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Generation of human regulatory T cells de novo with suppressive function prevent xenogeneic graft versus host disease

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Abstract

Treatment with rapamycin (RAPA) favorably affects regulatory T cells (Treg) in vivo, and RAPA induces the de novo expression of FOXP3 in murine alloantigen-specific T cells. Whether RAPA acts independently or with transforming growth factor beta (TGF- β) to produce ex vivo-induced Treg generation is unknown. Naïve CD4⁺ T cells isolated from peripheral blood mononuclear cells were stimulated with anti-CD3/CD28 coated beads in the presence of IL-2 for 5 to 7 days. Ten ng/ml of TGF- β (1 to 100 ng/mL RAPA) was added to some of the cultures. The phenotypes were analyzed with flow cytometry. The conditioned cells were cocultured with CFSE-labeled T cells in different ratios for 5 days. CFSE dilution indicating T response cell proliferation was analyzed by flow cytometry. Xenogeneic graft-versus-host disease (x-GVHD) was induced by transplanting human peripheral blood mononuclear cells into RAG2^{-/-} γ c^{-/-} mice exposed to total body irradiation, and various factors in the subjects were subsequently compared. CD4 cells induced by rapamycin and TGF- β (CD4_{RAPA/TGF- β}) expressed the natural Treg phenotypes and trafficking receptors, and no significant cytotoxicity was observed. CD4_{RAPA/TGF- β} was anergic and demonstrated potent suppressive activity in vitro. Although the transfer of human peripheral blood mononuclear cells into RAG2^{-/-} γ c^{-/-} mice caused x-GVHD, the cotransfer of CD4_{RAPA/TGF- β} decreased human cell engraftment and extended survival in mice. RAPA plus TGF- β induces human naïve T cells to become suppressor cells, a novel strategy for treating human autoimmune diseases and preventing allograft rejection.

Keywords

Regulatory T cells; TGF- β ; Rapamycin; Graft-versus host disease; Human

1. Introduction

The de novo generation of regulatory T cells (Treg) from conventional naïve T cells is a promising strategy for obtaining sufficient Treg for immunotherapy. The expression of FOXP3 is considered the definitive marker for mouse Treg but not for human Treg because T-cell receptor (TCR) stimulation with anti-CD3 and anti-CD28 has been reported to induce FOXP3 expression in human CD4⁺ CD25⁻ cells, which display no suppressive activity in

vitro [1–3]. TGF- β -induced Treg appear to resemble nTreg in all their phenotypic and functional properties in mice. TGF- β -induced Treg exert potent suppressor function in vitro and can prevent or control autoimmune and inflammatory diseases in vivo [4–6]. Tran and colleagues found that TCR stimulation alone was insufficient to induce FOXP3 expression in the absence of TGF- β but that high levels of FOXP3 expression could be induced in the presence of TGF- β [7]. Although in that study FOXP3 expression was stable, the TGF- β induced FOXP3⁺ T cells were neither anergic nor suppressive and produced high levels of inflammatory cytokines [7].

