Murine Regulatory T Cells Contain Hyperproliferative and Death-Prone Subsets with Differential ICOS Expression

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*Published online 9 January 2012*
http://www.jimmunol.org/content/early/2012/01/09/jimmunol.1102448

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Regulatory T cells (Treg) are crucial for self-tolerance. It has been an enigma that Treg exhibit an anergic phenotype reflected by hypoproliferation in vitro after TCR stimulation but undergo vigorous proliferation in vivo. We report in this study that murine Treg are prone to death but hyperproliferative in vitro and in vivo, which is different from conventional CD4+ Foxp3− T cells (Tcon). During in vitro culture, most Treg die with or without TCR stimulation, correlated with constitutive activation of the intrinsic death pathway. However, a small portion of the Treg population is more sensitive to TCR stimulation, particularly weak stimulation, proliferates more vigorously than CD4+ Tcon, and is resistant to activation-induced cell death. Treg proliferation is enhanced by IL-2 but is less dependent on CD28-mediated costimulation than that of Tcon. We demonstrate further that the surviving and proliferative Treg are ICOS+ whereas the death-prone Treg are ICOS−. Moreover, ICOS+ Treg contain much stronger suppressive activity than that of ICOS− Treg. Our data indicate that massive death contributes to the anergic phenotype of Treg in vitro and suggest modulation of Treg survival as a therapeutic strategy for treatment of autoimmune diseases and cancer. The Journal of Immunology, 2012, 188: 000–000.

Abbreviations used in this article: 4E-BP1, translational repressor 4 elongation factor binding protein 1; LN, lymph node; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; S6K1, 70-kDa ribosomal S6 kinase; Tcon, conventional CD4+ Foxp3− T cell; Treg, regulatory T cell; Treg-FBS, Treg in FBS-containing medium; Treg-PBS, Treg rested in PBS; WT, wild-type.

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Published January 9, 2012, doi:10.4049/jimmunol.1102448

Received for publication August 24, 2011. Accepted for publication December 6, 2011.

This work was supported by the National Institutes of Health (Grants R01AI076357, R01AI079088, and R21AI079873), the American Cancer Society (Grant RSG-08-186-01-LIB), the American Heart Association (to X.-P.Z.), and the Chinese National Science Foundation (31071237).

Materials and Methods

Mice

The C57BL/6J, Foxp3GFP (16), CD28−/−, and B6.PL-Thylα/ΔCyl congenic mice were all purchased from The Jackson Laboratory. Thy1.1-Foxp3GFP mice were generated by breeding B6.PL-Thylα/ΔCyl mice with Foxp3GFP mice. All mice were housed in an approved pathogen-free facility, and experiments were performed in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.

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Received for publication August 24, 2011. Accepted for publication December 6, 2011.

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Murine Regulatory T Cells Contain Hyperproliferative and Death-Prone Subsets with Differential ICOS Expression

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Regulatory T cells (Treg) actively suppress autoimmunity and play critical roles in self-tolerance. Natural Treg are generated in the thymus and are governed by the forkhead transcription factor Foxp3. Expression of Foxp3 is critical for Treg generation, maintenance, and function (1–5). Foxp3+ Treg are composed of a small portion of T cells with distinct molecular patterns and properties. Different from naive conventional CD4+ Foxp3+ T cells (Tcon), Foxp3+ Treg express signature molecules such as CD25, GITR, and CTLA4 that are usually expressed by activated Tcon (6–8). However, Treg do not express IL-2 or IFN-γ. Instead, they express suppressive cytokines such as IL-10 and TGF-β (9). Moreover, Treg have been marked as “anergic” in vitro and in vivo. In vitro, Treg have been found unable to proliferate after TCR engagement. In vivo, adoptively transferred CD4+CD25+ Treg that recognize OVA display decreased proliferative responses to Ag challenge in mice (10–13). Although these results point to an anergic property of Treg, Treg manifest normal or even stronger homeostatic proliferation after injection into lymphopenic hosts or TCR-induced proliferation in vivo (11, 14). Furthermore, Treg incorporate a higher level of BrdU than that of Tcon after administration of BrdU into mice, suggesting that Treg are more proliferative than Tcon in normal mice (15). The causes of these distinct observations and conclusions about Treg properties have been unclear. By tracking Treg proliferation and death simultaneously, we report in this study that CD4+Foxp3+ Treg are hyperproliferative but prone to death in vitro and in vivo, which differs from CD4+ Foxp3− Tcon. During in vitro culture with a TCR-stimulating Ab, the majority of Treg die before or during the first division. The remaining surviving subset of Treg is hyperproliferative to TCR stimulation, particularly when the stimulation is weak. Treg proliferation is enhanced by IL-2 and is less dependent on CD28 co-stimulation than that of Tcon. In addition, freshly isolated Treg show enhanced death and proliferation compared with Tcon. Whereas proliferative Tcon are sensitive to activation-induced death, the hyperproliferative Treg are resistant to activation-induced death. Lastly, we demonstrate that the living hyperproliferative Treg are ICOS+ and the death-prone Treg are ICOS−. Moreover, ICOS+ Treg have much stronger suppressive activity than ICOS− Treg. Together, our data indicate that Treg should not be simply defined as anergic. Rather, they contain a predominant ICOS+ death-prone subset and a minor ICOS− hyperproliferative subset. Our data provide an explanation for the aforementioned contradictory properties of Treg and suggest that promotion of Treg survival could be used as an effective strategy to enhance immune suppression and tolerance.

