Costimulation blockade is a promising strategy for preventing allograft rejection and inducing tolerance. Using a fully allogeneic mouse model, we tested the effectiveness of the combined blockade of the CD40 ligand and the inducible costimulator (ICOS) on islet allograft survival and in the prevention of autoimmune diabetes in the NOD mouse. Recipients treated with blocking monoclonal antibodies (mAbs) to ICOS and the CD40 ligand had significant prolongation of graft survival, with 26 of 28 functioning for >200 days. Long-term engrafted mice maintained antinor proliferative and cytotoxic responses, but donor-specific immunization did not induce graft rejection, and challenge with second, same donor but not third-party grafts resulted in long-term acceptance. The immunohistology of tolerant grafts demonstrated the presence of CD4+CD25+ T-cells expressing Foxp3, and islet/kidney composite grafts from tolerant mice, but not from mice lacking lymphocytes, were accepted indefinitely when transplanted into naive B6 mice, suggesting that recipient T-cells were necessary to generate dominant tolerance. Combined anti-ICOS and anti–CD40 ligand mAb therapy also prevented diabetes in NOD mice, with only 11% of treated recipients developing diabetes compared with 75% of controls. These data demonstrate that the blockade of CD40 ligand and ICOS signaling induces islet allograft tolerance involving a dominant mechanism associated with intragraft regulatory cells and prevents autoimmune diabetes in NOD mice. Diabetes 55:27–33, 2006

Tolerance induction is an attractive but elusive goal in transplantation, with many groups focusing on blockade of the various costimulatory pathways required for the initiation of immune responses. A key costimulatory pathway involves the binding of the T-cell–expressed CD40 ligand (CD154) to CD40 on antigen-presenting cells (1). CD40 ligand monoclonal antibody (mAb) therapy prolongs allograft survival in rodents and nonhuman primates (2,3), probably through mechanisms that vary depending on the model, concomitant therapy, type of transplant, and relative importance of CD4 versus CD8 host T-cell responses (1,4). For example, CD40 ligand mAbs and nonmyeloablative conditioning can induce mixed chimerism and tolerance in rodent and primate models (5,6), whereas donor-specific transfusion and CD40 ligand blockade can induce tolerance to islet allografts via T-cell anergy, which is dependent on cytotoxic lymphocyte (CTL) A-4 negative signaling (7,8). Others have demonstrated that CD40 ligand blockade combined with anti-CD8 mAbs involves the amplification of regulatory mechanisms as the primary mechanism of tolerance (9,10), and when both CD40 ligand and CD28 signaling are inhibited, long-term acceptance of allografts is mediated through apoptotic deletion of alloreactive T-cells (11). Selective depletion of activated T-cells by complement- and Fc receptor–mediated mechanisms may also contribute to the efficacy of CD40 ligand mAb therapy in some models (12). Taken collectively, the results of these studies emphasize the diversity of this molecule in influencing immune responses and the involvement of anergy, regulation, and/or deletion in mediating tolerance in differing models.

The inducible costimulator (ICOS) is a second costimulatory molecule of interest to transplant biologists, given its induction on CD4+ and CD8+ T-cells after CD28 signaling (13) and the finding that its ligation with B7RP-1 generates intracellular signals that regulate T helper 1 and 2 cell differentiation (14–16). Blockade of ICOS signaling is synergistic with other costimulatory blocking agents or conventional immunosuppression in achieving long-term graft acceptance and impairing chronic allograft rejection (17–22). We have recently shown that combined anti-ICOS and anti–CD40 ligand mAb therapy results in indefinite islet allograft acceptance across a fully major histocompatibility complex–mismatched barrier (19). In this study, we explored the mechanisms responsible and now report on the remarkable efficacy of this strategy in controlling alloimmune islet rejection and autoimmune incidence in NOD mice.

RESEARCH DESIGN AND METHODS

Adult C57BL/6 (H-2b), BALB/c (H-2d), and CBA/Cr (H-2k) mice were purchased from Charles River (St. Constant, Quebec, Canada) and housed under
DOMINANT TOLERANCE TO ISLET ALLOGRAFTS

standard conditions. Immunodeficient C57BL/6-RAG1-knockout mice (Jackson Laboratory, Bar Harbor, ME) and 4-week-old female NOD mice (Taconic, Mississauga, ON, Canada) were housed under specific pathogen–free conditions. All animals were cared for in accordance with the guidelines established by the Canadian Council on Animal Care.