There is in vivo evidence that treatment with RAPA has favorable effects on Treg [8,9]. RAPA also induces the de novo expression of FOXP3 in murine alloantigen-specific T cells, and this effect is related to TGF- β signaling pathway [10]. Because RAPA promotes the production of TGF- β in mice, it may be an important mechanism in the development of antigen-specific Treg [11]. A study by Valmori and colleagues suggested that in vitro RAPA exerts a regulatory effect on conventional human CD4⁺ T cells [12]. To our knowledge, the mechanism and characterization of RAPA-induced human Treg have not been reported. In this study, we assessed the effect of RAPA on the growth, phenotype, and function of human circulating naïve CD4⁺ T cells after TCR-mediated stimulation in the presence of TGF- β . We found that the stimulation of human CD4⁺ T cells in the presence of RAPA and TGF- β results in greatly increased suppressor function that is TGF- β dependent and that those cells demonstrated greater stable in vivo suppressor function than did CD4⁺ T cells stimulated without RAPA.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal fluorochrome-tagged antibodies against CD4 (RPA-T4), CD8 (HIT8a), CD28 (CD28.2), CD62L (Dreg 56), CD25 (M-A251), CD103 (Ber-ACT8), CTLA-4 (BNI3), CD122 (Mik- β 3), CCR4 (1G1), CCR7 (3D12), FOXP3 (259D/C7), Perforin (δ G9), Granzyme A (CB9) and Granzyme B (GB11), and control IgGs were all from BD PharMingen. PE-conjugated anti-GITR was purchased from R&D Systems. The Abs used were anti-CD8 (OKT8), anti-CD11b (OKM1), anti-CD16 (3G8), anti-CD74 (L243) (all hybridoma supernates, American Type Culture Collection, Manassas, VA). Anti-TGF- β 1 and IL-2 were from R&D Systems. ALK5 inhibitor (SB431542, Sigma-Aldrich) was dissolved in 0.1% DMSO, and was used as previously described [13,14].

2.2. Cell preparation and culture

Peripheral blood samples were obtained from health donors. The acquisition of blood products was approved according to the policies of the Nanjing Medical University in accordance with the Declaration of Health Ministry of China. Using a previous described cell separation protocol [15,16], we isolated mononuclear cells via density gradient sedimentation and T cells were separated by sheep red cells [17]. CD4⁺ T cells were negatively selected with a CD4⁺ T-cell isolation kit (Miltenyi Biotec, Auburn, CA), yielding populations of CD4⁺ cells (purity 96–99%). Next, CD4⁺CD25⁺ T cells were separated from CD4⁺CD25[−] T cells on the AutoMACS in two repetitive separation steps using CD4⁺CD25⁺ T Regulatory Cell Isolation Kit (Miltenyi Biotec). Negatively depleted CD4⁺CD25[−] T cell fractions (purity >98%) were immediately used for expansion protocols or suppression assays. Aliquots of the CD4⁺CD25[−] cells were used as autologous responder cells in suppression assays. CD4⁺CD25[−] T cells were cultured in AIM-V medium (Invitrogen) supplemented with 1% (vol/vol) human pooled serum (HPS), L-glutamine (2 μ M), penicillin (100 U/ml), streptomycin (100 μ g/ml) and mercaptoethanol (50 nM) in the presence of anti-human CD3/CD28 beads at 1:10 ratio. Cytokines for CD4⁺ T cell

differentiation were as follows: CD4_{Med}, IL-2 (100 U/ml); CD4_{TGF- β} , IL-2 (100 U/ml) and TGF- β (10 ng/ml); CD4_{RAPA/TGF- β} , IL-2 (100 U/ml), TGF- β (10 ng/ml) and RAPA (100 ng/ml) were added where indicated. Cultures were maintained for different days according to experiments needed.

2.3. Annexin V staining

CD4 conditioned T cells cultured as above were washed and resuspended in binding buffer in the presence of 50 μ l annexin-V buffer containing 5 μ l PE-annexin-V per 10⁶ cells and 25 μ g/ml 7-AAD for 15 min in the dark at room temperature. Cells were analyzed by flow cytometry within 30 min.

2.4. In vitro suppression assay

To determine the suppressive activity of the various conditioned T cells, we measured their ability to inhibit the generation of autologous responder T cells as previously described [13,14]. In briefly, responder T cells were stained with 1.5 μ M CFSE (Molecular Probes/Invitrogen, Carlsbad, CA) as previously described and co-cultured with different conditioned cells in a complete AIM-V medium, plate-bound anti-CD3 (1 μ g/mL) and soluble anti-CD28 Ab (1 μ g/mL) in wells of 96-well plates (at least 5 $\times$ 10⁵/well). Conditioned T cells were CD4_{Med}, CD4_{TGF- β} and CD4_{RAPA/TGF- β} activated with or without TGF- β (RAPA) for 5 days and rested for 24 h without IL-2. These cells were washed 3 times in phosphate-buffered saline before using them for the suppression assay. T conditioned cells were added to responder T cells at the condition: responder T cells ratios of 1:1, 1:5, 1:10 and 1:20. Co-cultures were incubated for 3–5 days (37 °C, 5% CO₂). After harvest, suppression of CFSE-labeled T cells proliferation was analyzed.