Materials and Methods

Mice

The C57BL/6J, Foxp3GFP (16), CD28−/−, and B6.PL-Thylα/ΔCyl congenic mice were all purchased from The Jackson Laboratory. Thy1.1-Foxp3GFP mice were generated by breeding B6.PL-Thylα/ΔCyl mice with Foxp3GFP mice. All mice were housed in an approved pathogen-free facility, and experiments were performed in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.
Flow cytometry

Single-cell suspensions of spleen and lymph nodes (LNs) were stained with Abs in PBS containing 2% FCS. The Abs include PE-Cy7–anti-CD4 (RM4-5),allophycocyanin–Cy7–anti-CD8α (53-6.7), PE-Cy7–anti-ICOS (398.4A), PE- or allophycocyanin-conjugated anti-CD90.1 (H1S1), and PE- or allophycocyanin-conjugated anti-CD90.2 (30-H12) (BD Biosciences). Biotin-conjugated Abs were developed with PE-conjugated, PE-Cy5–conjugated, or allophycocyanin-conjugated streptavidin. For intra-cellular staining, cells were permeabilized using the Foxp3 staining kit (eBioscience) after cell surface staining, followed by PE-anti-Foxp3 (FJK-16s; ebioscience) or unconjugated anti-Ki67 (B56; BD Biosciences). An Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen) was used to detect anti-Ki67 Ab. Dying cells were identified using 7-aminactinomycin D or the fixable Violet Live/Dead cell staining kit (Invitrogen). Allophycocyanin-conjugated annexin V (BD Biosciences) was used to detect apoptotic cells. Stained samples were collected on a Canto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Treg isolation

CD4 T cells were enriched using the APC selection kit (Stemcell Technologies) or MACS sorting with the LD column (Miltenyi Biotec) according to the manufacturers’ protocols with ~80% purity. The isolated CD4 cells were stained for CD4, CD25, ICOS, 7-aminactinomycin D, Thy1.1, and Thy1.2 when needed and sorted for live CD4*GFP* and CD4*GFP cells. The purities of sorting cells was usually >95%.

Proliferation assay

CFSE proliferation assay was performed as previously described (17). Single-cell suspension was made from spleens and LNs of mice in IMDM supplemented with 10% FBS, penicillin/streptomycin, and glutamine. Cells were stained with 10 μM CFSE for 9 min at room temperature in PBS-0.5% BSA. Labeled cells were left unstimulated or stimulated with an anti-CD3e Ab (2C11) at the indicated concentrations at 37°C for different times in a 5% CO2 incubator. Where indicated, IL-2 (1000 U/mL; Peprotech) or CTLA4Ig (10 μg/mL; BioXcell) were added at the beginning of the culture. When cultured with IL-2, CD5 cells were depleted using anti-CD5 microbeads and LD columns according to the manufacturer’s protocol (Miltenyi Biotec).

Treg contact inhibition assay

Sorted Thy1.1+Thy1.2+ Tcon were labeled with CFSE. Splenocytes from TCR β<sup>−/−</sup>β<sup>−</sup> mice were treated with 25 μg/mL mitomycin C for 30 min and used as APCs. In a 96-well U-bottom plate, 1.5 × 10<sup>5</sup> CFSE-labeled Tcon, the same number of APCs, and different numbers of sorted Thy1.2+ ICOS+ or ICOS− Treg according to the indicated ratios were added to each well. The cells were left unstimulated or stimulated with an anti-CD3e (2C11) Ab (1 μg/mL). After 72-h cultures, the cells were stained with PE-Cy7–anti-Thy1.1, allophycocyanin–anti-Thy1.2, and allophycocyanin–Cy7–anti-CD4 and analyzed by flow cytometry.

