In vivo expansion of T reg cells with IL-2–mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression

Kylie E. Webster,[1] Stacey Walters,[1] Rachel E. Kohler,[1] Tomas Mrkvan,[1] Onur Boyman,[2] Charles D. Surh,[3] Shane T. Grey,[1] and Jonathan Sprent[1]

[1]Immunology and Inflammation Division, Garvan Institute of Medical Research, Darlinghurst, NSW 2010, Australia
[2]Division of Immunology and Allergy, University Hospital of Lausanne, 1011 Lausanne, Switzerland
[3]The Scripps Research Institute, La Jolla, CA 92037

Via a transcription factor, Foxp3, immunoregulatory CD4$^+$CD25$^+$ T cells (T reg cells) play an important role in suppressing the function of other T cells. Adoptively transferring high numbers of T reg cells can reduce the intensity of the immune response, thereby providing an attractive prospect for inducing tolerance. Extending our previous findings, we describe an in vivo approach for inducing rapid expansion of T reg cells by injecting mice with interleukin (IL)-2 mixed with a particular IL-2 monoclonal antibody (mAb). Injection of these IL-2–IL-2 mAb complexes for a short period of 3 d induces a marked (>10-fold) increase in T reg cell numbers in many organs, including the liver and gut as well as the spleen and lymph nodes, and a modest increase in the thymus. The expanded T reg cells survive for 1–2 wk and are highly activated and display superior suppressive function. Pretreating with the IL-2–IL-2 mAb complexes renders the mice resistant to induction of experimental autoimmune encephalomyelitis; combined with rapamycin, the complexes can also be used to treat ongoing disease. In addition, pretreating mice with the complexes induces tolerance to fully major histocompatibility complex–incompatible pancreatic islets in the absence of immunosuppression. Tolerance is robust and the majority of grafts are accepted indefinitely. The approach described for T reg cell expansion has clinical potential for treating autoimmune disease and promoting organ transplantation.

CORRESPONDENCE
Jonathan Sprent:
j.sprent@garvan.org.au

IL-2 is a growth factor for T cells and drives these cells to proliferate and differentiate into effector cells. IL-2 predominantly activates cells expressing high-affinity receptors composed of three chains (IL-2Rα [CD25], IL-2Rβ [CD122], and γc [CD132]), such as activated CD4$^+$ and CD8$^+$ T cells, but can also activate cells with low-affinity βγ IL-2Rs, such as memory-phenotype (MP) CD8$^+$ cells and NK cells (1–3). In the case of CD4$^+$ cells, αβγ IL-2Rs are constitutively expressed by T regulatory cells (T reg cells), which, through expression of the transcription factor Foxp3, inhibit the function of other cells (4, 5). T reg cells are crucially dependent on IL-2 for their growth and survival (6, 7), and can be eliminated by the injection of neutralizing anti–IL-2 mAb (8, 9).

Selective enrichment of T reg cells has the potential to treat autoimmune disease and impair transplant rejection, and there is considerable interest in the idea of injecting T reg cells after previous expansion in vitro (10–12). An alternative approach would be to expand T reg cells in vivo. We have recently devised a method for inducing selective expansion of T reg cells under in vivo conditions in mice (13). This technique stemmed from the finding that the biological activity of IL-2 in vivo could be greatly enhanced by association with anti–IL-2 mAbs. For most IL-2 mAbs tested, injecting IL-2–mAb complexes led to marked and selective proliferation of MP CD8$^+$ cells and NK cells, i.e., cells expressing low-affinity βγ IL-2Rs. However, with one particular IL-2 mAb, JES6-1, injection of

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 206 No. 4 751-760
www.jem.org/cgi/doi/10.1084/jem.20082824

IL-2–mAb complexes caused selective expansion of T reg cells with little or no change in other cells. Recently, this approach was used successfully to treat asthma in a mouse model (14). In this report, we have defined the features of T reg cells expanded by IL-2–JES6-1 injections and present evidence that mice pretreated with these complexes are resistant to the induction of experimental autoimmune encephalomyelitis (EAE) and show long-term acceptance of MHC-incompatible pancreatic islet allografts.

RESULTS AND DISCUSSION

Features of T reg cells expanded by IL-2–JES6-1 injection

Previous evidence on T reg cell expansion after IL-2–JES6-1 injection was limited to the finding that daily i.p. injections of these complexes for 1 wk led to a mild (threefold) increase in the proportion of $CD4^+CD25^+Foxp3^+$ cells in the spleen (13). For these studies, a fourfold molar excess of mAb was used, i.e., 1.5 μg (87 pmol) IL-2 and 50 μg (330 pmol) mAb. To optimize the yield of T reg cells, we tested the effects of injecting different proportions of IL-2 and JES6-1 mAb. With three daily injections (days 0, 1, and 2) of IL-2 (1 μg/58 pmol) mixed with titrated concentrations of mAb, maximal T reg cell expansion in the spleen 1 d later (day 3) was observed with around 5 μg (33 pmol) mAb per injection, which was equivalent to an ∼1:2 molar ratio of mAb/IL-2, with neither reagent in excess (Fig. 1 A). With this ratio, the proportion of $CD4^+$ cells with a $CD25^+Foxp3^+$ phenotype rose to 50–60% compared with the baseline level of 5–10% in control mice. Increasing the total dose of mAb and IL-2 at this fixed 1:2 molar ratio resulted in a maximum T reg cell yield of 80% of the $CD4^+$ population (Fig. 1 B). A total dose of 6 μg per injection (5 μg mAb + 1 μg IL-2) gave near optimal T reg cell expansion and was chosen for all subsequent experiments.

At the optimal 1:2 ratio of JES6-1 mAb/IL-2, three daily injections of 6 μg led to a marked 10–20-fold increase in total numbers of T reg cells in the spleen on day 5. Injection of IL-2 alone caused only a very mild (50%) increase in T reg cells, which was not enhanced by coinjection with an isotype control (IC) mAb (Fig. 1 C and Fig. S1). When JES6-1 mAb was added in molar excess, T reg cell expansion by IL-2 was less marked (Fig. 1 A), a finding in concordance with in vitro data on the mild neutralizing effect of JES6-1 mAb on IL-2 presentation to high-affinity IL-2Rs (13). Similarly, injection of JES6-1 mAb alone caused a twofold reduction in the number of T reg cells, presumably reflecting neutralization of low levels of endogenous IL-2 (Fig. 1 C and Fig. S1).

Interestingly, the substantial increase in T reg cells observed after injection of IL-2–JES6-1 mAb was widespread, being also observed in the mesenteric LNs, liver, and BM, as well as Peyer's patches (PP) and the lamina propria (LP) of the gut; T reg cell expansion was especially prominent in the liver and LP (Fig. 1 D). There was also a moderate (threefold) increase in T reg cells in the thymus (Fig. 1 E). A comparable increase in $CD4^+CD8^+$ double-positive $Foxp3^+$ precursors implies that, at least in part, the expansion of thymic single-positive $CD4^+Foxp3^+$ cells occurred in situ. For T reg cells in the periphery, adoptive transfer of CFSE-labeled cells followed by injection of IL-2–JES6-1 mAb showed that the increase in numbers of T reg cells reflected substantial division in extrathymic sites rather than increased release of these cells from the thymus (Fig. 1 F). Proliferation was minimal with the transfer of CFSE-labeled resting $CD4^+CD25^-$ cells, indicating that the increase in T reg cell numbers did not reflect conversion from other $CD4^+$ cells.