C57BL/6 and immunodeficient C57BL/6-RAG1-knockout recipient mice were rendered chemically diabetic by a single injection of streptozotocin (STZ; 200 mg/kg i.v.; Sigma-Aldrich, Oakville, ON, Canada). Donor islets were isolated from fully major histocompatibility complex–mismatched BALB/c mice by collagenase digestion (1 mg/ml, Sigma-Aldrich) followed by Ficoll purification (Sigma-Aldrich) (23,24). Next ~500 islets were transplanted under the left renal capsule of diabetic recipient mice. Allograft function was monitored by serial blood glucose measurements. Successful engraftment was defined by the correction of the serum glucose level to <8 mmol/l by the third day after the transplant, and graft rejection was defined as a rise in serum glucose levels to >15 mmol/l for 2 consecutive days.

The CD40 ligand mAb (MR1) was purchased from Bio-Express (West Lebanon, NH). The production and characterization of a nondepleting anti-ICOS mAb (12A8) have been previously described (17). An isotype-matched IgG2a control mAb (Bio-Express) was used as the control for anti-ICOS mAb therapy. Islet allograft recipients were treated with CD40 ligand mAbs (0.25 mg; days 0, 2, 4, and 6) and/or anti-ICOS mAbs (0.1 mg/day × 14 days) beginning on the day of transplantation. Female NOD mice were treated with an IgG2a control mAb (0.1 mg/day × 14 days) or CD40 ligand mAb (0.25 mg; days 0, 2, 4, 6, and 10) with or without an anti-ICOS mAb (0.1 mg/day × 14 days). Treatment in NOD mice was initiated at age 10 weeks, during the development of insulitis but before the onset of spontaneous diabetes. All mAbs were given intraperitoneally.

**Mixed lymphocyte reactions.** Splenocytes were cultured in duplicate wells containing 2 × 10⁶ responder cells and dilutions of irradiated (1,500 Rad) stimulator cells. Responders were obtained from naive and long-term engrafted mice, and stimulator cells were derived from C57BL/6 (syngeneic), BALB/c (donor), and CBA/J (third party) mice. After being cultured at 37°C for 3 days, the cells were pulsed for 18 h with 1 γCi [³H]labeled thymidine/well and harvested, after which the thymidine incorporation was determined.

**Cytotoxic lymphocyte reactions.** Cytotoxic responses were assayed by the JAM test (25). Briefly described, 5 × 10⁵ spleen cells from C57BL/6 naive and tolerant mice (responders) were stimulated for 5 days with 2 × 10⁶ irradiated BALB/c (donor) spleen cells. Con A blast targets were set up 40 h before CTL assay by culturing 1.5 × 10⁶ naive syngeneic and BALB/c spleen cells with Con A (1.25 μg/ml), then labeling them with [³H]thymidine. The lysis of target cells was tested at various responder/target ratios.

**Immunization of tolerant mice with donor splenocytes.** A single-cell suspension of donor splenocytes from the spleen of BALB/c mice was passed through a nylon mesh. Viable cells were counted and adjusted to 20 × 10⁶ cells/ml. NOD mice previously treated with anti-ICOS and CD40 ligand mAbs were immunized with 5 × 10⁶ spleen cells (0.25 ml) intraperitoneally and followed for graft rejection.

**Confirmation of graft function and retransplantation.** Long-term graft function in mice maintaining normoglycemia for >200 days was confirmed by a return to hyperglycemia after nephrectomy of the kidney bearing the islet graft. To test specific donor-specific immunologic tolerance, nephrectomized mice underwent retransplantation of same donor strain (BALB/c) or third-party (CBA/J) islets into the remaining contralateral kidney. No immunosuppressive therapy was given, and blood glucose was monitored serially to detect graft rejection.