Western blot

CD4*GFP* and CD4*GFP cells were sorted into IMDM–10% FBS. Cell pellets of 0.5 to 1 million cells were lysed in 1% Nonidet P-40, 50 mM Tris, 150 mM NaCl) supplemented with protease and phosphatase inhibitors. Protein lysates were boiled for 5 min in denaturing sample buffer, separated by SDS-PAGE, and were transferred to a Trans-Blot Nitrocellulose membrane (Bio-Rad Laboratories). After blocking in TBST (10 mM Tris pH 8, 150 mM NaCl, 2% Tween 20) supplemented with 3% (w/v) dried skim milk powder, the membrane was probed with indicated specific Abs in 5% (w/v) BSA–TBST solution followed by HRP-conjugated secondary Abs in TBST supplemented with 3% (w/v) dried skim milk. Proteins on the membrane were detected by ECL. Abs for p-PLC-γ1 (no. 2821), p-RSK1/2 (S56/363; no. 9344), p-Foxo1 (no. 9461S), p-ERK1/2 (no. 9101S), p-JNK (no. 9202S), p-c-Src (no. 4838), S6 (no. 2217), p-4E-BP1 (no. 2855S), 4E-BP1 (no. 9644), cleaved caspase-3 (no. 9661), cleaved caspase 9 (no. 9509), and p-Akt (no. 4838) were probed with indicated primary Abs in 5% (w/v) BSA–TBST solution followed by HRP-conjugated secondary Abs in TBST supplemented with 3% (w/v) dried skim milk. Proteins on the membrane were detected by ECL. Abs for p-PLC-γ1 (no. 2821), p-RSK1/2 (S56/363; no. 9344), p-Foxo1 (no. 9461S), p-ERK1/2 (no. 9101S), p-JNK (no. 9202S), p-c-Src (no. 4838), S6 (no. 2217), p-4E-BP1 (no. 2855S), 4E-BP1 (no. 9644), cleaved caspase-3 (no. 9661), cleaved caspase 9 (no. 9509), and p-Akt (no. 4838) were probed with indicated primary Abs in 5% (w/v) BSA–TBST solution followed by HRP-conjugated secondary Abs in TBST supplemented with 3% (w/v) dried skim milk. Proteins on the membrane were detected by ECL.

Statistical analysis

For statistical analysis, two-tailed Student t test was performed (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Live Foxp3<sup>+</sup> Treg are hyperproliferative after TCR stimulation in vitro

We compared proliferative responses between CD4<sup>+</sup>Foxp3<sup>+</sup> Tcon and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg to different strengths of TCR stimulation in vitro using a CFSE dilution assay. CFSE-labeled splenocytes from wild-type (WT) mice were left unstimulated or stimulated with different concentrations of an anti-CD3 Ab for 72 h. After cell surface staining for CD4 and CD8 as well as fixable Live/Dead staining for dead cells, cultured cells were intracellularly stained for Foxp3. Comparison between total CD4<sup>+</sup>Foxp3<sup>+</sup> Treg and CD4<sup>+</sup>Foxp3<sup>+</sup> Tcon revealed much weaker proliferation of Treg than Tcon (Fig. 1A), which is consistent with previously published observations that Treg are “anergic” in vitro. However, if gated on live CD4<sup>+</sup>Foxp3<sup>+</sup> Treg and CD4<sup>+</sup>Foxp3<sup>+</sup> Tcon (Fig. 1B, 1C), we found that Treg had undergone more cell divisions than Tcon, particularly when the stimulation was weak (Fig. 1D).

Differential sensitivity to activation-induced death between proliferating Treg and Tcon

The differences of Treg proliferation between total Treg and live Treg prompted us to examine survival and death of Treg during in vitro culture. We analyzed the relationship of cell death versus proliferation of total CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> cells from the same experiments in Fig. 1A–D. As shown in Fig. 1E, most CD4<sup>+</sup>Foxp3<sup>+</sup> Treg died after multiple divisions, and live CD4<sup>+</sup>Foxp3<sup>+</sup> T cells had gone through less divisions than dead cells, particularly when the stimulation was strong. In contrast, most of the CD4<sup>+</sup>Foxp3<sup>+</sup> Treg died before the first cell division, and the hyperproliferating CD4<sup>+</sup>Foxp3<sup>+</sup> Treg were mostly live cells. Together, these observations suggest that proliferative CD4<sup>+</sup>Foxp3<sup>+</sup> Tcon are sensitive to activation-induced cell death and that most Treg are death prone, but proliferating Treg are resistant to activation-induced cell death in vitro.