Kinetics studies showed that T reg cell expansion after the three injections was rapid but quite brief, reaching a peak on day 5 and then declining rapidly to approach background levels by approximately day 15. These findings refer to cells in the spleen and apply to both the percentage of T reg cells (Fig. 1 G, left) and the total numbers of these cells (Fig. 1 G, right). Disappearance of most of the expanded cells by week 2 also applied to the liver, gut, and other tissues (unpublished data). T reg cell expansion was blocked by coinjection of CD25 mAb (PC61), indicating stimulation via high-affinity αβγ IL-2Rs (Fig. 1 G, left). In contrast, expansion of cells expressing the weaker βγ IL-2Rs, namely MP $CD8^+$ cells and NK cells, was minimal (Fig. 1 H).

With regard to surface phenotype, IL-2–JES6-1 mAb–expanded T reg cells in the spleen tested at 1 d after the injections (day 3) showed a considerable increase in the expression of molecules crucial to suppression, such as CD25, glucocorticoid-induced TNFR (GITR), cytotoxic T lymphocyte antigen 4 (CTLA-4), and inducible T cell co-stimulator (ICOS), and smaller increases in several other markers relative to T reg cells from control mice (Fig. 2 A); in contrast, injection of IL-2 alone had little or no effect on T reg cell phenotype. This highly activated state was brief, as by day 7 the majority of these molecules had returned to near-normal levels of expression (unpublished data). When their functional activity was tested by restimulation in vitro with CD3 mAb and IL-2, the expanded T reg cells showed enhanced synthesis of IL-10 mRNA but little change in TGF-β (Fig. 2 B). The activated phenotype of the IL-2–JES6-1 mAb–expanded T reg cells, in addition to the known IL-10–dependent inhibitory function of $Foxp3^+$ cells (15), was indicative of a possible superior suppressive function. This was indeed the case. Thus, IL-2–JES6-1 mAb–expanded T reg cells were more effective than normal or IL-2–stimulated T reg cells not only at inhibiting proliferation of normal $CD4^+$ cells in response to CD3 ligation in vitro (Fig. 2 C), but also in blocking homeostatic proliferation of naive $CD4^+$ cells in immunodeficient $RAG^{-/-}$ hosts (Fig. 2 D). Nevertheless, the superior suppressive effect of the expanded T reg cells was transient and had largely disappeared by day 7 (5 d after the last of the three injections).

Expansion of T reg cells impairs EAE induction

In view of evidence that T reg cells can impair immune responses of T cells in vivo (16, 17), we tested whether the expansion of T reg cells induced by IL-2–JES6-1 mAb injection could be used to inhibit the induction of T cell–mediated autoimmune disease. On this point, a recent study showed that continuous treatment of female nonobese diabetic mice

