CD28 Costimulation Is Essential for Human T Regulatory Expansion and Function


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CD28 Costimulation Is Essential for Human T Regulatory Expansion and Function

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The costimulatory requirements required for peripheral blood T regulatory cells (Tregs) are unclear. Using cell-based artificial APCs we found that CD28 but not ICOS, OX40, 4-1BB, CD27, or CD40 ligand costimulation maintained high levels of Foxp3 expression and in vitro suppressive function. Only CD28 costimulation in the presence of rapamycin consistently generated Tregs that consistently suppressed xenogeneic graft-vs-host disease in immunodeficient mice. Restimulation of Tregs after 8–12 days of culture with CD28 costimulation in the presence of rapamycin resulted in >1000-fold expansion of Tregs in <3 wk. Next, we determined whether other costimulatory pathways could augment the replicative potential of CD28-costimulated Tregs. We observed that while OX40 costimulation augmented the proliferative capacity of CD28-costimulated Tregs, Foxp3 expression and suppressive function were diminished. These studies indicate that the costimulatory requirements for expanding Tregs differ from those for T effector cells and, furthermore, they extend findings from mouse Tregs to demonstrate that human postthymic Tregs require CD28 costimulation to expand and maintain potent suppressive function in vivo. The Journal of Immunology, 2008, 181: 2855–2868.

regulatory T cells (Tregs) play a critical role in maintaining peripheral tolerance, and their loss results in severe autoimmune disease in both humans and in mice (1–3). In murine models of type 1 diabetes (4–9), experimental autoimmune encephalomyelitis (10, 11), and inflammatory bowel disease (12), adoptive transfer of Tregs has ameliorated autoimmune pathology. In humans, however, generation of therapeutic quantities of Tregs, particularly under GMP-compliant conditions, has proven problematic (13, 14). Due to the relative paucity of Tregs, their lack of unambiguous cell surface markers, and their hypoproliferative nature, ex vivo expansions for adoptive transfer will likely start with enriched but not pure populations of Tregs. Because T effector cells have a replicative advantage in most culture systems, low level contamination of input cultures by effector T cells after Treg enrichment presents an obstacle to developing therapeutic adoptive transfer strategies with Treg cultures after several weeks of ex vivo expansion. Thus, determination of the optimal costimulatory requirements of Tregs is essential to develop culture systems useful for therapeutic applications.

Several groups have demonstrated that rapamycin preserves the suppressive function of ex vivo expanded Tregs (15–18). However, the underlying mechanism remains controversial. One study suggested that rapamycin selects for Tregs in culture (15), while another study suggested that rapamycin induced transient suppressive function in T effector cells (19). Recently, our group demonstrated that Tregs are programmed to be rapamycin-resistant by the constitutive, Foxp3-dependent expression of the serine/threonine kinase pim 2 (20). Thus, Tregs are preferentially selected in rapamycin-containing cultures due to their decreased susceptibility to rapamycin’s blockade of the mTOR (mammalian target of rapamycin) pathway. However, Tregs are not completely resistant to the antiproliferative effects of rapamycin, and the overall level of Treg expansion in the presence of rapamycin may be insufficient to achieve therapeutic numbers of Tregs.

Tregs require costimulatory signals to become fully functional. This first became apparent in mice with the observation that spontaneous diabetes is exacerbated in CD28-deficient NOD mice (4, 21). Several other studies have addressed the role of CD28 costimulation in Treg formation and function in mice. For example, CD28 costimulation is required both for Foxp3 induction in thymocytes (22) and peripheral homeostasis (23). However, in vivo peripheral Treg function does not require CD28, nor is CD28 required for the acquisition of inducible Treg suppression in mice (24). Nonetheless, CD28 costimulation also serves as a survival factor for induced Tregs (24, 25). CD27 is another costimulatory molecule constitutively expressed on resting naïve T cells and Tregs. In humans, effector T cells tend to lose CD27 during

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CD28 costimulation is required for Treg ex vivo expansion

Materials and Methods
Cell isolation and purification
PBMCs were obtained by leukapheresis of healthy volunteer donors by the University of Pennsylvania Human Immunology Core (Philadelphia, PA). All specimens were collected under a University Institutional Review Board-approved protocol, and informed consent was obtained from each donor. CD4+ bulk T cells were purified from freshly elutriated human PBLs using the CD4 Negative Selection Kit II (Miltenyi Biotec) according to the manufacturer’s recommendations on the autoMACS cell separator (Miltenyi Biotec). Purified CD4+ T cells were resuspended at a concentration of 25 million per milliliter in running buffer (PBS containing 2 mM EDTA and 0.5% human serum albumin). Ten microliters of anti-human CD127-PE (BD Biosciences) were added per milliliter of cell suspension and the cells were incubated for 15 min at 4°C. The cells were washed once with running buffer, and CD127+ cells were removed using anti-PE beads (Miltenyi Biotec) following the manufacturer’s recommendations. CD127- cells were resuspended at a concentration of 10^8 cells per 80 μl of running buffer and 20 μl of CD25 beads (Miltenyi Biotec) per 10^7 cells were added. After 15 min of incubation at 4°C, the cells were washed once and CD25+ cells were separated on the autoMACS separator using the “Possels” (positive selection) program, enriching for the final population of CD4+CD25+CD127+ cells.

Mice
All animal experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. NOD/scid/IL2Rγnull (NOD) mice were purchased from The Jackson Laboratory. Animals were also bred in the Animal Services Unit of the University of Pennsylvania. The mice were housed under specific pathogen-free conditions in microisolation cages and given ad libium access to autoclaved food and acidified water. Animals of both sexes were used for experiments at 6–9 wk of age.