Hyperproliferative Treg are not converted from Tcon

Because some Tcon can convert to Foxp3<sup>+</sup> after TCR stimulation, this small population of hyperproliferative Foxp3<sup>+</sup> cells could be derived from Foxp3<sup>+</sup> Tcon during in vitro TCR stimulation. To rule out such a possibility, we performed a mixed culture experiment. In this experiment, we used the Foxp3<sup>+</sup>GFP mice in which an IRES-GFP cassette is knocked into the 3′ untranslated region of the Foxp3 gene so that GFP expression can be used to identify Foxp3<sup>+</sup> cells (16). Sorted Thy1.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup>GFP<sup>+</sup> Tcon from Thy1.1<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup>GFP<sup>+</sup> mice were mixed with Thy1.2<sup>+</sup> splenocytes in an amount so that the ratio of Thy1.1<sup>+</sup> to Thy1.2<sup>+</sup> Tcon was close to 1:1. The mixed cells were labeled with CFSE and stimulated with anti-CD3. While Thy1.1<sup>+</sup> and Thy1.2<sup>+</sup> CD4<sup>+</sup>Foxp3<sup>+</sup> T cells contributed almost equally, more than 95% of live and proliferating CD4<sup>+</sup>Foxp3<sup>+</sup> cells were Thy1.2<sup>+</sup> (Fig. 2). These observations indicate that while there was a low rate of conversion of CD4<sup>+</sup>Foxp3<sup>+</sup> to CD4<sup>+</sup>Foxp3<sup>+</sup> during in vitro TCR stimulation, the
contribution of such conversion to the proliferating Treg detected in response to TCR stimulation was minimal and that most of the hyperproliferating CD4+Foxp3+ cells were natural Treg.

Increased death and proliferation of freshly isolated Treg

To determine whether the aforementioned in vitro observations reflect properties of Treg in vivo, we examined cell death and proliferative status of freshly isolated T cells. CD4+Foxp3GFP+ Treg stained positive for annexin V 3–4 times more frequently than CD4+Foxp3GFP− Tcon (Fig. 3 A, B), suggesting more apoptosis of Treg than Tcon in vivo. About 1.5- to 2-fold more Treg than Tcon stained positive for Ki67 (Fig. 3 C, D), a marker of cell proliferation. These data are consistent with the observation that Treg incorporate more BrdU than do Tcon in vivo (15). Together, these data are consistent with the aforementioned in vitro data and suggest that Treg are hyperproliferative and prone to death in vivo.

Treg proliferation is less dependent on CD28 costimulation than that of Tcon

T cell activation requires signals from both the TCR and costimulatory receptors such as CD28. CD28-deficient mice contain fewer Treg than that of WT controls. CD28-mediated costimulation has been found to promote human CD4+CD25+ T cell expansion in vitro (18–20). We examined the role of CD28 costimulation for Treg proliferation using the same experimental system as in Fig. 1 except that CTLA4-Ig was added to the culture. CTLA4-Ig has high affinities to B7-1 and B7-2 costimulatory ligands and thus blocks CD28-mediated costimulation. As expected, CD4+Foxp3+ Tcon proliferated less in the presence of CTLA4-Ig than in the absence of this molecule. However, the inhibitory effect of CTLA4-Ig on TCR-induced Treg proliferation was much weaker than that on TCR-induced Tcon proliferation (Fig. 4 A).

To investigate further the importance of CD28-mediated costimulation on Treg proliferation, we analyzed proliferative responses of CD28−/− Tcon and Treg to TCR stimulation. As shown in Fig. 4 B, CD28−/− CD4+Foxp3− Tcon proliferated much more weakly than WT CD4+Foxp3− Tcon. However, CD28−/− CD4+ Foxp3+ Treg proliferation was only slightly decreased compared with that of WT CD4+Foxp3+ Treg. Together, these observations indicate that Treg are less dependent on CD28 costimulatory signal for their proliferation than are Tcon.
Altered mammalian target of rapamycin signaling and constitutive activation of the intrinsic death pathway in Treg

To investigate the mechanisms that may control Treg proliferation and survival, we compared signaling events between Treg and Tcon. CD4+Foxp3+ Treg and CD4+Foxp3− Tcon were sorted into serum-containing medium and lysed immediately after sorting. As shown in Fig. 5A, Treg contain higher levels of ERK1/2 phosphorylation than those of Tcon. ERK1/2 usually functions downstream of Ras to promote cell proliferation. The increased ERK1/2 activation correlates with increased proliferation of Treg.