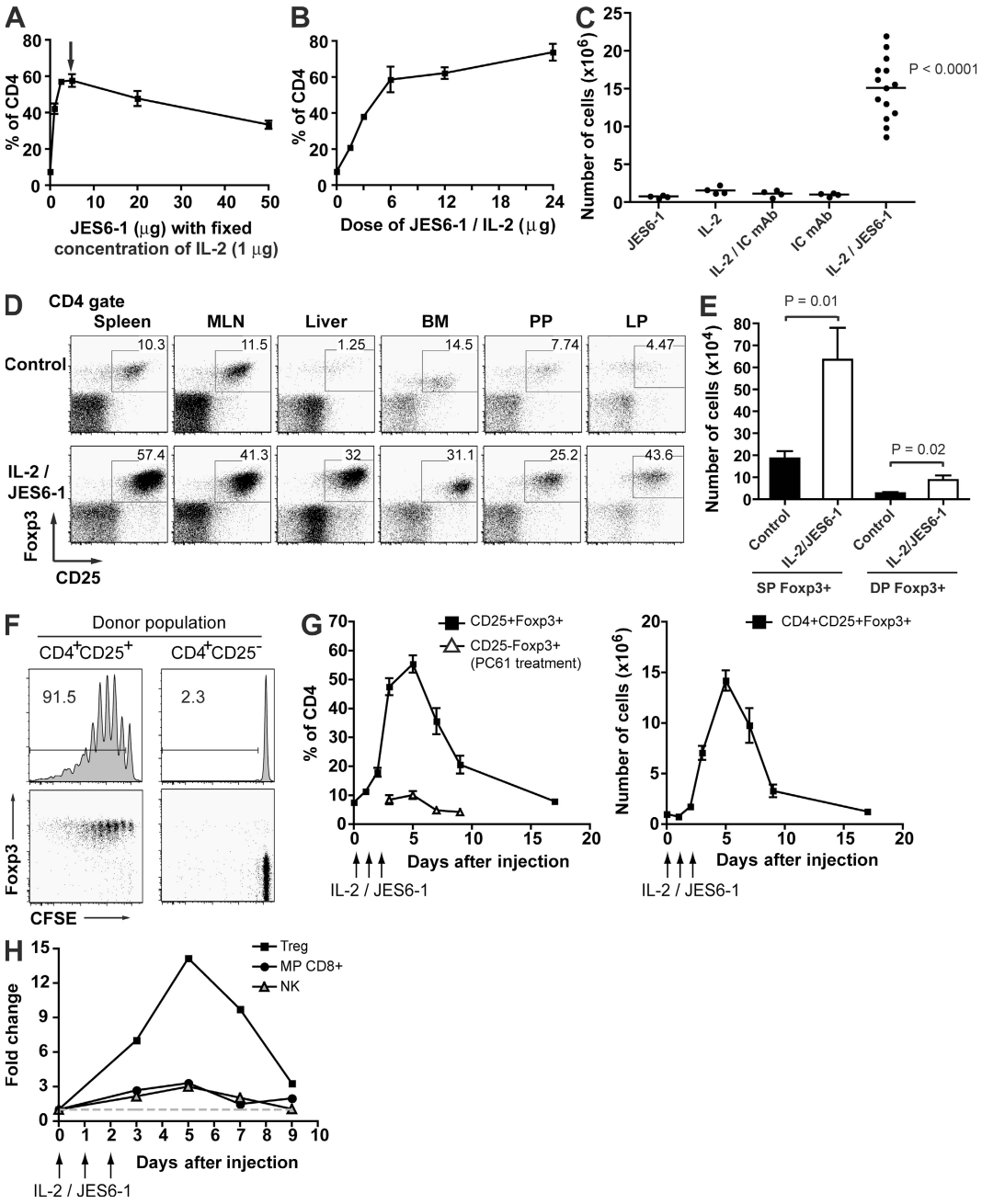

Figure 1. Rapid and widespread proliferation of T reg cells after IL-2–JES6-1 treatment. Mice were injected i.p. daily for 3 d (days 0, 1, and 2) with JES6-1 mAb and/or IL-2 and analyzed on day 3 (A and B), day 5 (C–E), day 6 (F), or where indicated (G and H), usually in the spleen. Doses were 1 μg IL-2 and 5 μg mAb unless otherwise specified. (A) The effect of varying the ratio of IL-2 and JES6-1 on $CD4^+CD25^+Foxp3^+$ (T reg cell) expansion was analyzed by injecting titrated doses (1–50 μg) of JES6-1 mAb, plus a fixed concentration (1 μg) of rmIL-2. The arrow indicates a 1:2 molar ratio of JES6-1 mAb to IL-2. (B) The effect of titrating the dose of IL-2 plus JES6-1 mAb (molar ratio constant at 2:1) was analyzed by varying the total dose from 0–24 μg per day. (C) The total numbers of T reg cells in the spleen were enumerated after treatment with JES6-1, IC mAb (rat IgG2a), IL-2, IL-2–IC mAb, or IL-2–JES6-1 mAb. Horizontal bars represent means. (D) Expansion of T reg cells in the spleen, MLN, liver, BM, PP, and small intestinal LP after injection of IL-2–JES6-1. (E) Number of $Foxp3^+$ cells in the $CD4^+$ single-positive and $CD4^+CD8^+$ double-positive thymocyte populations after IL-2–JES6-1 treatment. (F) Proliferation, as measured by CFSE dilution, of adoptively transferred $CD4^+CD25^+$ or $CD4^+CD25^-$ Ly5.1$^+$ T cells in LNs of mice injected with IL-2–JES6-1. Percentages of donor cells divided (as determined by CFSE incorporation) are shown. (G) Kinetics of T reg cell expansion in the spleen after three injections of IL-2–JES6-1. Percentages (left) and total cell numbers (right) are shown. The percentage of $Foxp3^+$ cells after 200 μg PC61 i.p. (day −1) followed by three injections of IL-2–JES6-1 is shown (arrows). (H) Expansion of cells with the βγ IL-2R, MP $CD8^+$ T cells and NK cells, compared with T reg cells (αβγ IL-2R) after IL-2–JES6-1 treatment. Data are shown as a fold change for each cell type compared with untreated mice over 9 d (the dashed line indicates a fold change of 1). Data in A, B, D, and G (left) are shown as percentages of $CD4^+$ T cells. Data are representative of two to five independent experiments. Data are means ± SEM. Flow cytometry plots depict $\log_{10}$ fluorescence.

with IL-2–JES6-1 for 10 wk prevented the onset of diabetes (18). We examined whether short-term (3-d) treatment of C57BL/6 mice with IL-2–JES6-1 mAb could impair induction of EAE, a model for multiple sclerosis (MS). EAE was induced by injection of an immunogenic peptide of myelin oligodendrocyte glycoprotein (MOG_{35-55}) in CFA (19). As expected, control C57BL/6 mice all developed severe disease by 14 d after MOG/CFA injection and had to be culled within 3 wk (Fig. 3 A). Significantly, pretreating the mice for 3 d with IL-2–JES6-1 mAb led only to mild neurological symptoms of EAE. No protection against EAE induction occurred when mice were pretreated with IL-2 alone.

The described experiment indicated that expanding T reg cells in mice led to strong resistance to subsequent induction of EAE. However, the clinical relevance of these findings is questionable because MS patients have an ongoing immune response. Hence, the effect of therapeutic administration of IL-2–JES6-1 mAb complexes on EAE progression was examined. When mice were immunized with MOG peptide

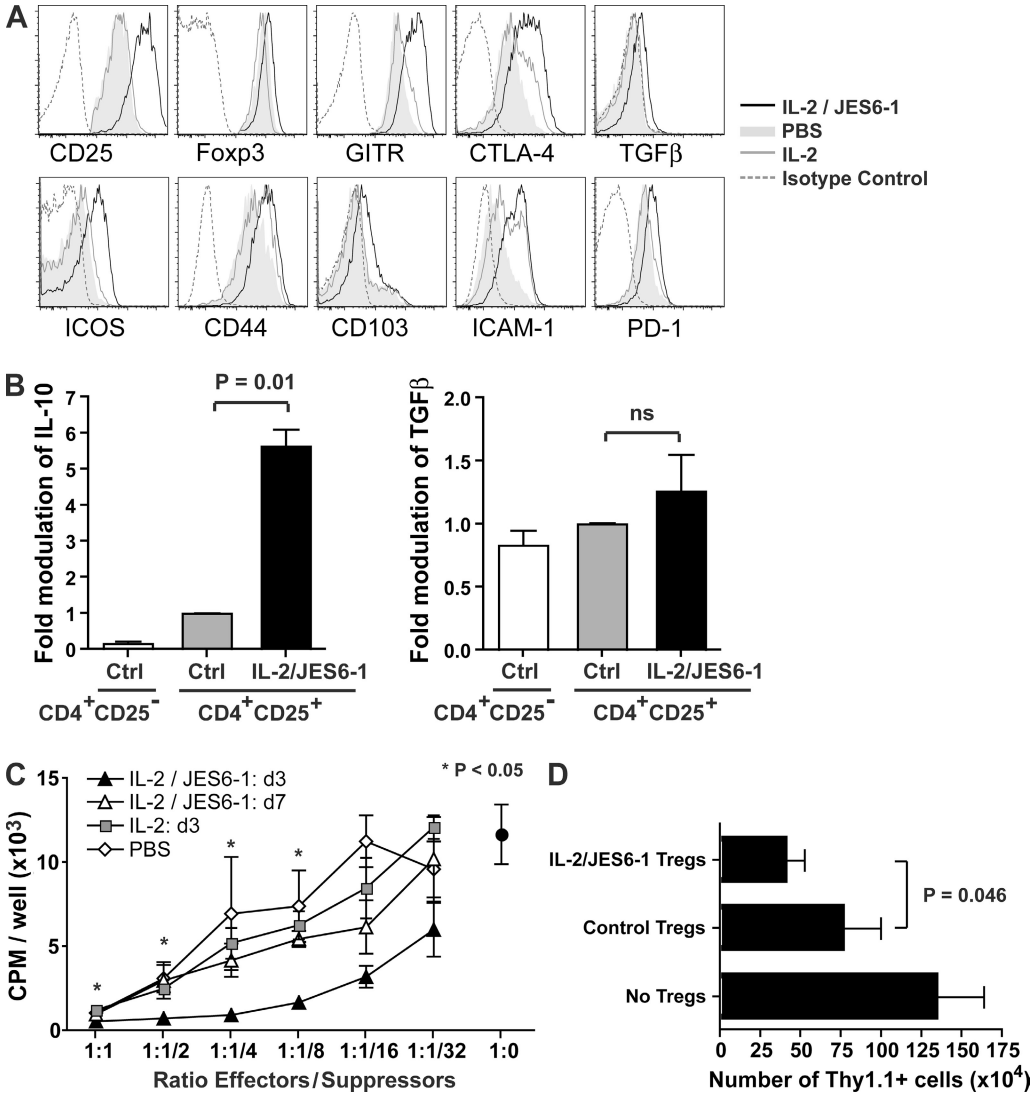

Figure 2. Phenotype of IL-2–JES6-1–expanded T reg cells. Mice were treated on days 0, 1, and 2 with 1 μg/5 μg IL-2–JES6-1, 1 μg IL-2, or PBS, and the phenotype of the expanded T reg cells was analyzed on day 3 or 7, as described. (A) Splenic $CD4^+CD25^+Foxp3^+$ T reg cells were analyzed on day 3 by flow cytometry for expression of cell-surface molecules, CD25, GITR, TGF-β, ICOS, CD44, CD103, ICAM-1, and PD-1, and intracellular molecules, Foxp3, and CTLA-4. (B) Quantification of IL-10 and TGF-β mRNA after in vitro restimulation of FACS-sorted $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells isolated from mice on day 3 after treatment (reference gene, 18s RNA). (C) In vitro suppression of $CD4^+CD25^-$ T cells (effectors) by FACS-sorted $CD4^+CD25^+$ T reg cells (suppressors; in indicated ratios) isolated from mice on day 3 or 7 after treatment. Proliferation of effectors was measured by incorporation of $[^3H]$thymidine. (D) In vivo suppression of homeostatic proliferation of $CD4^+CD25^-$ $Thy1.1^+$ T cells adoptively transferred into $RAG^{-/-}$ hosts in a 1:1 ratio with $CD4^+CD25^+$ T reg cells isolated from C57BL/6 mice on day 3 after treatment. $Thy1.1^+$ cell numbers were determined on day 7 after transfer. Data are representative of two to three independent experiments. Data are means ± SEM. Flow cytometry plots depict $\log_{10}$ fluorescence.

and treated with IL-2–JES6-1 mAb for 3 d after disease onset (days 7–9), there was no protection and the mice all developed severe disease (Fig. 3 B).