Preparation of aAPCs
The K562-based aAPC lines K32 (CD32 only), K32.86 (CD32 and CD86), K32.4-1BBL, K32, OX40 ligand (OX40L), K32.86-1BBL, K64, and K64.86 were previously described (51). K64.ICOS ligand (ICOSL), K64.70, and K32.40 were created as follows: ICOSL (GenBank accession no. NM_015259) was isolated by PCR from human DC cDNA, CD40 (GenBank accession no. X60592) was isolated by PCR from a transformed B cell line (Raji), and CD70 (GenBank accession no. NM_001252) was isolated by PCR from cDNA clone provided by Open Biosystems. All amplified gene products were cloned into the lentiviral vector pCLPS and high titer lentiviral vectors were produced as previously described (52). K562 cells were engineered to express multiple genes by sequential transduction with the appropriate vectors. Single-cell clones of each aAPC were generated using a MoFlo sorter (DakoCytomation). Clones were screened for uniform high expression by flow cytometry with the appropriate Ab (BD Biosciences) as previously described (51). aAPC culture medium consisted of AIM V medium (Invitrogen) containing 5% human AB serum (Valley Biomedical).

Stimulation and expansion of human CD4+ and CD4+CD25+ T cells
K562-based aAPCs (those using CD32 as a Fc receptor) were lethally irradiated with 100 Gy, washed once, and resuspended at 1 x 10^6 cells/ml in culture medium (X-VIVO 15 medium; Lonza) containing 10% heat-inactivated human AB serum (Valley Biomedical) and 0.2% N-acetylcysteine (Ben Venue Labs). One μg/ml either anti-human CD3 alone (OKT3; Ortho) or, where indicated, 1 μg/ml both anti-human CD3 and anti-human CD28 (9.3 μAb) were added to the aAPCs. After incubation for 10 min at 22°C, the Ab-loaded K32 cells were added to the cultures at a ratio of one K32 cell to two CD4 cells. K64-based aAPCs (those using CD64 as

differentiation (26), whereas functional Tregs maintain CD27 expression (18, 27). The ligand for CD27, CD70, is overexpressed in lymphomas, and blockade of CD70, but not CD80 or CD86, interferes with the induction of FoxP3 in CD4+CD25+ human T cells (28).

Tregs also express costimulatory molecules that are induced upon T cell activation (29). The role of OX40 costimulation is perhaps the most perplexing. OX40 costimulation is required to generate Tregs after NO exposure (30), and it promotes Treg survival in vivo (31). However, other studies indicate that OX40 costimulation exerts a negative effect on Tregs by inhibiting FoxP3 gene expression (32, 33) and blocking the generation of IL-10-producing murine Tr1 cells (34). More recently, OX40 costimulation was shown to inactivate Tregs and make effector T cells resistant to the effects of Tregs in a murine tumor model (35). The role of 4-1BB stimulation is also unclear. Engagement of 4-1BB on human Tregs blocks both their function and their expansion (36), and systemic treatment of mice with agonist 4-1BB Abs promotes viral and tumor clearance (37–39). Other studies, however, suggest that 4-1BB costimulation may augment the Treg response. Stimulation via a soluble form of the 4-1BB ligand (4-1BBL) promotes expansion of murine Tregs (40), and 4-1BB stimulation reduces autoimmune pathology in murine models of arthritis and asthma (41–43). Similarly, 4-1BB costimulation delays the antiviral response when provided early in the immune response (41). However, in these latter studies, the direct effect of 4-1BB stimulation on Treg number and function was not examined. Several studies have addressed the role the CD40-CD40 ligand (CD40L) costimulatory axis plays in Treg function and development. Both CD40- and CD154-deficient mice have reduced numbers of Tregs in both the thymus and the periphery (44–47), and CD154 blockade results in a loss of Tregs that can be overcome by administration of IL-2. Thus, in mice CD154 costimulation appears to be important for Treg function, but this pathway also promotes both B and T cell effector responses. Loss of ICOS expression results in greatly augmented resistance to mouse Chlamydia infection, due in part to the lack of a robust Treg response (48). A recent study indicated that while CD28 costimulation plays an important role in the generation of T effector and T regulatory responses in mice, ICOS costimulation may play a similar role in sustaining both T effector and regulatory responses (49).