**Composite islet-kidney transplantation.** C57BL/6 mice treated with anti-ICOS and anti-CD40 ligand mAbs and C57BL/6-RAG1-knockout immunodeficient mice underwent nephrectomy of their islet-bearing kidneys after maintaining normoglycemia for >100 days. The composite islet (BALB/c) and kidney (C57BL/6) grafts from both groups of mice were transplanted into STZ-administered diabetic, naive C57BL/6 mice, and the recipients’ blood glucose levels were monitored. Renal transplantation was performed as previously described (26) and involved flushing the donor kidney with cold saline immediately after nephrectomy followed by heterotopic implantation of the graft, with the donor renal artery and vein being anastomosed to the recipient aorta and inferior vena cava, respectively. An anastomosis between the donor ureter and recipient bladder was also created.

**Immunopathology.** After nephrectomy of graft-bearing kidneys, one half of the islet allograft was snap frozen, and cryostat sections were stained by immunoperoxidase using mAbs to CD4 and CD25 or isotype controls (Pharmingen, San Diego, CA), rabbit anti-mouse Foxp3 or control rabbit IgG, and an Envision Plus kit (Dako, Carpinteria, CA) (17). The rabbit antibody was generated against a 13-mer peptide TFFR5SPTPRKD5 corresponding to amino acids 169–181 of mouse Foxp3 and affinity purified. Control studies showed that this antibody produced a single band of ~52 kDa on Western blotting of mouse thymocytes and stained murine Foxp3⁺ cell transfectants but did not untrans-
ble that recipients were in fact “ignorant” of donor antigen but unable to reject a well-established graft that may not have delivered the necessary signals needed to recruit effector T-cells to the graft site (27). To more fully determine whether graft acceptance was due to immunologic ignorance or tolerance, we challenged long-term engrafted recipients with a second, same donor islet allograft.

Long-term allograft acceptance (>200 days) was first confirmed in mice treated with anti-ICOS and anti–CD40 ligand mAbs by nephrectomy of the graft-bearing kidney, which resulted in a prompt return of hyperglycemia in all cases (n = 11 of 11). Mice were then challenged with a second donor (BALB/c) islet allograft in the remaining contralateral kidney without further immunosuppressive therapy. All second donor allografts were accepted long term (>100 days), whereas a second cohort of tolerant mice challenged with third-party (CBA/J) islets rapidly rejected their grafts (Fig. 2C), demonstrating that donor-specific tolerance had been achieved. Because 60% of mice treated with anti–CD40 ligand mAbs alone also demonstrated long-term allograft survival, we challenged these mice with a second, same donor islet allograft and found that only two of four mice had long-term acceptance of the second graft. This suggested that in addition to increasing the proportion of mice achieving indefinite allograft survival, the combination of anti-ICOS with anti–CD40 ligand also impacts the quality of the tolerant state.

Intragraft T-cells with a regulatory phenotype are present in grafts from treated mice. We next sought to address the issue of long-term maintenance of immunologic tolerance in vivo despite the presence of strong antidonor proliferative and cytotoxic responses in vitro. Long-term allograft acceptance in our model could have been dependent on a regulatory mechanism that can actively constrain alloaggressive T-cell responses (28). Given that splenic lymphocytes from tolerant mice generated robust proliferative responses to donor antigen in vitro (Fig. 2A), it appeared unlikely that regulatory T-cells were present in the spleens of tolerant mice. To more fully rule out this possibility, a standard mixed-lymphocyte reaction assay was used to detect the presence of donor-specific regulatory T-cells. Lymphocytes from tolerant mice did not suppress the alloresponses of cocultured lymphocytes from naive mice (data not shown), suggesting that donor-specific regulatory T-cells are not present in the spleen of mice treated with combination therapy. However, it is possible that the specificity or effector function of these cells is restricted to islet antigen alone, rendering them undetectable in assays using splenocyte targets. Regulatory T-cells have also been identified in recent years within the grafts of tolerant mice and are capable of mediating a dominant-suppressive effect on naive T-cells (29). Therefore, a regulatory mechanism restricted to the graft site may have been operating in long-term engrafted mice treated with anti-ICOS and anti–CD40 ligand mAbs. Immunoperoxidase staining of tolerated islet allografts showed peri-islet staining of infiltrating mononuclear cells for CD4, CD25, and Foxp3, whereas expression of these markers was not seen in healed-in allografts from untreated B6-RAG– knockout mice (Fig. 3); sections labeled with control antibodies were unstained.

