Prolonged islet allograft survival in diabetic NOD mice by targeting CD45RB and CD154

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Abstract
Clinical islet transplantation is a successful procedure that can improve the quality of life in recipients with diabetes. A drawback of the procedure is the need for chronic administration of immunosuppressive drugs that, among other side effects, are potentially diabetogenic. Definition of immunosuppressive protocols that utilize nondiabetogenic compounds could further improve islet transplantation outcome. We used the NOD mouse to assess the effect of targeting the T-lymphocyte surface receptors CD45RB and CD154 in preventing loss of allogeneic islet grafts as a result of recurrence of autoimmunity and allorejection. Administration of the two antibodies led to significantly prolonged allograft survival, with a percentage of grafts surviving long-term. The therapeutic efficacy of the treatment was paralleled by a shift in CD45RB isoform expression on T-lymphocytes, increased in vitro responsiveness to interleukin-7, and increased in vitro gamma-interferon production after anti-CD3 antibody stimulation. Furthermore, graft infiltration by CD8+ T-cells was remarkably reduced. Recipient mice bearing functioning allografts [...]
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Recent clinical trials demonstrated that islet transplantation can result in remarkable improvement in the quality of life of patients with type 1 diabetes by maintaining tight glucose metabolic control in the absence of hypoglycemic episodes (1–4). However, the need for chronic immunosuppression with its side effects still limits the use of this procedure to a small cohort of patients with brittle diabetes and severe hypoglycemic episodes, where the risks associated with transplantation and chronic immunosuppression are justified. Furthermore, currently used immunosuppressive agents have intrinsic diabetogenicity, probably contributing to the observed need for more than one donor organ to achieve insulin independence (5).

Immunomodulatory compounds that allow for prolonged graft survival in the absence of diabetogenic effects could represent a valuable alternative for the treatment of transplant recipients. To this aim, blockade of signal 1 or 2 of T-cell activation by the use of biological modifiers such as monoclonal antibodies (mAb) and soluble receptor ligands has proved effective in preventing or delaying graft rejection as well as autoimmune diseases (6–21), in the absence of β-cell toxicity.

Modulation of signal 1 by administration of anti-CD45RB mAb has shown efficacy in preventing kidney (6), pancreas (7), and islet allograft rejection in murine models (8–11). CD45 is a family of protein phosphatases critically involved in T-cell receptor–mediated signal transduction (signal 1).

Blockade of signal 2 by selectively targeting co-stimulatory molecules has also yielded promising results in modulating immune responses and has provided a precious tool to explore the immunological mechanisms underlying transplant rejection and autoimmunity (12). Treatment with anti-CD154 mAb induced long-term allograft acceptance in several transplantation models (13–16) and efficiently prevented autoimmune diseases (17,18), including diabetes (19–21). CD154 is a tumor necrosis factor receptor family member involved (via binding to CD40) in T-cell co-stimulation (signal 2) after antigen recognition.

We have previously reported that simultaneous administration of mAb targeting CD45RB and CD154 protected islet allografts in mice and allowed for the induction of tolerance in a large proportion of recipients in a nonautoimmune background (21). In addition, monotherapy with anti-CD154 mAb significantly prolonged survival of syngeneic and allogeneic islet transplants in spontaneously diabetic NOD mice (21).

NOD mice spontaneously develop autoimmune diabetes, arguably representing the best available model for the study of allogeneic islet transplantation in type 1 diabetes. In NOD mice with already established autoimmune diabetes, few treatments lead to prolonged islet allograft survival, and even fewer lead to indefinite acceptance of the graft (22,23). Therapeutic approaches that result in long-term islet graft survival and even immunological tolerance...
in allogeneic combinations, in fact, often fail when tested in spontaneously diabetic NOD recipients (21, 24, 25).

In view of our preliminary data on CD154 monotherapy in NOD mice and on the synergy obtained by simultaneous targeting of CD45RB and CD154 in models of islet allograft-plantation into chemically diabetic recipients, we tested the efficacy of this combination treatment in NOD mice, in which allore cognition and recurrence of autoimmune concur to determine islet allograft failure.

RESEARCH DESIGN AND METHODS

Animals. Female NOD mice (Taconic Farms, Germantown, NY) were monitored for blood glucose levels until diabetes onset and were used as recipients of C57BL/6 (B6; Hilltop Laboratories, Scottsdale, PA) islet allografts after at least three nonfasting blood glucose readings >350 mg/dl. All mice were certified to be free of common laboratory animal pathogens and were housed in virus-free animal facilities, having free access to autoclaved feed and water. All animal manipulations were conducted under protocols approved by the Institutional Animal Care and Use Committee.

Islet isolation and transplantation. Murine islets were isolated from B6 donors as previously described (26). After overnight culture at 37°C, 5% CO2 in CMRL-1066 medium (Gibco, Long Island, NY) supplemented with 10% FCS (HyClone, Logan, UT), 2 mmol/l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mmol/l HEPES buffer (Mediatech, Herndon, VA), 600–700 IEQ were transplanted under the kidney capsule of NOD recipients (21). Blood glucose levels were then monitored biweekly using an Elite glucometer (LifeScan, Milpitas, CA). Diabetes recurrence was defined as two consecutive nonfasting blood glucose readings >250 mg/dl. Long-term graft survival was defined as graft function persisting >120 days.