Thus, to date, no single identified costimulatory pathway promotes Treg activity without simultaneously and similarly modulating T effector activity. Rather, the effect a particular costimulatory pathway has on promoting or attenuating the immune response will likely be context dependent. These factors make the development of agents that predictably modulate Treg activity in vivo challenging. This was unfortunately highlighted by the tragic outcome of the administration of superagonistic anti-CD28 Abs to healthy volunteers (50) that was not predicted by studies in rodents. In contrast, adoptive T cell transfer approaches in which autologous T cells are expanded ex vivo followed by reinfusion into the patient provide the opportunity for target cell enrichment and control over the environment in which costimulatory signals are delivered. In this study, we explore the costimulatory requirements for optimal ex vivo expansion of functional human postthymic Treg cells for adoptive T cell therapy. Using a Treg cell purification process that is GMP compatible along with cell-based artificial APCs (aAPCs) suitable for clinical use, we found that CD28 costimulation was unique in its ability to promote Treg expansion. Enriched Tregs that expanded with CD28 costimulation in the presence of rapamycin consistently maintained high FoxP3 expression in vitro and had potent in vivo suppressive function. In contrast to mouse T cells, other costimulatory pathways tested were unable to promote the expansion of functional Tregs even in the presence of CD28 costimulation and rapamycin. Importantly, by restimulating Tregs with aAPCs that deliver CD28 costimulation after 8–12 days of culture, we were able to consistently expand functional Tregs >1000-fold. These studies demonstrate that human postthymic Tregs are dependent upon CD28 costimulation for ex vivo expansion and provide the rationale for the design of culture systems to generate Tregs with sufficient potency for adoptive T cell therapy.
an Fc receptor) were washed and resuspended in serum-free culture medium (X-VIVO 15 containing 0.2% N-acetylcysteine) 24 h before Ab loading. The cells were irradiated with 100 Gy and washed, followed by the addition of 1 μg/ml anti-CD3 either alone or in combination with 1 μg/ml anti-CD28. The cells were rotated at 4°C for 30 min, after which unbound Ab was removed by washing three times. Ab-loaded K64 cells were resuspended in serum-free culture medium at a density of 1 × 10^6 cells/ml and combined with CD4 cells (also in serum-free medium) at a final ratio of one K64 cell to two CD4 cells. Where indicated, rapamycin (Calbiochem) was added on day 0 to a final concentration of 100 ng/ml. After 24 h of culture, human AB serum was added to a final concentration of 10%, whereas on day 2 of culture human IL-2 (Chiron Therapeutics) was added to a final concentration of 300 IU/ml. Cultures were monitored for cell volume and cell density using a Coulter Multisizer (Beckman Coulter) on days 5, 8, 12, 15, and 19 of culture. Following counting, the culture was adjusted to 3 × 10^6 cells/ml and 300 μM IL-2 was added. One hundred ng/ml rapamycin was added only to the medium used to feed the cultures.

In vitro suppression assay

Following the harvest of expanded T cells (Tregs and control CD4 cells), varying numbers of T cells were plated in 100 μl of medium in round-bottom, 96-well plates (Corning). Frozen autologous PBMCs were thawed and CFSE labeled. The 5 mM stock solution of CFSE (Invitrogen) in DMSO was diluted 1/100 in PBS. PBMCs were resuspended in PBS containing 5% FCS (PBS-FCS) at a concentration of 1 × 10^7 cells/ml. One hundred μl of CFSE solution in PBS were added per milliliter of cells and mixed rapidly; after 3.5 min at room temperature 10 volumes of PBS-FCS were added to the suspension, the cells were spun down for 5 min at 300 × g (20°C), and the supernatant was removed. Cells were washed two more times with PBS-FCS and one more time with the culture medium and then counted. CFSE-labeled PBMCs were resuspended in culture medium at 1 × 10^6/ml and anti-CD3 beads (Invitrogen Dynal) were added at a ratio of one bead per cell. One hundred μl of PBMC cell suspension (1 × 10^6 cells) was placed to one well in a 96-well plate. CFSE-labeled PBMCs without CD3 beads were used as negative controls; CFSE-labeled PBMCs stimulated with anti-CD3 were used as positive controls. The cultures were harvested 4 days later and stained with allophycocyanin-conjugated anti-CD3 Abs (BD Pharmingen). Data were acquired on a FACS caliber (BD Biosciences) flow cytometer using CellQuest Pro software and analyzed using FlowJo software (Tree Star). For quantitative analysis of Treg suppression capacity, the gates were placed on live CD8+ T cells. The amount of cells in each generation was analyzed by using FlowJo software. The percentage of undivided (PU) cells was calculated as percentage of cells in generation 0 at the end of the assay using the following equation: \( PU = \frac{N}{N_0} \) where \( N_0 \) is the number of cells in generation 0 at the beginning of the assay, \( N \) is the number of cells at the end of the assay divided by the number of cells at the beginning of the assay using the following equation: \( FE = \frac{(N_0 - N_0)}{(N_0 - N_0)} + \frac{N}{(N_0 - N_0)} \). Fold expansion (FE) was calculated as the number of offspring cells at the end of the assay divided by the number of cells at the beginning of the assay using the following equation: \( FE = \frac{(N_0 - N_0)}{(N_0 - N_0)} + \frac{N}{(N_0 - N_0)} \). In preliminary experiments we found that expanded CD4 bulk T cells show lower Treg activity than expanded Tregs in the thymus (22). For this reason, K562 cells were transduced with lentiviral vector(s) to express CD86 and/or an Fc receptor (CD32 or CD64). The Fc receptor permits the loading of anti-CD3 Abs onto the aAPC. Single-cell clones were isolated by sorting, expanded, and characterized for CD64 and CD86 expression (K64.86). In this study, K562 cells were transduced with lentiviral vector(s) to express CD86 and/or an Fc receptor (CD32 or CD64). The Fc receptor permits the loading of anti-CD3 Abs onto the aAPC. Single-cell clones were isolated by sorting, expanded, and characterized for CD64 and CD86 expression (K64.86). We have previously shown that lentiviral vector-engineered K562-based aAPCs stably express introduced molecules without the need for selection agents, and equivalent T cell proliferation is observed whether CD32 or CD64 was used to load anti-CD3 (51). The advantage of using genetically defined cell-based aAPCs as opposed to natural APCs is that we can study the influence of particular costimulatory pathways in isolation.