This finding was not unexpected because, like T reg cells, activated effector T cells express high-affinity IL-2Rs. Hence, injection of IL-2–JES6-1 mAb after EAE induction presumably

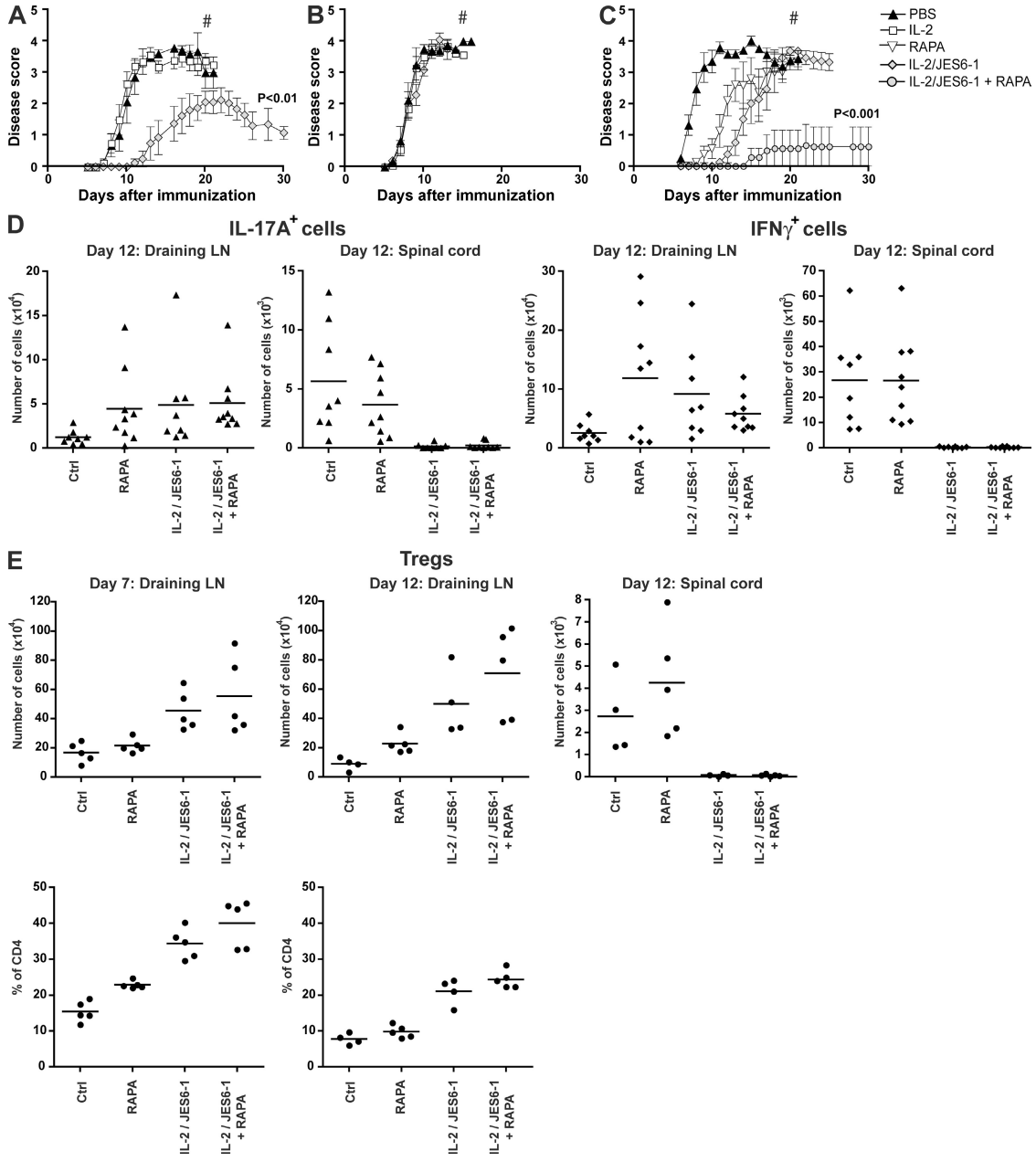

Figure 3. Impaired induction of EAE in mice treated with IL-2–JES6-1. EAE was induced on day 0 by immunization of MOG_{35-55} in CFA. Mice were treated with combinations of 1 µg IL-2, 5 µg JES6-1, and 1 mg/kg rapamycin, as described (n = 8–10 per group). (A) Disease score of mice treated on days −3, −2, and −1 with PBS, IL-2, or IL-2–JES6-1. (B) Disease score of mice treated with PBS, IL-2, or IL-2–JES6-1 at the onset of symptoms (typically days 7, 8, and 9). (C) Disease score of mice treated with PBS, IL-2–JES6-1 (days 2, 3, and 4), rapamycin (days 2, 3, and 4), or IL-2–JES6-1 (days 2, 3, and 4) plus rapamycin (days 2, 3, and 4). The treatment groups in C were further analyzed in D and E. (D) The number of $CD4^+IL\text{-}17^+$ (triangles) and $CD4^+IFN\text{-}\gamma^+$ (diamonds) in the draining LNs and spinal cords of mice at day 12 after MOG immunization. For the draining LNs, the percentage of cytokine-producing $CD4^+$ cells for the four groups ranged from a mean of 1.5–2% for $IL\text{-}17A^+$ cells and 2.5–3% for $IFN\text{-}\gamma^+$ cells; for the spinal cord, the mean percentage of positive cells in the control group was 11% for $IL\text{-}17A^+$ cells and 43% for $IFN\text{-}\gamma^+$ cells; and in the rapamycin group the mean was 5% for $IL\text{-}17A^+$ cells and 30% for $IFN\text{-}\gamma^+$ cells (n = 8–9 per group). (E) T reg cells quantified as cell numbers (top) and percentages (bottom) of $CD4^+$ T cells in the draining LNs and spinal cords of mice at days 7 and 12 after MOG immunization (n = 5 per group). Data are representative of two to three independent experiments. Data are means ± SEM (A–C). Symbols represent individual mice and horizontal bars represent means in D and E. #, mice euthanized for ethical reasons.

led to the expansion of effector T cells as well as T reg cells, thereby neutralizing the protective effects of the latter. To try to circumvent this problem, we studied the effects of coinjecting rapamycin, which is reported to inhibit proliferation of T effector cells while allowing, or even enhancing, the proliferation of T reg cells (20). Thus, after EAE induction, mice were treated with IL-2–JES6-1 mAb, rapamycin, or both for 3 d, starting on day 2 after priming, i.e., during the early stages of the immune response. Treatment at this time point was chosen in an effort to more closely resemble a therapeutic intervention during remission rather than active disease of MS patients. With this regimen, injecting either rapamycin or IL-2–JES6-1 mAb alone delayed the onset of clinical symptoms, but all of the mice eventually developed severe disease (Fig. 3 C). In contrast, the combined treatment of rapamycin and IL-2–JES6-1 mAb resulted in a marked reduction in disease severity, indicating a strong therapeutic effect.