The mammalian target of rapamycin (mTOR) integrates environmental stimuli including growth factors, nutrients, and stress-activated signals to regulate cell metabolism, survival, growth, and proliferation. mTOR signaling is mediated through two signaling complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is sensitive to rapamycin inhibition, whereas mTORC2 signaling is insensitive to acute rapamycin treatment. mTORC1 promotes cell growth and proliferation through phosphorylating two major substrates: the 70-kDa ribosomal S6 kinase (S6K1) and the translational repressor 4E-BP1. Activated S6K1 phosphorylates S6 to promote ribosome biogenesis. Phosphorylated 4E-BP1 dissociates from eukaryotic initiation factor 4E to promote protein translation. mTORC2 phosphorylates Akt at serine 473, leading to an increase of Akt activity (21–23).

FIGURE 2. Hyperproliferative Treg are not derived from Tcon. Sorted Thy1.1+CD4+Foxp3GFP− Tcon were mixed with Thy1.2+ splenocytes from mice carrying the Foxp3GFP transgene in a ratio in which Thy1.1+CD4+Foxp3GFP− Tcon were almost equal to Thy1.2+CD4+Foxp3GFP+ Tcon in the mixture. The mixed cells were labeled with CFSE, and proliferation of Tcon and Treg were similarly examined as for Fig. 1. A, Histograms show CFSE dilution of gated live CD4+Foxp3+ Tcon. Dot plots show Thy1.1 and Thy1.2 staining of gated live CD4+Foxp3− Tcon. B, Histograms show CFSE dilution of live gated CD4+Foxp3+ Treg. Dot plots show Thy1.1 and Thy1.2 staining of live gated CD4+Foxp3+ Treg. Data shown represent two experiments.

FIGURE 3. Assessment of proliferative and surviving status of freshly isolated Treg. A and B, Increased apoptosis of Treg. Freshly isolated splenocytes and LN cells from Foxp3GFP mice were stained with CD4, CD8, and annexin V. Annexin V staining of CD4+Foxp3−, CD4+Foxp3+, and CD8+ cells of mice was determined by FACS. A, Representative contour plots. B, Mean ± SEM presentation of data from multiple mice (n = 9). C and D, Ki67 staining of Treg, Tcon, and CD8 T cells. Freshly isolated splenocytes from C57BL/6/J mice were stained with CD4 and CD8 followed by intracellular staining for Ki67 and Foxp3. C, Representative dot plot of Ki67 expression in Treg, Tcon, and CD8 T cells. D, Mean ± SEM presentation of Ki67+ Treg, Tcon, and CD8+ cells in the spleen and LN from multiple mice (n = 5).
Both S6K1 and S6 phosphorylation was lower in CD4+Foxp3+ Treg than in Tcon. In contrast, 4E-BP1 phosphorylation was higher in Treg than in Tcon (Fig. 5B). These observations revealed that dissociation of phosphorylation of S6K1 and 4E-BP1 in Treg. Akt phosphorylation at serine 473 was lower in Treg than in Tcon. In addition, phosphorylation of Foxo1 and GSK3β, events mediated by Akt, was decreased in Treg compared with that in Tcon. Thus, mTORC2 and Akt activities are decreased in Treg (Fig. 5C).

Consistent with the decreased Akt activity, cleaved active caspase 9 and caspase 3 were both increased in Treg, suggesting enhanced activation of the intrinsic death pathway in Treg (Fig. 5D).

### TCR signaling in Treg

It has been reported that Treg display defective signaling transduction after TCR engagement (11). For example, decreased calcium influx and ERK1/2 phosphorylation were reported in TCR-stimulated CD4+CD25+ Treg (24). To compare TCR signaling between Treg and Tcon, we rested sorted CD4+Foxp3+ Treg and CD4+Foxp3− Tcon in PBS and then stimulated them with anti-CD3. As shown in Fig. 5E, phosphorylation of PLCγ1, ERK1/2, IκBα, and NF-κB was obviously decreased in Treg compared with that in Tcon after TCR stimulation, suggesting that proximal TCR signaling and downstream signaling cascades are impaired in Treg. Thus, the aforementioned increase of ERK1/2 phosphorylation in Treg in Fig. 5A is likely induced by cytokines or other growth factors existing in serum-containing medium.