Although these findings do not demonstrate a functional regulatory role of the intragraft T-cells, they do suggest the possibility that tolerance of the islet graft may be dominant and dependent on recipient T-cells.
Dominant tolerance of islet/kidney composite grafts from immunocompetent, treated mice transplanted into naïve B6 mice. To functionally evaluate the state of the tolerated graft and the possible involvement of dominant mechanisms, we performed a retransplant procedure involving the transfer of tolerated islet grafts from long-term engrafted mice to naïve mice. Composite islet (BALB/c) and kidney (C57BL/6) grafts from tolerant mice treated with anti-ICOS and anti-CD40 ligand mAbs were explanted and retransplanted into diabetic, naïve C57BL/6 mice. Control composite islet (BALB/c) and kidney (C57BL/6) composite grafts from STZ-administered C57BL/6-RAG–knockout mice were also harvested after long-term maintenance of normoglycemia (>50 days) and retransplanted into diabetic, naïve C57BL/6 mice. Islet allografts from both long-term engrafted (>100 days) wild-type and immunodeficient recipients were well preserved before the retransplantation (Fig. 4B–D). However, although grafts from immunocompetent, treated recipients were accepted over the long term in secondary naïve recipients mice, grafts from control, immunodeficient mice were rejected (Fig. 4A and E). Moreover, the expression of CD4, CD25, and Foxp3 in donor allografts from tolerant, treated mice was maintained long term (>100 days) after graft retransplantation into naïve mice (Fig. 4F). The rejection of the control islet grafts from immunodeficient mice demonstrated that it was not the healed-in characteristic of the graft or “parking” that

FIG. 3. Immunohistology of tolerated grafts (>100 days). Immunoperoxidase staining of BALB/c islet allografts on wild-type (A–C) or RAG–/– C57BL/6 (D–F) recipients treated with anti-ICOS and anti-CD40 ligand mAbs demonstrating peri-islet CD4, CD25, and Foxp3 mononuclear cells in wild-type but not in RAG–/– recipients. Asterisks indicate well-preserved islets and arrows indicate boundary of kidney versus islet grafts. C, inset: Lack of staining with Foxp3-peptide absorbed antibody (magnification ×300; representative of four grafts/group).

FIG. 4. Dominant regulatory tolerance in long-term engrafted mice treated with anti-ICOS and anti-CD40 ligand mAbs. A: Islet allografts harvested at 100 days after transplant from recipients treated with anti-ICOS and anti-CD40 ligand mAbs or immunodeficient recipients were transferred as islet-kidney composite grafts into STZ-administered naïve recipients; recipients were then followed for graft rejection. Histology of islet allografts: well-preserved islet allograft after anti-ICOS and anti-CD40 ligand mAb therapy (>100 days; B), well-preserved islet allograft after retransplantation (>100 days) from treated donor into naïve recipient (C); well-preserved islet allograft in B6-RAG recipient (>100 days; D), and acute rejection of islet allograft at day 13 after retransplantation from RAG mouse (>100 days) into naïve recipient (E; magnification ×250; asterisks indicate islets, representative of four grafts/group). F: Immunohistology of retransplanted kidney/islet composite grafts showing peri-islet accumulation of CD4+CD25+ mononuclear cells and associated Foxp3 expression; inset shows lack of Foxp3 staining using Foxp3 peptide–absorbed antibody. Shown are cryostat sections, magnification ×250, representative of four grafts/group.)
allowed for long-term acceptance of islet grafts from tolerant, treated mice. These results indicate that treatment with anti-ICOS and anti–CD40 ligand mAbs allowed donor islets to be accepted by a dominant mechanism involving recipient T-cells.