2.5. In vivo suppression assay

RAG2^{-/-} γ c^{-/-} mice were obtained from Jackson laboratories. They were bred and maintained in microisolator cages under specified pathogen-free conditions at the Central Laboratory Animal Institute of Nanjing University. Xenograft model for graft-versus-host disease was established by intravenous transfer of huPBMCs into RAG2^{-/-} γ c^{-/-} double-mutant mice by tail vein. Briefly, huPBMCs were isolated by Ficoll-Hypaque (Pharmacia) density centrifugation and washed twice in phosphate-buffered saline (PBS). Cells were then counted and resuspended in PBS/0.1% HPS in concentrations from 20 $\times$ 10⁶ cells/0.25 ml. Cell suspensions of 0.25 ml were injected intravenously into the mice via the tail vein. Mice received total body irradiation with a single dose of 350 cGy (gamma irradiation from a linear accelerator) before injection of huPBMCs on the same day.

2.6. In vivo monitoring of human T cells

The engraftment and expansion of human T cells were monitored in 50 μ l peripheral blood obtained weekly from the retro-orbital vein and analyzed by flow cytometry with BD TruCOUNT kit.

2.7. Histology and immunohistochemistry

At the time of animal sacrificed, livers were resected. Part of them were fixed in 10% formalin and embedded in paraffin. The other fresh tissues were embedded carefully in plastic mold with OCT. Paraffin block sections were stained with hematoxylin and eosin (H&E). Human leukocytes were detected by immunohistochemistry (IHC), using a mouse anti-human CD45 primary mAb, a goat anti-mouse biotinylated secondary Ab, a streptavidin–horseradish peroxidase (HRP) conjugate, and 3-amino-9-ethylcarbazole as chromogen. All reagents for IHC were purchased from Zymed (South San Francisco, CA).

2.8. Statistical analysis

Kaplan–Meier survival curves were analyzed using log rank test. Other statistical analyses were performed using a paired Student *t* test unless otherwise noted. *P* values below 0.05 were considered statistically significant.

3. Results

3.1. RAPA induces FOXP3⁺ cells but does not expand endogenous FOXP3⁺ T cells

Nikolaeva et al. [18] reported that RAPA inhibited the proliferative capacity of alloreactive CD4⁺ and CD8⁺ T cells. RAPA also has the ability to selectively expand nature regulatory T-cell population and to suppress the proliferation of effector cells [19]. To assess the relevance of those findings in our experimental system, CD25⁺ cells were depleted from the peripheral blood of healthy subjects to remove nature Treg population. The CD25[−] T cells were then labeled with CFSE and were stimulated with anti-CD3/CD28 coated beads with or without RAPA (100 ng/mL). After 14 days of stimulation, cell division and FOXP3 expression were measured by FACS. CD25[−] T-cell division, which was higher in the absence of RAPA, had reached a substantial fold expansion 2 weeks after the experiment (Fig. 1, A and B). Although RAPA significantly suppressed the proliferation of CD25[−] T cells, in 50 ng/mL concentration, it induced substantial amounts of FOXP3⁺ T cells that had experienced cell division (Fig. 1C); this suggests that the increased percentage of FOXP3⁺CD4⁺ T cells in RAPA-treated cultures was not due to the selective expansion of nature Treg but to the induction of FOXP3⁺ T cells in conventional T-cell populations. These results are in line with those in recently reported data [2,12]; this indicates that the expression of FOXP3 in human conventional CD25[−] T cells is induced by RAPA after TCR stimulation in vitro.