Antibodies and treatments. The hamster anti-mouse CD154 mAb (MR1) was purchased from Taconic Biotechnology. The rat anti-mouse CD45RB mAb was purified by affinity chromatography on protein-G columns from supernatants of cultured MB23G2 hybridoma cells (ATCC, Rockville, MD) (8). Recipients were treated with either 100 μg of anti-CD45RB mAb or 500 μg of anti-CD154 mAb alone or in combination, intraperitoneally injected on days −1, 0, and 5 after transplantation (acute treatment regimen). Alternatively, anti-CD154 mAb was administered weekly (chronic treatment), and/or anti-CD45RB mAb was given repeating the induction protocol semimonthly (two injections on consecutive days followed by a third injection on the fifth day) until day 50 or indefinitely.

Histology and immunohistochemistry. At selected time points, paraffin-embedded sections of grafted kidneys were stained with hematoxylin and eosin to assess graft morphology. Immunostaining on frozen sections was performed by the use of biotinylated antibodies directed to CD4 (L3T4) or CD8a (Ly-2; BD-PharMingen, San Diego, CA), followed by streptavidin-peroxidase and aminoethyl carbazole (AEC). Control slides, where the primary antibody was omitted, were always processed in parallel. Flow cytometry analysis. Phenotypic analysis of splenocytes was performed 10–12 days after transplantation by the use of mAb directed to CD3 (ε-chain), CD4 (L3T4), CD8 (Ly-2), CD25, and CD45RB (16A clone; BD-PharMingen). Antibodies were either directly conjugated to fluorochromes (APC, FITC, and PE) or biotinylated. Biotinylated antibody binding was detected by incubation with PE-conjugated streptavidin. Analysis of the samples was performed on a FACScalibur flow cytometer (BD-PharMingen), acquiring at least 5,000 events per sample. Unstained and control isotype-treated samples were always processed in parallel.

Response to phorbol-myristate-acetate and cytokines. For assessing the effect of the different treatments on the response to selected cytokines, spleen cells obtained from treated and control animals 10–12 days after transplantation were cultured in Iscove's media (Gibco) supplemented with 10% heat-inactivated FCS, and 2-μg-mercaptoethanol (2-μE, TCM), in the presence of phorbol-myristate-acetate (PMA) alone or with interleukin (IL)-2, IL-4, or IL-7 (10 ng/ml; Peprotech, Rocky Hill, NJ) at 2 × 106 cells/well. This time point was selected for this and all following mechanistic studies to compare immunological profiles of treated mice bearing a functional graft with control mice at graft rejection. After 48 h of incubation, plates were pulsed with tritiated thymidine ([3H]Thy; 1 μCi/well), and incorporation was measured 6 h later on a liquid scintillation β-counter and expressed as counts per minute.

Anti-CD3 stimulation. Splenocytes from NOD recipients (4 × 106 cells) were harvested 10–12 days after transplantation and cultured in 24-well plates in the presence of anti-CD3 mAb (1 μg/ml) for 72 h in TCM. After incubation, 20,000 cpm were plated in 96-well plates in the presence of 10 ng/ml of IL-2, IL-4, or IL-7. After overnight culture, plates were pulsed with [3H]Thy, and its incorporation was measured after 6 h.

In other experiments, splenocyte supernatants were collected after 72 h of culture in the presence of anti-CD3 mAb and were assayed by enzyme-linked immunosorbent assay for IL-4, IL-10 (Biosource, Camarillo, CA), IL-2, and IFN-γ (R&D Systems, Minneapolis, MN).

Mixed lymphocyte reaction. Splenocytes from NOD recipients (2 × 106) were cultured in the presence of mitomycin-treated stimulator splenocytes obtained from B6 (donor), NOD (self), or Balb/c (third party) mice at a 1:1 ratio. After 3 days (37°C, 5% CO2), plates were pulsed with [3H]Thy, and incorporation was measured 8 h later.