**Combined anti-ICOS and anti–CD40 ligand mAb therapy prevents diabetes in NOD mice.** Successful tolerance protocols in islet transplantation must be effective at preventing alloimmune rejection and also in overcoming the underlying autoimmune process of diabetes. Therefore, having shown that combined therapy can prevent islet allograft rejection, we tested the effectiveness of this therapy in preventing autoimmune islet destruction by treating female NOD mice with anti-ICOS and anti–CD40 ligand mAbs, either alone or in combination, for 14 days, beginning at age 10 weeks. Female NOD mice in our colony develop islet infiltration by leukocytes by about age 6–7 weeks. Without immunomodulation, ~50% of these mice develop diabetes by age 25 weeks and 75% develop diabetes by age 33 weeks. In this study, treatment of NOD mice with an IgG2b control mAb did not alter the rate of diabetes as compared with untreated mice (Fig. 5A). Monotherapy with either anti-ICOS or anti–CD40 ligand mAbs resulted in a marked but nonsignificant reduction in diabetes onset compared with mice treated with control mAbs (37 and 35 vs. 63%, respectively; P > 0.05). The combination of anti-ICOS mAbs with anti–CD40 ligand mAbs led to a more potent reduction in the onset of diabetes, with only 11% of mice (2 of 19) becoming diabetic (P = 0.065 vs. individual monotherapies; P < 0.001 vs. control mAbs) (Fig. 5B). Taken together, these results indicate that dual blockade of ICOS and CD40 ligand signaling is highly effective at preventing alloimmune rejection and autoimmune destruction of islet cells.

**DISCUSSION**

T-cell activation and allograft rejection can occur despite blockade of individual costimulatory molecules such as CD28, CD40 ligand, or ICOS. Furthermore, although renal allografts in nonhuman primates can survive for >1 year after discontinuation of anti–CD40 ligand treatment, islet allografts are rapidly rejected within several months (2,3), suggesting that more robust strategies involving two or more agents may be required to induce islet transplant tolerance. The results of the current study support this concept by demonstrating that the blockade of ICOS and CD40 ligand signaling is significantly more effective in facilitating islet allograft acceptance when used in combination rather than as individual monotherapies.

A rationale for combining ICOS and CD40 ligand targeting is suggested by studies showing that although anti-ICOS monotherapy provides some protection to heart and liver allografts from rejection (18,21), it is ineffective at promoting islet graft acceptance (30). In contrast, when anti-ICOS mAbs are combined with blockade of CD28–B7 signaling, FK506 therapy, or rapamycin treatment, islet allograft survival is significantly improved (19,22). In a similar fashion, although blockade of CD40 ligand signaling alone is not effective at inducing tolerance in stringent models of islet and skin allotransplantation, adjunctive strategies such as donor-specific transfusion (31), CD45 signaling blockade (32), blockade of the adhesion/homing receptor LFA-1 (33), or concurrent stimulation of negative signaling through programmed cell death 1 (34) can all lead to indefinite islet allograft acceptance.

The synergy we observed between ICOS and CD40 ligand blockade may involve complementary inhibition of CD4+ and CD8+ T-cell responses. Inhibition of CD40 ligand signaling can effectively prevent donor-specific CD4+ T-cell responses but has little effect on alloreactive CD8+ T-cells (35), which can still mediate allograft rejection (36,37). In stringent models, strategies to induce tolerance through CD40 ligand blockade require adjuncts, such as CD8 mAbs or CTLA4-Ig, that can provide direct anti-CD8+ T-cell activity (10,11,36). In our model, the combination of anti–CD40 ligand mAbs with anti-ICOS mAbs provided a similar beneficial effect, consistent with a key role of ICOS in regulating the expansion and differentiation of CD4 and CD8 effector cells (19,21). The benefit of this combination is of particular interest in islet transplantation given that CD8+ cells have been reported to be important effectors of rejection in murine models of islet transplantation (38).

After demonstrating the efficacy of combined ICOS and CD40 ligand blockade, we assessed whether tolerance had been achieved in this model. In vitro analysis of T-cells...
harvested from long-term engrafted mice showed potent proliferative and cytotoxic responses to donor alloantigens, indicating that clonal deletion of donor-reactive T-cells was neither achieved nor required for the long-term acceptance of islet allografts after combination therapy. To test for immunologic ignorance as a mechanism for allograft survival, immunization with donor splenocytes was performed in long-term engrafted mice, with the result that it failed to trigger allograft rejection. Because a well-established allograft may be refractory to rejection despite immunization (39), we challenged long-term engrafted mice with a second donor islet allograft to more thoroughly exclude ignorance as a mechanism and to test for immunologic tolerance. These mice accepted same donor but promptly rejected third-party allografts, indicating that anti-ICOS and anti–CD40 ligand mAb treatment induces donor-specific tolerance. One possibility for these divergent findings may be the generation of split tolerance, wherein the recipient may be tolerant to the islet allograft but not to donor splenocytes (40). Another possible explanation is that tolerance in this model is dependent on a dominant regulatory mechanism that controls peripheral alloreactivity.