3.2. TGF-β/TGF-β receptor signaling pathway is crucial for the differentiation of FOXP3⁺ cells induced by RAPA

A study by Valmori et al. [12] provided evidence that the stimulation of human CD4⁺ T cells in the presence of rapamycin results in a greater increase in suppressor function than that produced in the absence of RAPA. TGF-β has been shown to induce mouse CD4⁺FOXP3⁺ cells with suppressor function in vitro, but it has been demonstrated that TGF-β-induced human CD4⁺FOXP3⁺ cells are neither anergic nor suppressive [7]. To determine whether the combination of RAPA and TGF-β induces naïve human CD4⁺ T cells to become Treg, CD4⁺CD25[−] T cells isolated from the peripheral blood of healthy donors were cultured in the presence of IL-2. TGF-β and RAPA were added to some of those cultures. When RAPA was titrated with constant levels of TGF-β (5 ng/mL) and IL-2, RAPA did not produce an increase in FOXP3 conversion at different concentrations (Fig. 2A). Nonetheless, exogenous TGF-β (0 to 20 ng/mL) enhanced FOXP3 expression by RAPA (Fig. 2B). In our study, we examined whether the effects of RAPA were associated with the induction of TGF-β. As shown in Fig. 2C, the addition of 50 ng/mL of RAPA to TCR-stimulated CD4⁺ T cells induced an almost 5-fold increase in the production of TGF-β found in vehicle-treated controls (*P*<.05). When TGF-β receptor I (ALK5) inhibitor (ALK5i) (10 ng/mL) was added to the culture medium at day 0, there was no increased FOXP3 expression in CD4⁺ T cells cultured with RAPA after 5 days of TCR stimulation (Fig. 2D). The addition of a similar dose of DMSO did not alter the FOXP3 expression induced by RAPA; this suggests that ALK5 inhibitor specifically inhibits FOXP3 conversion rather than the nonspecific suppression of T cell activation and proliferation. The ability of RAPA to induce FOXP3 expression without the addition of exogenous TGF-β and the ability of ALK5 inhibitor to abolish that FOXP3 induction indicate that conversion is not dependent on exogenous TGF-β but rather that conversion involves TGF-β signaling.

3.3. Combination of RAPA and TGF- β produces a synergistic effect on FOXP3⁺ cell induction

The phenotypes of CD4_{RAPA/TGF- β} cells were significantly different from those of CD4_{Med} and CD4_{TGF- β} cells. CD4 cells cultured primarily with RAPA expressed the characteristic nTreg markers, including FOXP3, CD62L, CTLA-4, and GITR (Fig. 3A). In our experiment, FOXP3 expression was up-regulated in CD4_{Med} cells after culture of 7 days' duration. However, after 72 h of resting, the FOXP3 level in CD4_{TGF- β} and CD4_{Med} cells decreased, but the FOXP3 level in CD4_{RAPA/TGF- β} cells remained constitutively higher, and most of the cells were CD127^{dim} (Fig. 3B). These data support the findings of Valmori et al. [12]. The combination of RAPA and TGF- β is more effective in Treg induction than is either RAPA or TGF- β alone. Because RAPA alone can induce FOXP3⁺ Treg, the addition of TGF- β can enhance that effect.

3.4. Characteristics of CD4⁺ Treg induced by RAPA and exogenous TGF- β

To determine whether RAPA-induced Treg were resistant to apoptosis, we examined annexin-V expression by conditioned T cells that had been rested for 48 h. The level of annexin-V binding to CD4⁺ cells cultured in the presence of TGF- β was very high, but a significantly lower level of annexin-V binding to CD4⁺ cells ($P < .001$) was evident in proliferating RAPA-plus-TGF- β cultures (Fig. 3C). Significantly higher percentages of annexin-V⁺ 7AAD⁺ T cells were observed in the cultures without RAPA (Fig. 3D). In contrast, CD4_{RAPA/TGF- β} became highly resistant to apoptosis on TCR- and IL-2-mediated activation in the presence of RAPA.