To investigate the mechanism of suppression, we examined T cell subsets in the spleen, draining LNs and spinal cord. Because EAE is mediated in part by local release of IFN-γ and IL-17 in the central nervous system (CNS) (21, 22), cells synthesizing these cytokines were quantitated after restimulation in vitro. When $CD4^+$ cells were examined on day 7 after immunization, significant numbers of IFN-γ^+ and IL-17^+ cells were found in the draining LNs and spleen (unpublished data), and were most prominent in the group treated with IL-2–JES6-1 mAb alone, presumably reflecting IL-2 stimulation via their high-affinity IL-2Rs. At this early stage, neurological symptoms were not yet detectable and very few cells were found in the spinal cord. By day 12 after immunization, IFN-γ^+ and IL-17^+ cells were prominent in the spinal cord of the control mice and those given rapamycin alone (Fig. 3 D); the presence of these cells in the spinal cord correlated with the onset of symptoms and paralleled a reduction of cells in the draining LNs, especially in the control group. In comparison, there was no accumulation of effector cells in the spinal cord of mice treated with IL-2–JES6-1 mAb with or without rapamycin (Fig. 3 D), which correlated with the lack of symptoms in these groups. At day 15, however, the mice given IL-2–JES6-1 mAb without rapamycin developed clinical symptoms, and infiltration of effector cells in the spinal cord was observed (unpublished data). In contrast, combined treatment of IL-2–JES6-1 mAb plus rapamycin inhibited effector cell accumulation in the CNS and the mice remained symptom free.

With regard to the distribution of T reg cells, expanded numbers were found in the draining LNs and spleen of the groups that received IL-2–JES6-1 mAb, and were especially prominent in the symptom-free group given the combination of IL-2–JES6-1 mAb plus rapamycin (Fig. 3 E and not depicted); this finding applied both to total numbers of T reg cells (Fig. 3 E, top) and the percentage of T reg cells (Fig. 3 E, bottom). T reg cells entered the spinal cord of the mice that developed symptoms along with effector cells but, like the latter, were excluded from entering the spinal cord of the symptom-free mice (Fig. 3 E).

These findings on EAE induction suggest that short-term treatment with IL-2–JES6-1 mAb complexes plus rapamycin after MOG immunization is highly effective in blocking disease. Because T reg cells failed to enter the spinal cord of the protected group, the expanded T reg cells presumably acted peripherally, apparently by somehow impairing homing of effector cells to the CNS. The relevance of the data to the treatment of MS remains to be investigated.

Expansion of T reg cells prevents rejection of pancreatic islet allografts

The data on EAE induction indicate that, in the absence of immunosuppression, the inhibitory activity of T reg cells was most effective when these cells were expanded before, rather than after, activation of effector cells. It would therefore follow that previous expansion of T reg cells might be particularly effective in preventing rejection of organ allografts. In fact, there are several reports demonstrating that allograft rejection can be delayed by the infusion of in vitro–expanded T reg cell populations, both polyclonal and antigen specific (11, 12, 23), or in transgenic mice in which T reg cell numbers are constitutively elevated (24). In contrast, attempts at expanding T reg cells in vivo for transplantation tolerance have required either a lymphopenic environment for expansion (11) or combination with immunosuppression (25) for success. To examine this issue, we tested the effects of T reg cell expansion on the rejection of allogeneic pancreatic islets. In this case, C57BL/6 ($H2^b$) mice were first treated with streptozotocin to destroy pancreatic insulin-secreting β cells. Once diabetic (as indicated by elevated blood glucose levels; BGLs), the mice were treated for 3 d with IL-2–JES6-1 mAb complexes (days −3, −2, and −1) as in the above experiments. 1 d later (day 0), the mice were grafted under the kidney capsule with fully MHC-mismatched BALB/c ($H2^d$) islets by standard techniques (26).

As expected, blood glucose measurement showed that PBS-treated control mice all rejected their grafts rapidly with a mean survival time of 17 d ($n = 12$; Fig. 4 A). Pretreating the mice with IL-2 alone had no demonstrable effect on graft survival (mean survival time of 16.5 d; $n = 4$). In marked contrast, pretreatment with IL-2–JES6-1 mAb complexes led to prominent long-term graft survival. A small proportion of the mice rejected their grafts around days 20–25, but the remaining 82% ($n = 28$ out of 34) of the mice retained their grafts indefinitely (>100 d), until they were eventually used for other experiments (see below). In fact, for a total of 34 mice tested in nine separate experiments, not a single graft was rejected after day 25. Histology of long-term grafts revealed well-preserved islets with robust insulin production in situ and negligible leukocyte infiltration. Only minor, peri-islet mononuclear accumulations were observed (Fig. 4 B, arrow). To confirm that euglycemia and inferred long-term graft acceptance were indeed graft dependent, a nephrectomy was performed of the graft-bearing kidney after day 100. All mice ($n = 5$) rapidly reverted to hyperglycemia (Fig. S2), verifying their graft dependence for insulin production.

The mechanism of tolerance was investigated in vitro and in vivo. Mixed lymphocyte reactions revealed that T cells from long-term grafted mice retained their capacity to proliferate in response to BALB/c spleen cells (versus third party CBA [$H2^k$] spleen) in vitro (Fig. 4 C). More importantly, the transfer of spleen cells from these long-term grafted mice into immunodeficient B6.RAG$^{-/-}$ hosts bearing established BALB/c islets caused graft rejection in 78% of cases ($n = 9$; Fig. 4 D). Moreover, grafting three of the long-term grafted mice with BALB/c skin led to rapid rejection of these skin grafts (unpublished data). Collectively, these findings indicated that, despite long-term acceptance of the islet allografts in situ, host lymphoid cells showed little or no evidence of tolerance to BALB/c alloantigens. Also, injection of anti-CD25 mAb to deplete $CD25^+$ cells failed to cause graft rejection (Fig. S3). To test the possibility that long-term islet graft acceptance had led to a state of "ignorance" to the graft, some of the recipients were injected i.v. with a large dose of 2×10^7 T-depleted BALB/c spleen cells as a source of donor APCs. In this case, 9 out of 11 recipients retained their islet grafts (Fig. 4 E); for the 2 mice that did reject their grafts, the mice were tested at <100 d after grafting.

These findings indicated that long-term acceptance of islet allografts led to a robust form of operational tolerance in which the grafts were not rejected even after strong stimulation of

