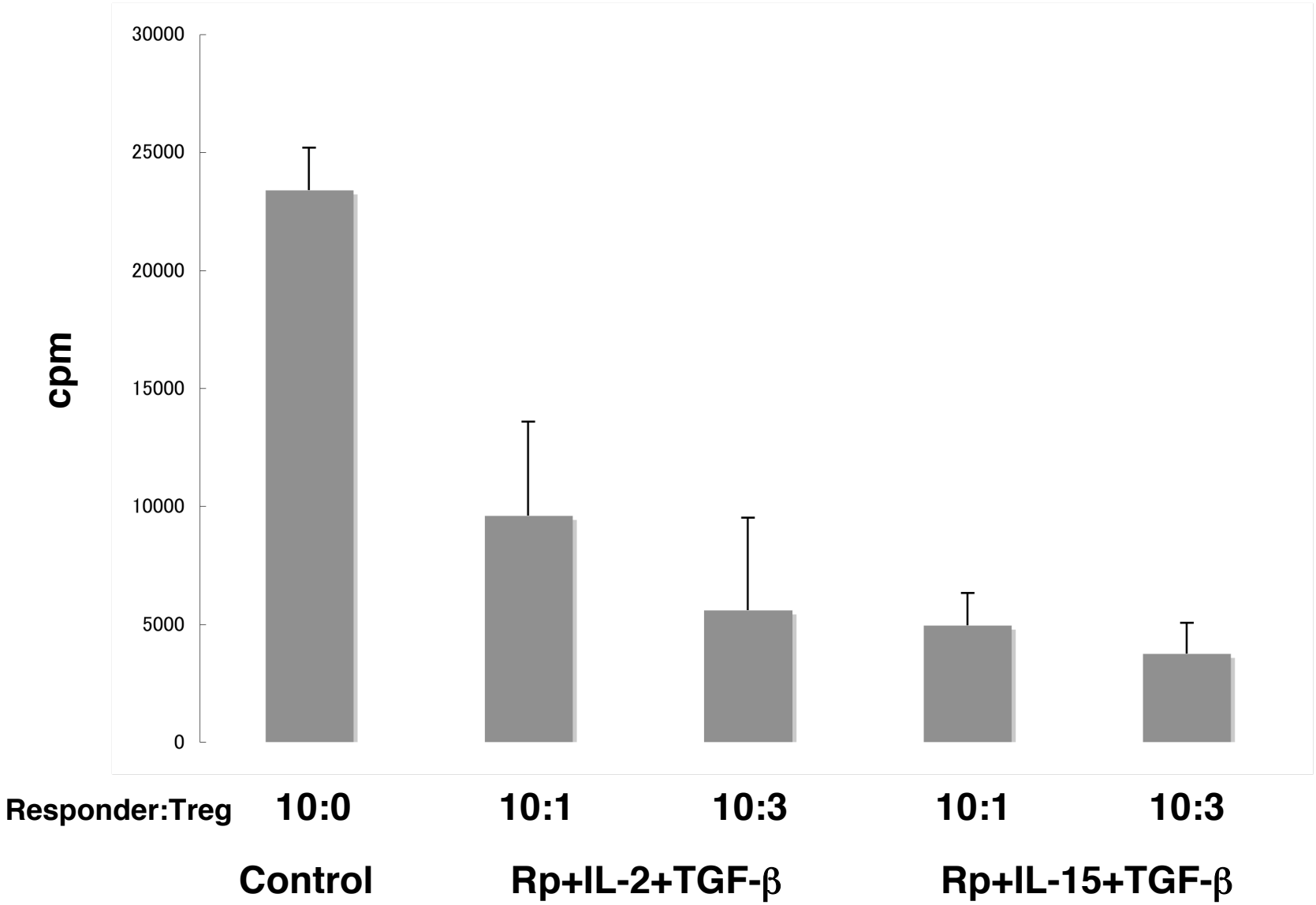
FL1-H
FoxP3

Fig.2.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Rp+IL-2+TGF-β
Rp+IL-15+TGF-β