Treg, which are hypoproliferative, suppress the proliferation of effector cells. We also assessed the proliferative and suppressive activities of CD4_{RAPA/TGF- β} cells. We found that like nTreg, CD4_{RAPA/TGF- β} cells had lower proliferation potentials than did CD4_{Med} or CD4_{TGF- β} cells. When IL-2 was added to the culture at day 0, all three conditioned cells proliferated (Fig. 4A). CD4_{RAPA/TGF- β} cells suppressed the proliferation of T cells, and TGF- β -treated T cells and IL-2-treated T cells exerted no suppressive function (Fig. 4, B and C). In the next step, we investigated the mechanism of the suppression of T lymphocyte proliferation by CD4_{RAPA/TGF- β} cells. We examined whether the suppressive effect of CD4_{RAPA/TGF- β} cells on responder T-cell proliferation is mediated by soluble factor(s) because we found that RAPA stimulates TGF- β production in T cells. Responder T-cell proliferation in the cocultures with CD4_{RAPA/TGF- β} cells was different from that in the transwell experiment; this suggests that the suppression of T cells by CD4_{RAPA/TGF- β} cells depends on cell-to-cell contact rather than on soluble factors (data not shown).

3.5. RAPA- and TGF- β primed CD4⁺ T cells express membrane-bound TGF- β (mTGF- β), which mediates the suppressive ability of those cells

Because the regulatory effect of the RAPA- and TGF- β -induced Treg was cell-to-cell-contact dependent, we tested the expression of mTGF- β on the conditioned cell surface by Flow cytometry after 5 days of restimulation with 1:1 antihuman CD3/CD28 beads (Fig. 4D). To further test the involvement of TGF- β in suppression, we targeted the TGF- β type 1 receptor by using an ALK5 inhibitor. The addition of ALK5i reversed the inhibition of Treg induced by RAPA and TGF- β cells, but the proliferation of control cells remained unaffected (Fig. 4E). Those data show that the suppressor function of CD4 Treg induced by RAPA plus TGF- β is partially dependent on TGF- β , because blocking the TGF- β signaling pathway partially reversed suppression.

3.6. Coadministration of CD4_{RAPA/TGF- β} with autologous PBMC significantly reduced the incidence of lethal x-GVHD in the RAG2^{-/-} γ c^{-/-} mice

In RAG2^{-/-} γ c^{-/-} mice, the severity of x-GVHD induced by the transfer of huPBMCs depends on the dose of human cells administered [20,21]. When huPBMCs were cotransferred with conditioned cells into NOG mice, only the CD4_{RAPA/TGF- β} group demonstrated improved survival (Fig. 5A). All RAG2^{-/-} γ c^{-/-} mice that received only huPBMCs died within 20 days of acute x-GVHD. In all mice that were protected from lethal x-GVHD after CD4_{RAPA/TGF- β} , the expansion rates of human T cells were significantly lower than those in the other 2 groups (Fig. 5B). The mice were killed after 2 weeks, and immunohistochemical analysis showed human cells in all organs, especially in the lungs, liver, and kidneys. The subjects in the CD4_{Med} and CD4_{TGF- β} groups exhibited more extensive liver infiltration by mononuclear cells that stained positive for human CD45 on immunohistochemical analysis (Fig. 5, C and D).