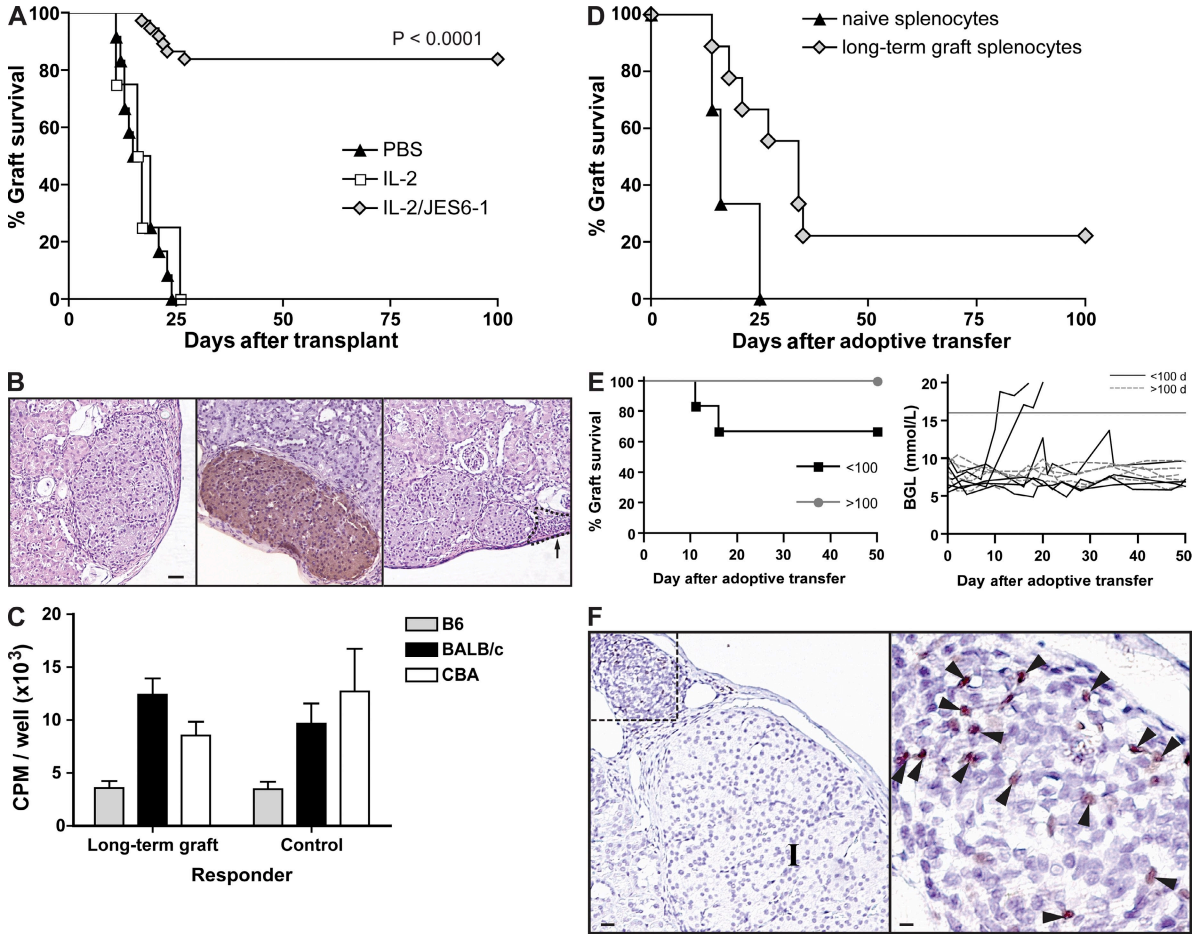

Figure 4. Long-term allograft survival in IL-2–JES6-1–treated mice. (A) Streptozotocin-induced diabetic C57BL/6 ($H2^b$) mice were treated with PBS ($n = 10$), 1 µg IL-2 ($n = 4$), or 1 µg/5 µg IL-2–JES6-1 ($n = 34$) on three consecutive days (days −3, −2, and −1). On day 0, mice were transplanted with BALB/c ($H2^d$) islets, and BGLs were monitored as a measure of graft function and survival. Grafts were considered rejected after two consecutive BGLs >16 mmol/liter after a period of normoglycemia. Cumulative data from nine independent experiments are shown. (B) Representative hematoxylin-eosin–stained islet graft under the kidney capsule (>100 POD; left), islet graft stained for insulin (middle), and graft with minor peri-islet mononuclear accumulation (arrow; right; $n = 5$). (C) Mixed lymphocyte reaction using T cells from long-term engrafted mice or control naive mice as responders, and syngeneic C57BL/6, donor BALB/c, or third party CBA/Ca-irradiated splenocytes as stimulators. The proliferation of responders was measured by incorporation of [^{3}H]thymidine. Data are means ± SEM. (D) Survival of BALB/c islet grafts in B6.RAG$^{-/-}$ hosts after adoptive transfer of 2×10^7 splenocytes i.v. from long-term engrafted mice ($n = 9$) or control naive C57BL/6 mice ($n = 3$). (E) Survival of BALB/c islet grafts in C57BL/6 hosts after challenge with BALB/c T-depleted splenocytes at either <100 d ($n = 6$) or >100 d ($n = 5$) after grafting. Data are shown as a Kaplan-Meier graft survival (left) or blood glucose measurement (the horizontal gray line indicates a BGL of 16 mmol/liter, the threshold for rejection; right). (F) Representative immunohistochemical staining for Foxp3$^+$ cells in peri-islet mononuclear accumulation (inset; $n = 5$). Foxp3$^+$ cells are denoted by horseradish peroxidase staining (arrowheads). I, islet graft. Data are representative of three independent experiments unless otherwise specified. Bars: (B) 100 µm; (F, left) 750 µm; (F, right) 20 µm.

nontolerant host T cells by APCs of donor graft origin. Neither clonal ignorance nor active suppression appear to be responsible for maintaining tolerance in this situation. In particular, as mentioned earlier, most T reg cells induced by the IL-2–JES6-1 mAb injections disappeared rapidly, which makes it unlikely that the grafts were maintained by local infiltration with T reg cells. This possibility cannot be excluded, however, because in long-term grafts, scattered T reg cells were detected in the small pockets of lymphoid cells observed adjacent to the grafts (Fig. 4 B, arrow; and Fig. 4 F). The role of these Foxp3$^+$ cells is difficult to determine in an islet model, although it has been shown in other transplantation settings, such as the skin, that T reg cells present at the tolerated tissue can prevent rejection (27).

Our data clearly illustrate the potential for using IL-2–JES6-1 complexes for selectively expanding T reg cells in autoimmune disease and organ transplantation. The heightened suppressive ability of these expanded cells coupled with the widespread upsurge in numbers provides a transient environment of superior suppression. The finding that short-term pretreatment with IL-2–JES6-1 mAb complexes led to the indefinite survival of pancreatic islets across the strong BALB/c→B6 MHC barrier in the absence of immunosuppression may have clinical implications for organ transplantation.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), BALB/c, CBA/Ca, and RAG$^{-/-}$ (B6 background) mice were obtained from the Animal Resources Centre and were used at the age of 6–12 wk. Animals were housed under conventional barrier protection and handled in accordance with protocols approved by the Garvan Institute of Medical Research and St. Vincent's Hospital Animal Experimentation and Ethics Committee, which comply with the Australian code of practice for the care and use of animals for scientific purposes.

Injection of IL-2–IL-2 mAb complexes. IL-2–anti–IL-2 mAb (JES6-1) complexes were prepared as previously described (13). Recombinant mouse IL-2 (PeproTech) was mixed with anti–IL-2 mAb (clone JES6-1; purified from hybridomas) and incubated at 37°C for 30 min. Mice were injected i.p. Concentrations and duration of injections are specified in Results.