Recently Graca et al. (29) demonstrated that CD4+ regulatory T-cells were present in tolerated skin allografts and were capable of mediating dominant transplantation tolerance. Moreover, Hori et al. (41) reported on a novel transcription factor specifically expressed by CD4+ CD25+ regulatory T-cells, namely Foxp3. This gene represents a specific marker for regulatory T-cells, unlike CD25, CD45RB, and GITR, which are also expressed on activated effector or memory T-cells. More recently, Cobbold et al. (42) demonstrated that tolerant recipients of skin allografts after CD4 Ab blockade had intragraft regulatory T-cells that expressed high levels of Foxp3 mRNA. In the current study, immunohistology of tolerated islet allografts showed peri-islet staining for CD4+ CD25+ T-cells and the presence of Foxp3, suggesting a possible intragraft regulatory mechanism for the maintenance of tolerance. This hypothesis is supported by the fact that composite islet and kidney grafts from tolerant mice were accepted indefinitely when retransplanted into naive B6 mice, whereas healed-in composite islet and kidney grafts from control B6-RAG–knockout mice devoid of CD4+ CD25+ Foxp3+ T-cells were rejected. These findings indicate that acceptance of composite grafts from treated recipients is not a consequence of reduced immunogenicity of the graft secondary to being healed in (27) and/or depleted of passenger leukocytes (43) but that treatment with anti-ICOS and anti–CD40 ligand alters the graft microenvironment in facilitating tolerance. The presence of CD4+CD25+ Foxp3+ T-cells in the graft, together with the dependence on recipient lymphocytes for tolerance of the retransplanted islets, suggests that regulatory T-cells may have been acting on anti-donor T-cells. However, even though the treated recipients were clearly tolerant rather than ignorant, we cannot fully exclude the additional possibility that the recipient T-cells acted directly on the graft to reduce the graft’s immunogenicity. Nevertheless, these observations provide important initial data regarding a role for recipient T-cells in generating a transferable operationally tolerant state. The association between the presence of intragraft lymphocytes and the maintenance of tolerance could have been further strengthened by comparing allografts that were rejected to those that were accepted indefinitely in recipients treated with anti-ICOS and anti–CD40 ligand mAbs; however, there were too few recipient mice that actually rejected their allograft (2 of 28) for this comparison to be made. Further study is required to determine if in fact the CD4+CD25+ Foxp3+ intragraft T-cells have a functional regulatory role.

Having demonstrated the efficacy of combined anti-ICOS and anti–CD40 ligand blockade in the regulation of alloimmune responses, we proceeded to test this strategy in the prevention of autoimmune diabetes in the NOD mouse. Consistent with previous reports (44), we found that monotherapy with the CD40 ligand reduced the onset of diabetes. In an interesting finding, the blockade of ICOS signaling alone demonstrated a superior effect over CD40 ligand blockade at delaying diabetes onset, although the incidence of diabetes was similar in both groups by age 30 weeks. Although monotherapy with either agent demonstrated efficacy, the combination of these agents resulted in a more profound reduction in diabetes. In our study, therapy was administered after the onset of insulinitis but before the onset of diabetes. The timing of costimulation blockade was based on evidence that blockade of ICOS signaling during antigen priming can result in more severe disease, whereas blockade during the effector immune response (i.e., immediately before disease onset) can prevent its progression (45).

In summary, we have demonstrated that combination treatment with anti-ICOS and anti–CD40 ligand is a potent strategy in inducing long-term islet allograft acceptance. The maintenance of donor-specific tolerance despite the presence of alloreactive T-cells includes the presence of T-cells at the site of the tolerated allograft that express the regulatory markers CD4, CD25, and Foxp3. In addition, we have shown that this combination therapy can also significantly reduce the onset of primary autoimmune diabetes in NOD mice, indicating that its effectiveness is not limited to controlling alloimmune responses. These findings underscore the efficacy of simultaneous blockade of ICOS and CD40 ligand signaling as a potential therapy in clinical islet transplantation and emphasize the need for further studies in large animal models.

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