4. Discussion

The immunosuppressive action of RAPA occurs primarily by inhibition of the cytokine-induced responses of T lymphocytes, an event that results from the blockade of signaling that occurs downstream from the mammalian target of rapamycin (mTOR) [22]. Like other investigators, we found that the prolonged administration of RAPA decreased the absolute number of (but did not alter the proportion of) the CD4 single positive cells that unregulated their expression of FOXP3 in the thymus [8,23]. This indicates that the ontogeny of natural Treg is unaffected by RAPA, reversely RAPA favored Treg expansion and survival [21]. Battaglia et al. [19] reported that the in vitro long-term exposure of murine CD4⁺ T cells to RAPA induced the expansion of the naturally occurring CD4⁺ CD25⁺ FOXP3⁺ Treg, which retained their suppressive function in vitro and in vivo. In addition, Valmori et al. [12] showed that the RAPA-mediated enrichment of T cells with regulatory activity in stimulated CD4⁺ T cell cultures is not due to the selective expansion of naturally occurring Treg but rather to the induction of regulatory functions in conventional CD4⁺ T cells. Like Valmori et al. [12], we observed that the TCR-mediated stimulation of CD25⁻ T cells in the presence of RAPA resulted in greatly increased FOXP3 expression.

It has been shown that in mice, RAPA induces the de novo expression of FOXP3 alloantigen-specific T cells dose dependently, this ability was abolished by anti-TGF- β antibody, suggesting a potential link between RAPA effect and TGF- β signaling [10]. We demonstrated similar results in human Treg induction. We found that RAPA-induced FOXP3 expression in human CD4 T cells is completely blocked by ALK5 inhibitor, this suggests a potential link between the effect of RAPA and TGF- β signaling in human CD4⁺ T cells. TGF- β might be the factor responsible for the RAPA-induced conversion of naïve CD4 T cells to FOXP3⁺ T cells, because ALK5i blocks the induction of FOXP3 (Fig. 2D). However, the effect of RAPA on the induction of FOXP3⁺ cell differentiation cannot be supplemented by increasing the TGF- β dose (Fig. 2B). Although the effect of RAPA is TGF- β dependent, TGF- β might not be the sole factor responsible for RAPA-induced conversion. In contrast to TGF- β -induced mouse FOXP3⁺ cells that were both anergic and suppressive in vitro, human induced FOXP3⁺ cells have been shown to be neither anergic nor suppressive [7]. In our study, we found that the combination of RAPA and TGF- β had a synergistic effect in inducing human Treg with suppressive function both in vitro and in vivo. The phenotypic characteristics of CD4_{RAPA/TGF- β} cells are qualitatively similar to those of freshly isolated CD4⁺CD25^{high} T cells. We have shown that CD4_{RAPA/TGF- β} , like fresh nTreg, demonstrate high expression levels of FOXP3. CD4_{RAPA/TGF- β} also expresses higher levels of chemokine receptors CCR4 and CCR7 than do CD4_{TGF- β} . The specific expression of CD62L, CCR4, and CCR7 on Treg may allow the migration of Treg toward APCs and activated T cells, which in turn leads to the inhibition of APC function or the

suppression of responding T cells. Thus for successful Treg therapy, it is imperative that the ex vivo-generated Treg migrate to those sites. Although little is known about the in vivo trafficking characteristic of ex vivo-generated Treg, the increased migration receptor pattern in CD4_{RAPA/TGF-β} suggests that it might function at distinct sites. CD4_{RAPA/TGF-β} cells resisted actively induced cell death and still expressed higher levels of FOXP3 than CD4_{Med} or CD4_{TGF-β} cells did.