Akt phosphorylation at S473 and Foxo1a phosphorylation were diminished in Treg before and after TCR stimulation (Fig. 6B), suggesting that TCR-induced mTORC2 activation is impaired in Treg. Whereas S6K1 phosphorylation was decreased before and after TCR stimulation in Treg, 4E-BP1 phosphorylation in Treg was higher than that in Tcon before and after TCR stimulation, suggesting again that Treg are distinctive from other cells in mTORC1 signaling.

### Promoting Treg proliferation by IL-2

IL-2 plays important roles for Treg homeostasis. Deficiency of IL-2 causes decreased Treg numbers in mice, which can lead to autoimmune diseases (25–27). In contrast, injection of IL-2 plus an anti–IL-2 Ab increases Treg numbers in mice, which can promote immune suppression (28). We tested how IL-2 may affect the hyperproliferative and death-prone properties of Treg. IL-2 can significantly increase proliferation of Tcon (Fig. 6A, 6C). IL-2 showed different effects on Tcon survival dependent on the strengths of TCR stimulation. Without TCR stimulation, IL-2 promotes Tcon survival. When TCR stimulation was weak, IL-2 drastically promoted Tcon proliferation as well as death. When TCR stimulation was strong, IL-2 could inhibit Tcon death (Fig. 6A, 6B). IL-2 also enhanced Treg proliferation. However, the effect of IL-2 to promote Treg survival was not obvious and could only be observed when stimulated with a high concentration (1 μg/ml) of anti-CD3.

### Distinguishing hyperproliferative and death-prone Treg subsets by ICOS expression

An important issue that has arisen from our aforementioned data are whether the hyperproliferative Treg and death-prone Treg represent different subsets of Treg. To address this question, it is critical to identify markers that can be used to define Treg with these distinct properties. It has recently been reported that human Treg can be divided into ICOS+ and ICOS− subsets and that the suppressive activity of the ICOS+ Treg appears to be superior to that of the ICOS− Treg (29–31). ICOS has also been found to be able to promote NKT cell survival (32). We examined the death and proliferative properties of ICOS+ and ICOS− Treg. Freshly isolated ICOS+ Treg displayed higher death rates than those of ICOS− Treg (Fig. 7A). Moreover, ICOS+ Treg stained more intensively for Ki67 than that for ICOS− Treg, suggesting that more
ICOS+ Treg and Tcon than ICOS− Treg and Tcon are cycling in vivo (Fig. 7B). After in vitro TCR stimulation, the majority of ICOS− Treg died and did not proliferate. However, most ICOS+ Treg were alive and proliferated (Fig. 7C). ICOS expression on Treg may alleviate the requirement of CD28 for TCR-induced proliferation. ICOS+ Treg expressed a higher level of the proapoptotic molecule Bad than that of ICOS− Treg, although they express similar levels of Bax (Fig. 7D). Further studies should determine whether differential Bad expression controls survival of these two Treg subsets. Using a contact inhibition assay, we further compared the suppressive activities between these two subsets of Treg. As shown in Fig. 7E, ICOS+ Treg displayed much stronger suppressive activity than that of ICOS− Treg. The inhibitory effect of ICOS+ Treg at 16:1 Tcon to Treg ratio was similar to that of ICOS− Treg at 1:1 Tcon to Treg ratio. Together, these data indicate that ICOS expression defines two subsets of Treg with distinct survival and proliferating properties as well as obvious differences of suppressive activities.

Discussion
In this report, we simultaneously analyzed Treg proliferation and death using fixable Live/Dead staining and the CFSE dilution assay. We demonstrated that Treg contain two subsets with distinct death and proliferation properties. The predominant subset is ICOS− and is prone to death, defective in proliferation, and weak in suppression. The rare subset is ICOS+, which is characterized by superior survival, hyperproliferative, and highly suppressive properties. The massive death of ICOS− Treg after in vitro TCR stimulation may be due to the activation of the intrinsic death pathway and contribute to the hypoproliferative phenotype of Treg during in vitro TCR stimulation. Many studies on Treg proliferation and survival have been performed with CD4+CD25+ cells. Because most Treg die in vitro, our data suggest that it is critical to use Foxp3 rather than CD25 whenever possible as the marker for Treg isolation when studying Treg, particularly when examining their proliferative responses and death. A small contamination of Tcon in the CD4+CD25+ population could well outnumber the rare proliferative ICOS+ Treg and thus significantly impact data interpretation.