Flow cytometry and antibodies. Cell suspensions of spleen, LNs, thymus, PP, and BM were prepared according to standard protocols. Lymphocytes from single-cell suspensions of perfused livers were separated by a 33% isotonic Percoll density gradient (GE Healthcare). To isolate LP lymphocytes, small intestine segments were treated with PBS plus 5% FCS, 1 mM dithiothreitol, 1 mM EDTA, 50 U/ml penicillin, and 50 μg/ml streptomycin to remove epithelial cells, after which they were digested in 0.5 mg/ml collagenase and 0.01% DNase (Roche) at 37°C; lymphocytes were isolated from the interphase of a 40%/70% Percoll density gradient. Cell suspensions were stained for FACS analysis using the following antibodies (obtained from eBioscience unless otherwise stated): PE-Cy5.5 anti-CD4 (RM4-5); allophycocyanin or biotin anti-CD25 (PC61.5); PE or allophycocyanin anti-Foxp3 (FJK-16s); PerCP-Cy5.5 NK1.1 (PK136; BD); FITC–anti-CD54 (YN1/1.7.4), –anti-CD44 (IM7), –anti-CD8 (53-6.7), and –anti–IL-17A (eBio17B7); PE–anti-CD152 (CTLA-4; UC10-4B9), –anti-PD1 (J43), –anti-ICOS (7E.17G9; BD), and –anti–IFN-γ (XMG1.2); biotin–anti-CD103 (2E7), –anti-GITR (DTA-1), and –anti-CD122 (TM-β1; BD), and biotin-conjugated chicken anti–TGF-β (R&D Systems); and FITC–rat IgG2aκ, FITC–rat IgG2bκ, PE–rat IgG2aκ, PE–rat IgG2bκ, biotin–rat IgG2a, PE–Ar Ham IgG1, biotin-conjugated chicken IgY (IgG; Jackson ImmunoResearch Laboratories), and Pacific blue streptavidin (Invitrogen). Foxp3 staining sets were purchased from eBioscience. Samples were analyzed on an LSRII or sorted using a FACSAria (BD). Data were analyzed using FloJo software (Tree Star, Inc.).

CFSE labeling. Lymphocytes were washed once in 0.1% BSA in PBS, and labeled with 1 μl of 5 mM CFSE (Invitrogen) at a density of 10^6 cells/ml 0.1% BSA in PBS for 10 min at 37°C in the dark. The reaction was stopped with RPMI 1640 with 10% FCS and washed twice. To examine the possibility of conversion, 5×10^6 CD4$^+$CD25$^-$ cells or 10^6 CD4$^+$CD25$^+$ cells were adoptively transferred to normal immunocompetent B6 hosts; these mice were treated with IL-2–JES6-1 on three consecutive days (1 μg/5 μg each) and examined for proliferation on day 6.

In vitro suppression assay. Cell-culture media was RPMI 1640 supplemented with 10% fetal calf serum, 10 mM Hepes, 1 mM Na pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 55 μm β-mercaptoethanol. CD4$^+$CD25$^-$ (responders) and CD4$^+$CD25$^+$ (suppressors) cells were purified from spleen and LN suspensions by cell sorting after first enriching for CD4$^+$ cells by complement-mediated killing using anti-HSA (J11d), anti-IAb (28.16.8S), and anti-CD8 (3.168) and guinea pig complement (Cedarlane Laboratories). T-depleted splenocytes were used as APCs and prepared by complement-mediated killing using anti-Thy1.2 (J1j), followed by irradiation (30 Gy). In suppression assays, responders (2×10^4 cells/well) were cultured in a U-bottom 96-well plate with 8×10^4 APCs and 2 μg/ml of soluble anti-CD3 antibody (clone 145-2C11). CD4$^+$CD25$^+$ T cells were added to the well in decreasing numbers (responder/suppressor ratios = 1:1, 1:1/2, 1:1/4, 1:1/8, 1:1/16, and 1:1/32). Cells were pulsed with 1 μCi [^{3}H]thymidine (GE Healthcare) per well for the last 8 h of the 72-h culture period. All data are shown as mean [^{3}H]thymidine incorporation in triplicate cultures.

Quantitative RT-PCR. RNA was isolated from FACS-sorted cells using the RNeasy kit (QIAGEN), checked for integrity on a 2100 Bioanalyzer (Agilent Technologies), and cDNA synthesized using Superscript III (Invitrogen) and random hexamers according to manufacturer's instructions. Relative abundance of cDNAs was determined in triplicate by quantitative RT-PCR analysis using the ABI Prism 7700 Sequence Detection System (Applied Biosystems), with results validity confirmed by samples located in the linear range of standard curve analysis. Primer pairs and TaqMan probes for IL-10 and TGF-β were obtained from Applied Biosystems. Values were normalized to 18s rRNA expression in each sample, and fold modulation was calculated by a comparative CT method.

Homeostatic proliferation. Congenic Thy1.1$^+$CD4$^+$CD25$^-$ T cells and Thy1.2$^+$CD4$^+$CD25$^+$ T reg cells from PBS- or IL-2–JES6-1–treated donors (three injections of 1 μg/5 μg each) were isolated using MACS columns (Miltenyi Biotec). Cells were mixed in a 1:1 ratio (10^6 each) and adoptively transferred into RAG$^{-/-}$ recipient mice. Spleens and LNs were harvested on day 7, and the total number of Thy1.1$^+$ T cells was enumerated using flow cytometry.

EAE induction. C57BL/6 mice were immunized subcutaneously (into the flanks) with 100 μg MOG$_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK; Prospec-Tany Technogene Ltd.) in CFA containing 400 μg of heat-inactivated *Mycobacterium tuberculosis* H37 RA (Difco) and 25 μg *M. butyricum* (Difco). They received two i.v. injections of 200 ng pertussis toxin (List Biological Laboratories, Inc.) on days 0 and 2 after immunization. Animals were monitored daily for symptoms and were scored as follows: 0, no disease; 1, loss of tail tone; 2, tail paralysis; 3, hind limb weakness; 4, hind limb paralysis; and 5, hind limb paralysis and forelimb weakness (19). Mice were euthanized if they reached a score >4, according to Garvan Institute guidelines. To investigate the effect of IL-2–IL-2 mAb (1 μg/5 μg), mice were injected i.p. daily on days −3, −2, and −1 or days 2, 3, and 4, or days 7, 8, and 9 (or at commencement of initial symptoms). Control mice were injected with IL-2 alone or PBS. Rapamycin (EMD) was dissolved in 1% wt/vol DMSO and administered i.p. at a dose of 1 mg/kg on days 2, 3, and 4. To analyze CNS mononuclear cells, mice were perfused and spinal cords were extracted. Lymphocytes were separated by a 33% isotonic Percoll gradient at room temperature and restimulated in vitro with 100 ng/ml PMA and 2 μg/ml ionomycin for 3.5 h, with brefeldin A added after 1 h 20 min of incubation.

Islet transplantation. To induce diabetes, recipient mice (C57BL/6) were injected with 200 mg/kg streptozotocin (Sigma-Aldrich) in 10 mM citrate buffer, pH 4.2 (28), and BGLs were determined using an ACCU-CHECK Advantage (Roche). Mice with a blood glucose value >16 mmol/liter were selected as transplant recipients. Islets were prepared from the pancreata of donor (BALB/c) mice at a ratio of three pancreata per recipient, as we have previously described (26). For the transplant, the kidney was accessed by a left-flank incision and brought into the wound by gentle blunt dissection. A small nick was made in the kidney capsule at the inferior renal pole, and the islets were deposited through the nick toward the superior pole of the kidney. BGLs were analyzed on postoperative days (POD) 1, 2, and 5, and then three times per week until rejection. Graft rejection was defined as an increase of blood glucose to >16 mmol/liter after a period of euglycemia. Nephrectomy was performed a POD ≥100 to determine if the euglycemia was graft dependent.

Assessment of tolerance. To assess donor-specific tolerance, $RAG^{-/-}$ ($H\text{-}2^b$) mice were rendered diabetic with streptozotocin and grafted with BALB/c islets, as outlined in the previous section. After 30 d, the mice received 2×10^7 spleen cells i.v. from long-term graft survivors or naive age-matched C57BL/6 controls. To assess immunological ignorance, C57BL/6 long-term graft survivors were challenged with 2×10^7 T-depleted spleen cells from BALB/c mice. Blood glucose was measured to determine the outcome of the challenges.