A study by Nakamura and colleagues demonstrated the important role of TGF-β in the maintenance of the suppressor function of CD4⁺CD25⁺ Treg [24]. CD4⁺CD25⁺ T cells can produce TGF-β as either soluble protein or a cell-surface protein, depending on the type and/or strength of TCR stimulation [25]. The addition of anti-TGF-β Ab directly to the suppression assays had little effect in our study, even at the high concentrations (50 μg/mL) reported to be necessary for inhibition. Our transwell experiment showed that CD4_{RAPA/TGF-β} cells mediate their suppressive function by cell-to-cell contact. The importance of TGF-β as a key immunoregulatory mediator was first described in the context of TGF-β-secreting Th3 cells in studies of oral tolerance [26], and CD4⁺CD25⁺ T cells expressing membrane-bound TGF-β with a suppressive function were subsequently described [25]. In our study, we analyzed the specific aspect of membrane-bound TGF-β that mediates its immunosuppressive effect and found that the function of CD4_{RAPA/TGF-β} was dependent on mTGF-β and not on soluble TGF-β.

We also assessed the ability of CD4_{RAPA/TGF-β} cells to block acute x-GVHD and prevent human T-cell proliferation in RAG2^{-/-} γc^{-/-} mice. Other investigators used RAG^{-/-} SCID cy chain^{-/-} mice to induce human x-GVHD and demonstrated the protective effect of expanded endogenous nTreg [20,21,27,28]. To cause the development of GVHD in NOG mice, the use of toxic liposomes is not necessary [29]. We aimed to have a rapid readout for our suppressor cell assay and injected the subjects with a large dose of human CD25-depleted human PBMC, which caused death within 14 days. The addition of CD4⁺ cells conditioned with RAPA and TGF-β, but not the addition of TGF-β alone, delayed the onset of weight loss (data not shown) and extended the survival of the subjects so treated for 2 additional months. We found that CD4_{RAPA/TGF-β} significantly suppressed human cell engraftment.

In conclusion, we have shown that RAPA and TGF-β have a synergistic effect in inducing Treg in vitro. CD4_{RAPA/TGF-β} has almost the same phenotypes as those found in naturally occurring Treg. CD4_{RAPA/TGF-β} significantly inhibits T-cell proliferation in vitro and in vivo. Our data and those of other investigators suggest that the combination of RAPA and TGF-β can be used in vitro to generate therapeutic numbers of iTreg for cellular therapy or in vivo to induce and/or enhance peripheral tolerance.

Acknowledgments

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Fig. 1.

RAPA inhibits T-cell proliferation and induces the generation of FOXP3⁺ T cells. Human T cells were purified by sheep red cells from human PBMCs, and CD25⁺ T cells were depleted with anti-CD25 beads. The CD25⁺ T cells were labeled with CFSE and were stimulated with antihuman CD3/CD28 beads (bead-to-cell ratio, 1:10) with or without RAPA (100 ng/mL). The proliferation of CD25⁺ T cells was analyzed by FACS (A). The data for 6 donors are shown as the mean value including the SEM (B). When the concentration of RAPA changed to 50 ng/mL, the intracellular FOXP3 were analyzed via flow cytometry (C).

Fig. 2.

The generation of FOXP3⁺CD4⁺ Treg is dependent on the TGF-β signaling pathway. Naïve CD4⁺ T cells were isolated as described in the Materials and methods section of the text. (A) TGF-β-mediated FOXP3 expression as a result of titrating concentrations of RAPA. With constant levels of IL-2 and TGF-β, RAPA was titrated in serial dilutions (100 ng/mL, 10 ng/mL, 1 ng/mL, and 0 ng/mL). (B) FOXP3 expression by TCR-stimulated CD4⁺ cells as a function of titrating concentrations of TGF-β. IL-2 and RAPA concentrations were kept constant, and TGF-β (15 ng/mL, 10 ng/mL, 5 ng/mL, 1 ng/mL, and 0 ng/mL) was titrated. (C) Naïve CD4⁺ T cells were stimulated in vitro with antihuman CD3/CD28 beads in the presence or absence of 0 ng/mL, 10 ng/mL, or 50 ng/mL of RAPA for 72 h. The supernatants were harvested and assessed for the production of total TGF-β by ELISA. The results are presented as the mean ± SEM. (D) ALK5 inhibitor (10 ng/mL) was added to naïve CD4⁺ T cells treated with IL-2 (20 U/mL) or IL-2 (20 U/mL) plus RAPA (10 ng/mL), and the FOXP3⁺ cells was analyzed by FCAS on day 3. All experiments shown are representative of at least 3 independent experiments.