ICOS+ and ICOS− Treg have been noted for their difference in several aspects. Human ICOS+ Treg use IL-10 to suppress dendritic cells and TGF-β to suppress T cells, whereas ICOS− Treg only use TGF-β for suppression (30). Human ICOS+ Treg have also been found to contain stronger suppressive activity than that of ICOS− Treg (29, 31, 33). Our data showing that murine ICOS+ Treg are much more suppressive than ICOS− Treg are consistent with the data of human Treg. Of note, human ICOS+ Treg were found to be more apoptotic than ICOS− Treg (30). At present, the reason for the difference in survival between human and murine ICOS+ Treg is unclear. Murine ICOS− Treg become apoptotic within a few hours after in vitro TCR stimulation (data not shown). Because the human Treg were purified based on CD25 expression, it would be of interest to determine whether these CD25+ and ICOS+ Treg contain activated conventional T cells, which may have a high death rate. ICOS appears to be unimportant for Treg generation in the thymus but is involved in Treg homeostasis. Deficiency of either ICOS or its ligand results in decreases of Treg numbers in the peripheral lymphoid organs (31, 34). In addition, expression of ICOS ligand by tumor cells promotes Treg expansion, which may contribute to tumor immune suppression (35). Together, these observations and our data suggest that ICOS may directly promote Treg survival. The importance of ICOS to maintain Treg pool size and survival may help to explain the recent finding that inhibition of ICOS signal in mice suggests Treg expansion, which may contribute to tumor immune suppression (34). In addition, expression of ICOS ligand by tumor cells promote Treg expansion, which may contribute to tumor immune suppression (35). Together, these observations and our data suggest that ICOS may directly promote Treg survival. The importance of ICOS to maintain Treg pool size and survival may help to explain the recent finding that inhibition of ICOS signal in mice may reduce Treg number, promote autoimmunity, and exacerbate airway hyperresponsiveness (36–39).

Human CD4+ Treg can be divided into resting and activating/memory subsets. It has been reported that the human CD4+ CD45RO−Foxp3+CD25hi Treg are highly proliferative but susceptible to apoptosis (40). In another report, human CD4+ Treg were divided into CD45RA−Foxp3low resting, CD45RA+Foxp3hi activated, and CD45RA− Foxp3low cytokine-secreting subpopulations. Both CD45RA−Foxp3low and CD45RA+Foxp3hi Treg...
are suppressive, whereas CD45RA⁺Foxp³low Treg are nonsuppressive. In addition, CD45RA⁺Foxp³low Treg appear to be the precursors of CD45RA⁺Foxp³hi Treg, which are terminally differentiated and death prone (41). The CD45RA⁺Foxp³low Treg are more proliferative and survive better than the CD45RA⁺Foxp³hi Treg after TCR stimulation, which mimics the murine ICOS⁺ and ICOS⁻ Treg to certain extents. However, the differences between these human Treg subsets defined by Foxp3 levels and CD45RA expression are not as drastic as those between the murine Treg subsets defined by ICOS expression.

Our study has revealed common and different signaling features between freshly isolated Treg in FBS-containing medium and in PBS with or without TCR stimulation. Treg in FBS-containing medium (Treg-FBS) display increased phosphorylation of ERK1/2 and Rsk1, substrate for ERK1/2, but decreased phosphorylation of PLCγ1 compared with those of Tcon. However, in Treg rested in PBS (Treg-PBS), ERK1/2 and Rsk1 phosphorylation is drastically decreased and is much lower than that in Tcon in PBS. The same is true for Treg-PBS stimulated with anti-CD3. These observations suggest that Treg may be hyperresponsive to growth factors or cytokines in the serum-containing medium such as recently reported with leptin (42), and such signaling is independent of PLCγ1 activation. Additionally, distal signaling events that lead to ERK1/2 activation should not be defective in Treg as ERK1/2 phosphorylation occurs at a high level in Treg-FBS. Although Treg are hyperresponsive to yet to be identified stimuli in the serum-containing medium, they are defective in TCR signaling. The defects of TCR signaling in Treg are likely proximal to PLCγ1, as its phosphorylation is significantly decreased. Diminished IκBα and NF-κB phosphorylation as well as ERK1/2 phosphorylation is likely caused by diminished PLCγ1-derived diacylglycerol, which is the upstream activator for both the NF-κB pathway and the Ras-ERK1/2 pathway (43).