Previous studies have shown that CD28 is required for the development of Tregs in the thymus (22). First, we evaluated whether costimulation was required for human postthymic Treg expansion. We have previously shown that anti-CD3-loaded cell-based aAPCs deliver a more potent signal to T cells than plate- or bead-bound anti-CD3 (53), so we asked whether cell-based anti-CD3 stimulation, in the presence of exogenous IL-2, was sufficient to promote Treg expansion. Although rapamycin preserves human T regulatory activity (15–18), it also limits the extent of Treg expansion. Therefore, we tested whether Treg expansion, either in the presence or absence of rapamycin, required costimulatory molecules. In cultures stimulated by cell-bound anti-CD3 Ab alone, a considerable lag time preceded Treg expansion, and the addition of rapamycin markedly reduced this already modest level of expansion (Fig. 1C). In contrast, Treg cultures that received CD28 co-stimulation via either anti-CD28 Ab (data not shown) or CD86 (K32.86) entered exponential growth phase upon stimulation. However, inclusion of rapamycin in these cultures resulted in a 5–10-fold reduction in Treg expansion (Fig. 1C). Rapamycin is reported to select for Foxp3+ cells (15–17). Therefore, it was surprising to observe (Fig. 1, D and E) that rapamycin in the absence of CD28 costimulation was insufficient to select for cells expressing Foxp3 and naive markers that have been correlated to potent Treg activity (6, 18, 27, 55).
FIGURE 1. CD28 costimulation is required to consistently obtain expanded cultures enriched for Foxp3 expression. A, Analysis of CD25 expression in purified CD4 T cells (left panel) and CD25 (middle panel) and Foxp3 (right panel) expression after CD127 depletion and CD25 selection of CD4 T cells. These data are representative of the enrichment of all input Treg populations used in this study. B, Expression of CD64 and CD86 was analyzed on K64 (left panel) and K64.86 (right panel) aAPCs by flow cytometry. C, Two hundred thousand enriched Tregs were stimulated with anti-CD3 Ab-loaded K32 aAPCs and anti-CD3-loaded K32.86 aAPCs cultured in the presence and absence of rapamycin (RAPA) for 2 wk and the population doubling rate was measured by cell counting. Each data point represents the average of four independent experiments (error bars represent SD). D and E, Analysis of Foxp3 (D) or CD27 and CD62L (double positive) (E) expression on Tregs expanded with anti-CD3 Ab-loaded K32 or K32.86 aAPCs in the presence of rapamycin. The panels on the left show a representative experiment and the box plots on the right contain data compiled from four independent experiments.
Collectively, these studies indicate that both CD28 costimulation and rapamycin are required to consistently maintain the Treg phenotype during ex vivo expansion. However, in ~25% of the cultures that were expanded by using CD28 costimulation, rapamycin was not required to maintain high levels of Foxp3 expression and in vitro suppressive function (data not shown), indicating that in some donors Tregs can be expanded without rapamycin providing that sufficient CD28 costimulation is present. The experimental basis for the donor to donor heterogeneity vis-à-vis the requirement for rapamycin remain unclear; however, for consistent generation of suppressive Tregs by ex vivo expansion, the routine incorporation of rapamycin seems justified.

**FIGURE 2.** CD28 ligands on cell-based aAPCs expand Tregs more efficiently than bead-based aAPCs. A. Enriched Tregs (2 x 10^5) were stimulated with either CD3/28 Ab-coated beads or anti-CD3-loaded K64.86 aAPCs and cultured in the presence or absence of rapamycin (RAPA) for 2 wk. Expansion was measured as described in the Materials and Methods. B. Box plot showing fold expansion for each culture condition using data collected from four donors. Fold expansion was determined by dividing the total number of cells at the end of culture by the initial starting number stimulated by the indicated aAPC in the presence (+) or absence (−) of rapamycin (Rapa). C. Analysis of Foxp3 and CD25 expression in Tregs expanded with either CD3/28 Ab coated beads (BD) or anti-CD3-loaded K64.86 aAPCs. Data are representative of four independent experiments.
The population doubling rates were measured by cell counting. Each data point represents the average of three independent experiments (error bars represent SD). However, in the presence of rapamycin, cell-based aAPCs expanded Tregs to a much greater degree than bead-based aAPCs (Fig. 2A and B). In contrast, our previous studies of bulk human CD4 cells cultured in the absence of rapamycin, bead, and K562-based aAPCs were equivalent in the ability to promote the expansion of effector CD4 T cells (53). We observed no statistically significant difference (p = 0.2) in Foxp3 expression in cells expanded with anti-CD28 coated beads (59 ± 18%) and anti-CD28-loaded K64.86 aAPCs (66 ± 23%) (Fig. 2C). However, it should be noted that in every experiment (n > 5), we observed more Foxp3+ cells in cultures expanded with the cell-based aAPC vs beads (data not shown). Thus in the presence of rapamycin, cell-based aAPCs promote more efficient expansion of human Tregs than anti-CD28/Ab-coated beads.

**CD28, 4-1BB, CD40L, OX40, or ICOS costimulation cannot substitute for CD28 costimulation to promote Treg expansion**

Little is known about how costimulatory molecules other than CD28 affect the expansion and function of adult human postthymic Tregs. In the mouse, OX40 contributes to efficient Treg-mediated...
suppression. However, T effectors became insensitive to Treg-mediated suppression when they were exposed to OX40L-expressing cells (61). Furthermore, ICOS signaling is required to maintain tolerance in the NOD mouse (62). To determine whether other costimulatory molecules could substitute for CD28 or augment the ability of CD28 to expand Tregs, we created aAPCs expressing the ligands for CD154, CD27, 4-1BB, OX40, and ICOS by transducing CD32- or CD64-expressing K562 cells with lentiviral vectors