Mixed lymphocyte reaction. T cells from naive and long-term engrafted mice (responders; 2.5×10^5 cells/well) were cultured with irradiated T-depleted splenocytes (stimulators; 5×10^5/well) derived from C57BL/6 (syngeneic), BALB/c (donor), and CBA/Ca (third party) mice. Cells were pulsed with 1 μCi [^{3}H]thymidine per well for the last 8 h of a 5-d culture period. All data are shown as mean [^{3}H]thymidine incorporation in triplicate cultures.

Immunohistochemistry. Tissues were fixed in 10% buffered formalin, and paraffin sections were stained with hematoxylin-eosin. Parallel sections were stained for insulin or Foxp3 using polyclonal anti–swine insulin (Dako) or anti–mouse/rat Foxp3 (FJK-16s;eBioscience). Foxp3 detection required antigen retrieval with 10 mM citrate, pH 6. Secondary antibodies, biotinylated anti–guinea pig and anti–rat (Jackson ImmunoResearch Laboratories), were used to detect insulin and Foxp3, respectively. The Vectastain Elite ABC kit (Vector Laboratories) was used to amplify the signal, and DAB (Sigma-Aldrich) was used for visualization. Sections were counterstained with hematoxylin.

Statistical analysis. Results are presented as means ± SEM. The Student's *t* test was used to assess statistical significance between two groups, and one-way analysis of variance was used to assess statistical significance between EAE scores. Graft survival was analyzed using the Kaplan-Meier method, and survival curves were compared using the log-rank test (Prism software; GraphPad Software, Inc.).

Online supplemental material. Fig. S1 contains flow cytometry data of the splenic T reg cell population after administration of combinations of control antibody, IL-2, or IL-2 mAb. Fig. S2 documents the increase in BGLs after nephrectomy of the graft-bearing kidney, demonstrating that euglycemia depends on the presence of the allograft. Fig. S3 shows the depletion of $CD25^+$ cells after anti-CD25 (PC61) administration, and the subsequent BGL and graft maintenance. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082824/DC1.

The authors thank the staff at the Biological Testing Facility at the Garvan Institute Australia for help with animal breeding and care. We also thank J. Darakdjian and C. Brownlee for cell sorting; D. Kim, J.H. Cho, H.O. Kim, A. Hong, and A. Weinberg for technical help; C. King for antibodies; and A. Basten for critical reading of this manuscript.

This work was supported by grants from the National Health and Medical Research Council of Australia; the National Institutes of Health, and the Swiss National Science Foundation.

Three of the authors (J. Sprent, O. Boyman, and C.D. Surh) are co-founders in a company for developing the therapeutic use of cytokine–mAb complexes. The authors have no other competing financial interests.

Submitted: 17 December 2008
Accepted: 4 March 2009

REFERENCES

1. Cantrell, D.A., and K.A. Smith. 1983. Transient expression of interleukin 2 receptors. Consequences for T cell growth. *J. Exp. Med.* 158:1895–1911.
2. Cantrell, D.A., and K.A. Smith. 1984. The interleukin-2 T-cell system: a new cell growth model. *Science.* 224:1312–1316.
3. Taniguchi, T., and Y. Minami. 1993. The IL-2/IL-2 receptor system: a current overview. *Cell.* 73:5–8.
4. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 299:1057–1061.
5. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 4:330–336.
6. D'Cruz, L.M., and L. Klein. 2005. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat. Immunol.* 6:1152–1159.
7. Fontenot, J.D., J.P. Rasmussen, M.A. Gavin, and A.Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6:1142–1151.
8. Murakami, M., A. Sakamoto, J. Bender, J. Kappler, and P. Marrack. 2002. CD25+CD4+ T cells contribute to the control of memory CD8+ T cells. *Proc. Natl. Acad. Sci. USA.* 99:8832–8837.
9. Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural $Foxp3^+$ $CD25^+$ $CD4^+$ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201:723–735.
10. Tang, Q., K.J. Henriksen, M. Bi, E.B. Finger, G. Szot, J. Ye, E.L. Masteller, H. McDevitt, M. Bonyhadi, and J.A. Bluestone. 2004. In vitro–expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J. Exp. Med.* 199:1455–1465.
11. Nishimura, E., T. Sakihama, R. Setoguchi, K. Tanaka, and S. Sakaguchi. 2004. Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25-+CD4+ regulatory T cells. *Int. Immunol.* 16:1189–1201.
12. Golshayan, D., S. Jiang, J. Tsang, M.I. Garin, C. Mottet, and R.I. Lechler. 2007. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood.* 109:827–835.
13. Boyman, O., M. Kovar, M.P. Rubinstein, C.D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science.* 311:1924–1927.
14. Wilson, M.S., J.T. Pesce, T.R. Ramalingam, R.W. Thompson, A. Cheever, and T.A. Wynn. 2008. Suppression of murine allergic airway disease by IL-2:anti-IL-2 monoclonal antibody-induced regulatory T cells. *J. Immunol.* 181:6942–6954.
15. Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190:995–1004.
16. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155:1151–1164.
17. Wood, K.J., and S. Sakaguchi. 2003. Regulatory T cells in transplantation tolerance. *Nat. Rev. Immunol.* 3:199–210.
18. Tang, Q., J.Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C.A. Piccirillo, B.L. Salomon, and J.A. Bluestone. 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity.* 28:687–697.
19. Mendel, I., N. Kerlero de Rosbo, and A. Ben-Nun. 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. *Eur. J. Immunol.* 25:1951–1959.

20. Battaglia, M., A. Stabilini, and M.G. Roncarolo. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood*. 105:4743–4748.
21. Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J. Immunol*. 177:566–573.
22. Korn, T., J. Reddy, W. Gao, E. Bettelli, A. Awasthi, T.R. Petersen, B.T. Backstrom, R.A. Sobel, K.W. Wucherpfennig, T.B. Strom, et al. 2007. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat. Med*. 13:423–431.
23. Joffre, O., T. Santolaria, D. Calise, T. Al Saati, D. Hudrisier, P. Romagnoli, and J.P. van Meerwijk. 2008. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat. Med*. 14:88–92.
24. Walters, S., K.E. Webster, A. Sutherland, S. Gardam, J. Groom, D. Liuwantara, E. Marino, J. Thaxton, A. Weinberg, F. Mackay, et al. 2009. Increased CD4+Foxp3+ T cells in BAFF-transgenic mice suppress T cell effector responses. *J. Immunol*. 182:793–801.
25. Tao, R., E.F. de Zoeten, E. Ozkaynak, C. Chen, L. Wang, P.M. Porrett, B. Li, L.A. Turka, E.N. Olson, M.I. Greene, et al. 2007. Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat. Med*. 13:1299–1307.
26. Liuwantara, D., M. Elliot, M.W. Smith, A.O. Yam, S.N. Walters, E. Marino, A. McShea, and S.T. Grey. 2006. Nuclear factor-kappaB regulates beta-cell death: a critical role for A20 in beta-cell protection. *Diabetes*. 55:2491–2501.
27. Graca, L., S.P. Cobbold, and H. Waldmann. 2002. Identification of regulatory T cells in tolerated allografts. *J. Exp. Med*. 195:1641–1646.
28. Like, A.A., and A.A. Rossini. 1976. Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science*. 193:415–417.