Multiple reports have demonstrated that PI3K and mTOR signaling inhibits Treg differentiation and/or function and that Treg display decreased mTOR and Akt activities (42, 44–49). Our

**FIGURE 6.** Effects of IL-2 on Treg proliferation and survival. Experiments were performed and analyzed similarly to those of Fig. 1 except that IL-2 was added at a concentration of 1000 U/ml in the test group. A. CFSE and Live/Dead staining on gated total Tcon and Treg. B. Death rates of Tcon and Treg. C. Overlay of CFSE staining in live Tcon and Treg. Data shown are representative of three experiments.
studies are consistent with the notion that mTOR signaling is differentially regulated in Treg and Tcon. mTORC2 activation is decreased in both Treg-FBS and Treg-PBS with or without TCR stimulation, indicating that Treg are defective in mTORC2–Akt signaling. At present, it is unclear how much the decreased Akt activity in Treg may contribute to Treg death. In both Treg-FBS and Treg-PBS with or without TCR stimulation, S6K1 phosphorylation is decreased but 4E-BP1 phosphorylation is increased compared with those in Tcon. The dissociation of S6K1 and 4E-BP1 phosphorylation is quite striking and intriguing, as phosphorylation of these two proteins is usually mediated by mTORC1 and occurs concordantly in other cell types (23). Such dissociation could be caused by altered composition of mTORC1 leading to change of substrate preference and differential expression of phosphatases for S6K1 and 4E-BP1 in Treg.

FIGURE 7. Differential expression of ICOS defines proliferative and death-prone Treg subsets. A, Assessment of Treg death and survival of freshly isolated Treg based on ICOS expression. Top panels, CD4 and Foxp3 staining and ICOS expression on CD4+Foxp3+ Treg. Bottom panels, Live/Dead staining of ICOS+ and ICOS− Treg. B, Ki67 staining of ICOS+ and ICOS− Treg and Tcon. C, CFSE-labeled splenocytes were stimulated with an anti-CD3 Ab (1 μg/ml; 2C11) for 24 or 48 h. Treg proliferation and survival were examined as for Fig. 1 except that Treg were gated for ICOS+ and ICOS− subsets. Histogram shows overlay of CFSE staining of ICOS+ and ICOS− Treg after 48 h TCR stimulation. D, Increased Bad expression in ICOS− Treg. Bad and Bax mRNA levels were determined by real-time quantitative PCR with cDNA synthesized from total RNA isolated from sorted ICOS+CD4+Foxp3GFP+ and ICOS−CD4+Foxp3GFP− Treg as templates. E, Assessment of suppressive function of ICOS+ and ICOS− Treg using the contact inhibition assay. CFSE-labeled Thy1.1+Thy1.2+ Tcon were mixed with Thy1.2+ ICOS+ or ICOS− Treg at the indicated ratios and mitomycin C-treated TCRβ−/−δ−/− splenocytes. Cells were left unstimulated or stimulated with an anti-CD3 Ab for 72 h and then stained for Thy1.1, Thy1.2, and CD4. Top panels show CFSE intensity in gated Thy1.2+ Tcon from unstimulated or anti-CD3–stimulated cultures. Bottom panels are overlaid histograms of CFSE intensity in gated Thy1.1+Thy1.2+ Tcon from anti-CD3–stimulated cultures in the presence of either ICOS− Treg or ICOS+ Treg. At the 1:1 Tcon to Treg ratio, only ICOS− Treg were tested.
In summary, we have demonstrated that Treg display both hyperproliferative and death-prone properties and that ICOS can define Treg into two subsets based on these properties. The ICOS\(^+\) Treg are rare but are superior in survival, are hyperproliferative in response to TCR stimulation, and contain strong suppressive activity. Our data suggest modulating Treg death prone, exhibit defective proliferation capabilities, and contain weak suppressive activity. We data suggest modulating Treg death prone, exhibit defective proliferation capabilities, and contain weak suppressive activity. Our data suggest modulating Treg death prone, exhibit defective proliferation capabilities, and contain weak suppressive activity. Our data suggest modulating Treg death prone, exhibit defective proliferation capabilities, and contain weak suppressive activity. Our data suggest modulating Treg death prone, exhibit defective proliferation capabilities, and contain weak suppressive activity. 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