Fig. 3.

Phenotypic characterization of human RAPA-and-TGF- β -induced CD4⁺ T cells. Naïve CD25-depleted CD4⁺ cells were stimulated with antihuman CD3/CD28 beads and the additives listed above for 5 days. (A) The staining intensity of the marker of different conditioned cells is shown. * $P < .05$, ** $P < .001$ (B) When administered in combination with TGF- β , RAPA markedly increased the proportion of FOXP3⁺ cells that became CD127^{dim}. (C) Both annexin-V and 7AAD staining were used to determine the levels of apoptosis in 3 different conditioned CD4 cells by FACS after 48 h of resting. An increased incidence of late apoptotic CD4⁺ cells (annexin-V⁺ 7AAD⁺) and a decreased incidence of viable cells (annexin-V⁻ 7AAD⁻) were detected in CD4_{RAPA/TGF- β} . Those data are shown in panel D.

Fig. 4.

CD4^{RAPA/TGF-β} cells are anergic and suppress function according to the level of TGF-β that is present. (A) Three different conditioned cells were rested for 24 h and were then labeled with CFSE. Those cells were stimulated with antihuman CD3/CD28 beads (1:10) with or without IL-2 (20 U/mL) for 3 days. The dilution of CFSE was analyzed by FACS. (B) Three conditioned cells were cultured in different conditions as described in the Materials and methods section of the text for 5 days and were then rested for 24 h. Those cells were harvested and cocultured with CD25⁻ T cells labeled with CFSE (0.4×10^5) at different ratios (1:1, 1:5, 1:10, and 1:20) and were then stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) for 3 days. (C) Flow cytometric analysis of cell division by CFSE dilution. (D) Naïve CD25-depleted CD4⁺ cells were stimulated with antihuman CD3/CD28 beads and the additives listed above for 5 days. The cells were then rested in fresh medium containing 10% FCS for 24 h. All conditioned cells were harvested, washed, and restimulated with antihuman CD3/CD28 beads (1:1) and IL-2 (20 U/mL) for 72 h without any external soluble TGF-β. Those cells were then harvested and washed, and mTGF-β expression was assayed via FACS. The results are representative of 3 separate experiments. (E) The inhibition of TGF-β signaling suppresses CD4^{RAPA/TGF-β} cell suppression. The addition of ALK5 inhibitor (ALK5i) (SB431542), which inhibits TGF-β RI enzyme activity, reversed the suppression of CD4⁺ T cells by CD4^{T RAPA/TGF-β}.

Fig. 5.

The effect of CD4_{RAPA/TGF-β} coadministration on x-GVHD induced by autologous huPBMCs. (A) Kaplan–Meier survival estimates of RAG2^{-/-} γc^{-/-} mice that received either a high dose of CD25⁻ cell-depleted huPBMCs (20×10⁶) only or a cotransfer with different conditioned total CD4⁺ T cells (5×10⁶ cells/mouse, n=6). (B) The mean percentages of circulating human T cells after human cell transfer. (C) Liver histologic characteristics of CD4_{RAPA/TGF-β}-treated RAG2^{-/-}γc^{-/-} mice compared with similar mice treated with CD4_{Med} or CD4_{TGF-β} on day 14 of the experiment. The arrows indicate the central vein of the liver lobule surrounded by lymphocytes (H&E stain, original magnification ×100). (D) Immunohistochemical evaluation was performed in 3 mice in each group. Note the infiltration of human CD45⁺ cells in the livers of the RAG2^{-/-}γc^{-/-} mice. The infiltration of human cells on day 14 of the experiment is shown in the livers of those mice (original magnification ×200).