FIGURE 4. Consistent suppression in vitro by Tregs expanded with CD28 costimulation in the presence of rapamycin (rapa). A, Tregs were expanded using the indicated cell-based aAPC. In a suppression assay, autologous PBMCs were labeled with CFSE and stimulated using anti-CD3 Ab-coated beads and mixed with either no Tregs (top panel) or expanded Tregs at a ratio of 1:4 (Treg:PBMC). Histograms show the expansion of CD8+ cells. Fold expansion and percentage of suppression (in parentheses) are indicated in the upper left-hand corner. B and C, Scatter plots showing the degree by which CD28-costimulated Tregs expanded with and without rapamycin (B) or by which anti-CD3 Ab- or anti-CD3 and CD28-costimulated Tregs expanded in rapamycin (C) suppress CD8+ T cell proliferation. Each point represents a separate culture; the average (±SD) suppressive activity is indicated. Paired t test was performed to determine the significance of differences between each group.
FIGURE 5. In vivo prevention of xenogeneic GVHD of Tregs expanded with CD28 and rapamycin. A, A starting cell population (SCP) of 2 × 10^5 CD4 bulk T cells (CD4) or enriched Tregs (CD25^+) were stimulated with K64.86 aAPCs in the presence or absence of rapamycin (Rapa). After 2 wk of culture, Foxp3 (filled histogram) expression and isotype control Ab (open histogram) staining was measured by flow cytometry. B, In vitro suppression of each population described in A was measured as described in Fig 4. Data represents four independent experiments. C, Two million cells from each culture shown in A were mixed with 10 million autologous PBMCs and injected into NOG mice (six mice per group). After 8 wk the mice were bled and the number of human CD8^+ T cells per microliter of blood was determined. D, Kaplan-Meier survival analysis (log rank) was performed with the indicated cohorts of mice. The Holm-Sidak method for multiple comparisons (significance level = 0.05) was performed in all groups and significant differences were found between the mice that were treated with enriched Tregs expanded in the presence of rapamycin and all other groups.
expressing each of the costimulatory ligands. Each aAPC was loaded with anti-CD3 Ab and used to expand Tregs in the presence of rapamycin. Only CD28-costimulated Tregs did not undergo a lag phase before expansion (Fig. 3A). Importantly, only those Tregs that expanded in the presence of CD28 costimulation maintained high levels of Foxp3 expression and naive T cell markers (CD27 and CD62L) (Fig. 3B). These data suggest that CD28 costimulation is required to augment the expansion of Tregs and that this signal for Treg expansion cannot be delivered by the other costimulatory pathways that were tested.

**CD28 costimulation and rapamycin are required to consistently obtain cultures that suppress in vitro**

Tregs are defined functionally by their ability to suppress immune responses. In the preceding experiments we used the level of expression of Foxp3 and Treg-related cell surface markers to demonstrate that CD28 costimulation and rapamycin maintain a Treg-like phenotype. Next, we determined whether both CD28 and rapamycin are required for Treg function by using an in vitro suppression assay. Autologous PBMCs were labeled with CFSE and stimulated with anti-CD3 coated beads. Ex vivo expanded Tregs were added at various dilutions and their ability to suppress CD8+ T cell expansion was measured. To quantify the ability of expanded Tregs to suppress, we determined the extent of CD8+ T cell suppression by counting the number of daughter cells and dividing by the number of input cells. Thus, in the absence of added expanded Tregs, we observed a 5.5-fold expansion of CD8+ T cells during 4 days of culture (Fig. 4A). Tregs that expanded by anti-CD3 stimulation alone, either with or without rapamycin, only had modest (20%) inhibitory activity on CD8+ T cell proliferation. In contrast, Tregs that expanded with CD28 costimulation (either by Ab engagement or CD86 ligation) exhibited significant suppressive activity, reducing CD8 T cell expansion by an order of magnitude. The inhibitory properties of these Tregs were further augmented by rapamycin addition. We compared the ability of rapamycin to augment the functional activity of CD28-costimulated Tregs in 11 donors. As shown in Fig. 4B, CD28-costimulated Tregs that expanded in the absence of rapamycin maintained a high level of functional activity in only a subset of the cultures. However, in all cases rapamycin augmented the functional activity of CD28-costimulated Tregs. Importantly, Tregs from all of the donors that expanded by CD28 costimulation in the presence of rapamycin displayed robust functional activity (Fig. 4A). Next, we determined whether CD28 costimulation was required for functionally active Tregs or whether expanding the cells in rapamycin would be sufficient. For this, we compared five donors (Fig. 4C). In addition to proliferating to ~10-fold higher levels (Fig. 2A), Treg cultures that expanded by using CD28 costimulation in the presence of rapamycin displayed more functional activity than cultures that expanded in the presence of rapamycin but in the absence of CD28 costimulation. Thus, the culture conditions that yielded Tregs expressing high levels of Foxp3 and naive T cell markers also yielded Tregs with the highest functional activity (compare Fig. 1, D and E with Fig. 4C). This correlation between Treg marker expression and Treg functional activity was also observed in Tregs cultured by costimulatory molecules other than CD28. None of these cultures shown in Fig. 3 functioned as well as CD28-costimulated Tregs (data not shown; see Fig. 6B). Thus, both CD28 costimulation and rapamycin are required for optimal and consistent expansion of functional Tregs.

**FIGURE 6.** OX40 costimulation promotes CD28-mediated T regulatory expansion in the presence of rapamycin but often results in the loss of suppressive activity. A, Enriched Tregs (2 x 10^5) were stimulated with cell-based aAPCs that delivered the indicated costimulatory signals in the presence of rapamycin and the population doubling rate was measured by cell counting. Each data point represents the average of three independent experiments (error bars represent SD). B and C, Scatter plots of the percentage of Foxp3-expressing cells (B) and in vitro suppressive activity (C) measured after 2 wk of culture following stimulation with the indicated cell-based aAPC. Each dot represents a separate experiment and cell donor. Mean values and SD are also indicated.

* T regulatory cultures expanded with CD28 and rapamycin are able to prevent xenogeneic GVHD

Tregs have been posited to control T effector responses by many mechanisms, including cytokine starvation, production of IDO,
TGF-β, IL-10, and IL-35, or by directly killing effectors (63). Presently, it is not clear which mechanism or mechanisms predominate in vivo. Furthermore, it is unclear whether in vitro suppression assays predict in vivo Treg function. To test whether CD28-costimulated Tregs could function in vivo, we used a xenogeneic acute GVHD model in which we could test whether expanded, autologous Tregs could block GVHD induced by human PBMC transferred into NOG mice. Enriched Treg cells and bulk CD4 T control cells were expanded by anti-CD3-loaded K64.86 aAPCs in the presence or absence of rapamycin. Interestingly, rapamycin-treated bulk CD4 T cells and nonrapamycin-treated enriched Treg cultures had equivalent levels of Foxp3 expression at the end of culture. Enriched Tregs expanded with rapamycin had substantially more Foxp3-expressing cells, whereas bulk CD4 T cells that were expanded without rapamycin had the least number of Foxp3-expressing cells (Fig. 5A). We tested these cells for their ability to suppress in vitro CD8 T cell expansion (Fig. 5B). Surprisingly, CD4 T cells that expanded in the presence of rapamycin had significantly more in vitro suppression than CD4 T cells that expanded in the absence of rapamycin. As previously shown in Fig. 4, the functional activity of enriched Tregs that expanded in the absence of rapamycin was variable, whereas the functional activity of enriched Tregs expanded with rapamycin was consistent and robust (Fig. 5B). Two million expanded T cells from each culture shown in Fig. 5A were mixed with 1 × 10^7 autologous PBMCs and injected into NOG mice (six mice per group). After 8 wk, the mice were bled and the number of human CD8 T cells per microliter of blood was determined.

**FIGURE 7.** Restimulation of T regulatory cells enables >1000-fold expansion without loss of in vitro or in vivo suppressive activity. **A**, Enriched Tregs (2 × 10^5) were stimulated by K64.86 aAPCs and expanded in the presence of rapamycin (RAPA). The population doubling rate was measured by cell counting. Each data point represents the average of three independent experiments (error bars represent SD). When cell expansion reached the plateau phase the culture was restimulated (restim) with K64.86 cell-based aAPCs (open circles) or left alone (filled circles). **B**, Analysis of Foxp3 and CD25 expression in enriched Tregs stimulated with K64.86 aAPCs once or twice in the presence of rapamycin after 14 days of culture. Data are representative of four independent experiments. **C**, The in vitro suppressive activity of cell populations shown in A and B was measured by mixing CFSE-labeled autologous PBMCs, anti-CD3 Ab-coated beads, and the indicated ratio of expanded Treg and PBMCs. After 4 days of culture, CFSE dilution was measured by flow cytometry. **D**, Cells (2 × 10^6) from each culture shown in A were mixed with 1 × 10^7 autologous PBMCs and injected into NOG mice (six mice per group). After 8 wk, the mice were bled and the number of human CD8 T cells per microliter of blood was determined.
lethal xenogeneic GVHD, indicating that these ex vivo expanded Treg cells can function in vivo. It should be emphasized that rapamycin was not given to the mice, indicating that Tregs expanded with rapamycin retain in vivo function in the absence of rapamycin. In addition, these results show that the results from in vitro suppression assays do not necessary correlate with those from in vivo GVHD assays, particularly in the case of bulk CD4 T cells that had low-level but consistent suppressive activity after culture in rapamycin, while failing to suppress in vivo. Finally, the level of CD8 T cells measured in the peripheral blood of the mice correlated well with survival (compare Fig. 5, C and D).

OX40 costimulation promotes CD28-mediated T regulatory expansion in the presence of rapamycin but often results in the loss of suppressive activity

As shown above in Fig. 1, Tregs underwent six to seven population doublings (100–300-fold) after a single round of CD28 costimulation in the presence of rapamycin. This amount of expansion may be insufficient for therapeutic applications, as studies with effector CD4 T cells have found that infusions of 1 × 10^6 to 1 × 10^10 cells are required to affect CD4 T cell homeostasis (13). To determine whether additional costimulatory pathways could augment CD28-mediated Treg expansion in the presence of rapamycin, we created aAPCs expressing CD86 and ICOSL, CD40, OX40L, 4-1BBL, or CD70. Of these, only K64.86.OX40L aAPCs consistently expanded Treg cultures in the presence of rapamycin 5–10-fold greater than CD28 costimulation alone (Fig. 6A and data not shown). However, OX40 costimulation appeared to have a dominant effect over CD28 costimulation in that it reduced the number of Foxp3^+ cells (Fig. 6B) and the in vitro suppressive activity of these cells (p = 0.08) (Fig. 6C). Thus, consistent with the results in Fig. 3, the engagement of additional costimulatory pathways in Tregs provided no benefit to CD28 costimulation and hindered the ability of CD28 costimulation to promote the expansion of Tregs.

Distinct restimulation requirements of effector T cells and Tregs

Once recruited into the cell cycle, T cells require repeated cycles of exposure to Ag and rest to maintain exponential expansion. For human CD8 T cells the cultures require restimulation at weekly intervals (64), whereas effector CD4 T cells require less frequent restimulation at 2–3-wk intervals (65). Moreover, if effector cells are restimulated prematurely they undergo activation-induced cell death. Thus, the timing of restimulation is crucial to optimize T cell expansion. It is unclear whether Tregs follow the same rules of ex vivo expansion as T effector cells. We stimulated enriched Tregs in the presence of rapamycin using anti-CD3-loaded K64.86 aAPCs and monitored cell volume and expansion using a Coulter counter. When the Tregs stopped expanding exponentially (day 8) the culture was split, with a portion being restimulated by anti-CD3-loaded K64.86 aAPCs while the remaining cells were maintained undisturbed with continued culture in IL-2. We observed that the restimulated Tregs reentered exponential growth phase and continued to expand until day 20. Superficially at least, the culture requirements for Tregs appear more to resemble CD8 T cells rather than CD4 effector T cells with respect to the timing of restimulation. Using this modified approach, Tregs were consistently expanded to >10 population doublings (1000-fold) (Fig. 7A). Next, we examined Foxp3 expression in Treg cultures receiving one and two stimulations. Restimulated Tregs reproducibly had more Foxp3^+ cells (Fig. 7B). The mechanism behind this observation is unclear presently, but it may indicate that residual contaminating effector cells from the input population were killed by being restimulated too early. Importantly, twice -stimulated Tregs were able to function as well as singly stimulated Tregs in vitro and in vivo by protecting against GVHD lethality (Fig. 7, C and D).

Discussion

Based on results from numerous studies in mice (1), human Tregs have considerable therapeutic potential in adoptive transfer settings (14), provided that they can be generated in therapeutic quantities. In this study we compared the ability of K562 cell-based and bead-based aAPCs expressing a panel of costimulatory ligands to expand functional Tregs from enriched but still impure starting populations. In the absence of rapamycin we observed an overall decline in the frequency of Foxp3-expressing cells in both cell- and bead-based culture systems, suggesting that both systems preferentially expanded T effectors rather than Tregs. In the presence of rapamycin, however, cell-based aAPCs expanded functional Treg populations more efficiently than bead-based aAPCs. Several mechanisms could account for the superiority of the cell-based APC culture approach. Our previous data showed that Foxp3 expression promotes pim 2 kinase expression, which mediates rapamycin resistance (20). Thus, if cell-based aAPCs induced higher levels of Foxp3 than bead-based aAPCs, greater pim 2-mediated rapamycin resistance would lead to increased Treg proliferation. However, we were unable to observe a significant difference in the level of Foxp3 expression or in the number of Foxp3-expressing cells between bead-based and cell-based aAPC-stimulated cultures (Fig. 2). However, it is possible that the expression level or stability of other molecules associated with Foxp3 and pim 2 may be promoted by cell-based aAPCs. For example, the expression of TIP60 and histone deacetylase 7 (HDAC7) may differ, as Foxp3 must form higher order complexes with these molecules to inhibit IL-2 production (66). If stimulation with cell-based aAPCs promoted or stabilized the formation of these complexes within Tregs more efficiently than stimulation with bead-based aAPCs, then this could explain why more Foxp3 activity, i.e., rapamycin resistance via pim 2 expression, is not revealed simply by absolute measurements of Foxp3 levels.

Regardless of the precise mechanism, our observation that cell-based aAPCs promote the expansion of Tregs in the presence of rapamycin has important therapeutic implications. If rapamycin is included in the culture system to expand Tregs, it will be advantageous to use cell-based aAPCs delivering CD28 costimulation either by anti-CD28 Ab or CD86. Although cell-based aAPCs are superior to bead-based aAPCs or soluble anti-CD28 Ab (data not shown) in expanding rapamycin-resistant Tregs, they possess other advantages for the expansion of Tregs. K562 cell-based aAPCs express multiple adhesion molecules, facilitating aAPC-T cell interactions. Moreover, these aAPCs participate in T cell/APC crosstalk by up-regulating multiple modulators of T cell activation (53). Because they are lethally irradiated, K562-based aAPCs disappear from culture after ~5 days, whereas bead-based aAPCs must be removed from culture at the time of cell harvest by passage through magnetic fields or other procedures. Furthermore, the self-replicating nature of K562-based aAPCs likely makes them more cost effective than bead-based aAPCs. Lastly, irradiated GM-CSF-expressing K562 cells were incorporated in a cancer vaccine protocol (67), suggesting that K562-based aAPCs can be used safely in humans.

The use of cell-based aAPCs facilitated the identification of culture conditions for the optimal expansion of functional Tregs. T cell activation requires two signals; one signal is delivered through the TCR/CD3 complex and the second is delivered by one or more members of the costimulatory receptor families. To deliver signal one, we have used Fc receptors to bind anti-CD3 Ab. Both CD32
and CD64 bind anti-CD3 Ab to promote Treg expansion; however, the higher affinity of CD64 for Fc allows excess Ab to be washed away before use, reducing the amount of anti-CD3 Ab infused into patients and the likelihood of inducing human anti-mouse Ab responses after multiple T cell infusions. Thus, CD64 is superior for clinical applications. Starting from enriched but not pure Treg populations, we found that CD27, CD40L, 4-1BB, OX40, and ICOS costimulation all failed to support Treg expansion. Multiple mechanisms could account for these results, including the inactivation of Tregs or the conversion of Tregs to T effectors. Alternatively, these costimulatory pathways could favor the expansion of T effectors at the expense of Tregs. We favor the latter interpretation, because our studies using Tregs purified from cord blood (CB) revealed that in addition to CD28 costimulation, 4-1BB and OX40 costimulation significantly augmented CB Treg expansion and function (68). Because CB CD4 T cells are Ag inexperienced and resting, highly enriched Treg populations can be selected by simply isolating CD4^+ CD25^+ cells (57). Importantly, rapamycin was not required to maintain Foxp3-expressing, functional CB Tregs. Although these findings could reflect fundamental growth differences between CB and peripheral blood Tregs, they more likely indicate that if sufficiently pure populations of Tregs can be obtained from any source, costimulatory pathways such as OX40 and 4-1BB will promote Treg expansion and rapamycin might not be required to check T effector replication. However, in peripheral blood-derived Treg populations containing T effectors, costimulatory pathways other than CD28 promote the expansion of T effectors more efficiently than they promote the expansion of Tregs and therefore are not optimal for the reproducible expansion of Tregs.

In contrast to other costimulatory molecules, CD28 costimulation proves to be necessary for peripheral blood Treg expansion, as enriched Treg populations are unable to maintain their Treg phenotype (Foxp3 and naive T cell marker expression) or suppressive activity in the absence of CD28 costimulation even when expanded in the presence of rapamycin. The CD28-dependent signaling pathways that promote Treg expansion are currently unknown. However, the inability of ICOS costimulation to promote Treg expansion provides some clues. In CD4 T cells, both CD28 and ICOS costimulation induce similar genetic programs that transition resting T cells into activated effector cells. However, differential regulation of several transcripts, including IL-2, ICOS, IL-9, MAL, and MYO1F is observed, suggesting the existence of both overlapping and distinct pathways (69). One shared pathway is PI3K activation (52) which likely rules this pathway out as being solely required for Treg expansion. Additionally, Singer and colleagues have identified a proline-rich sequence within the murine CD28 cytoplasmic tail that is crucial for thymic Treg differentiation (22). It will be interesting to determine whether this motif is required for human Treg expansion ex vivo and, if so, what signaling molecules are recruited to this motif.

In all of our culture experiments using enriched peripheral blood Tregs (n > 20) we found that CD28 costimulation in the presence of rapamycin resulted in the expansion of functional Tregs. However, in the absence of rapamycin CD28 costimulation did not consistently yield functional Tregs. There has been some debate as to the mechanism(s) by which rapamycin maintains the Treg phenotype in culture. One study indicated that in the presence of rapamycin Tregs preferentially expand at the expense of T effector (15), whereas another study suggested that rapamycin temporally imparts Treg-like activity to effector cells and, upon rapamycin removal, the cells revert to an effector phenotype (19). Resolving this controversy is crucial, because if rapamycin only produces “pseudo Tregs,” then its therapeutic utility is suspect. Our data help clarify this issue. We previously demonstrated that Foxp3 expression induces pim 2 (20), a serine/threonine kinase that mediates rapamycin resistance (70). Thus, Tregs are programmed to preferentially expand in the presence of rapamycin. In the present study we observed that CD4 T cells, when expanded in the presence of rapamycin, exhibited suppressive activity in vitro (Fig. 5). However, when the function of these cells was assayed in an in vivo mouse model, these cells failed to prevent xenogeneic GVHD. We interpret these data to indicate that rapamycin can induce a suppressive phenotype, albeit transient, in effector T cells. These observations highlight the dangers of relying solely on in vitro suppression assays to measure Treg activity. Thus, our data show that rapamycin can simultaneously select for Tregs and confer a transient Treg-like phenotype upon T effector cells. An important implication of this dual effect of rapamycin is that Tregs must be sufficiently purified (~60% Foxp3-expressing cells) before initiating culture. Insufficient purity may result in the predominance of rapamycin-induced “pseudo Tregs” within the culture.

The lack of a unique cell surface marker to identify Tregs and a clear understanding of how they function to control immune responses in vivo has hindered the development of Treg therapies. Although Foxp3 is the best marker, it has limitations. For one, it is a transcription factor and there is currently no way to measure its expression in live cells. Secondly, especially in humans, Foxp3 expression has not always correlated with suppressive activity. Upon T cell activation Foxp3 is transiently induced (71–73), and this induction does not result in suppressive activity (74). More striking is the ability of TGF-β-treated human T effector cells to express high levels Foxp3 for sustained periods of time and yet have absolutely no suppressive activity (75, 76). This disconnect between Foxp3 expression and suppressive activity may reflect specific modifications of the Foxp3 promoter (77) or the higher order ensembles Foxp3 forms with other molecules within the cell (78). In any case, the definition of a Treg remains a functional one. In this study we show that a xenogeneic GVHD mouse model provides a robust measurement of Treg activity, as it accurately distinguishes between “pseudo Tregs” (control bulk CD4 T cells expanded with rapamycin) and true Tregs. It should be noted again that no rapamycin was given to the mice, suggesting that functional Tregs expanded with rapamycin retain a suppressive phenotype in the absence of rapamycin.

In conclusion, our data indicate that CD28 provides an essential signal for mature Tregs, extending its role previously demonstrated in the mouse (4). Human enriched peripheral blood Tregs can be expanded >1000-fold within 3 wk of ex vivo culture in the presence of rapamycin by two stimulations using cell-based aAPCs that deliver CD28 costimulation. These cells retain potent in vitro and in vivo suppressive function and are an attractive population of cells for the prevention and perhaps treatment of GVHD and autoimmunity in humans.

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References


CD28 COSTIMULATION IS REQUIRED FOR TREG EX VIVO EXPANSION