Graft cytokine analysis. Islet grafts dissected free from the kidney surface (10–12 days posttransplantation) were snap-frozen and stored at −80°C until RNA extraction (RNA NOW-LM kit; Biogentex, Seabrook, TX). cDNA was created using Superscript reverse transcriptase (Gibco), and quantitative PCR analysis of cytokine mRNA steady-state levels was performed with the LightCycler instrument (Roche) (26). PCR conditions were 1 min at 94°C, 35 cycles of 1 s at 94°C, 9 s at 55°C, and 12 s at 72°C. Cytokine and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages were amplified using the following primer pairs: IL-2 5′-TCTCTGGAGGATGGAGAATT3′ (forward), 5′-CGCAAGGTCAAGTGTAGCT3′ (reverse); IL-4 5′-GACATTTGGAAGCTGACAA3′ (forward), 5′-AGGAAGTTGCCACATCATAC3′ (reverse); IL-10 5′-TTTGAATCCCCGTTGAGAA3′ (forward), 5′-ACAGGGAGAAATCGTGACAA3′ (reverse); IFN-γ 5′-CTCTTCTGATCATCGGAGATGG3′ (forward), 5′-CATGAACTCCTGGCCGACTCTG3′ (reverse); transforming growth factor-β 5′-GAGGACAAATGTGGAACTTCCAG3′ (forward), 5′-GAGCTCAAAGAGCGCCACCTGG3′ (reverse); GAPDH 5′-CTCAACTACATGTTGCTCACA3′ (forward), 5′-CCATTCTGCGCTTACGCTG3′ (reverse). Initial starting concentrations of cDNA were determined by arbitrary units, and the ratio of cytokine to GAPDH was used to normalize the data to the control sample.

Statistical analysis. The Statistica software (Statsoft, Tulsa, OK) was used for statistical analysis. Data are expressed as mean and SD or SE. Kaplan-Meier analysis was performed for diabetes-free survival determination, and differences were assessed with the Mantel-Cox log-rank test. Two-tailed, unpaired, or paired Student’s t test was used, whenever appropriate. P < 0.05 was considered significant.

RESULTS

Peritransplant administration of antibodies directed against CD45RB and CD154 significantly prolongs survival of islet allografts in NOD mice. Allogeneic islets transplanted in untreated spontaneously diabetic NOD mice (controls) were invariably lost within 15 days, with a mean survival time (±SD) of 10 ± 2.2 days (n = 8). A short treatment course with anti-CD154 mAb (days −1, 0, and 5) resulted in a measurable, although not dramatic, prolongation of graft survival (18.6 ± 3.8 days, n = 5; Cox-Mantel test P = 0.002 versus controls). Peritransplant treatment with anti-CD45RB mAb alone resulted in comparable prolongation of graft survival (18.4 ± 2.5 days; n = 5; P = 0.0008; Fig. 1A).

Administration of both mAb in combination during the peritransplant period led to remarkable prolongation of graft survival (47.2 ± 4.3 days; n = 5). Statistical analysis showed highly significant differences between the group that received the combination treatment and the groups that received either antibody alone or no treatment. This result is consistent with the reported synergistic effect observed in an allogeneic setting when a similar combination of mAb was used (11).

Extended administration of anti-CD45RB and anti-CD154 mAb results in further prolongation of graft survival. We recently reported that extended administration of anti-CD45RB mAb seemed more effective than a short-course treatment in prolonging allograft acceptance in spontaneously diabetic NOD mice (21). On the basis of this observation, we explored the effect of weekly administrations of anti-CD154 mAb after the peritransplant treatment with both mAb. With this regimen, graft survival was prolonged (59 ± 32 days; n = 5) but was not significantly
transplant combination therapy with anti-CD154 plus anti-CD45RB mAb (Fig. 1B). In this group, graft loss occurred after discontinuation of the anti-CD45RB therapy (50 days posttransplantation), in 4 of 5 recipients, suggesting that extended administration of the antibody was required for maintenance of islet graft function. Therefore, we examined the effects of indefinite anti-CD45RB therapy, in addition to peritransplant anti-CD154 treatment. This strategy resulted in a significant prolongation of graft survival and long-term graft acceptance (>120 days) in 2 of 5 recipients (88.7 ± 33.3 days; P = 0.00015 versus controls; P = 0.04 versus all treated groups; Fig. 1B).

**Treatments with anti-CD45RB and anti-CD154 mAb induces a CD45RB isoform shift.** Analysis of the T-cell subset distribution in splenocytes and peripheral blood of NOD mice assessed by flow cytometry revealed that none of the treatments altered the overall proportion of CD3+ T-cells or the ratio of CD4+CD8+ cells, when compared with untreated NOD mice (not shown). Similarly, no difference in the expression of the IL-2 high-affinity receptor (CD25) was observed on CD4+ and CD8+ T-cells (data not shown), suggesting that the protection afforded by the treatment was not associated with alterations in T-regulatory cells (e.g., CD4+CD25+) or T-cell activation or with a change in the normal proportion of T-cell subsets.

It has been previously reported that treatment with anti-CD45RB mAb in B6 mice resulted in a shift of CD45RB isoform from the high (CD45RBhi) to the low (CD45RBlow) expression on CD4+ T-cells (8,10) and that the concomitant use of anti-CD154 mAb did not alter this phenomenon (11). In the present study, no measurable shift in the CD45RB expression on CD4+ T-cells was observed in NOD recipients that were treated with the anti-CD45RB mAb alone. Although the proportion of CD4+ T-cells that expressed a CD45RBlow phenotype was slightly higher than that observed in control animals that received a transplant, this was not statistically significant (mean ± SD = 64 ± 17.8% vs. 50 ± 15.3%, respectively; two-tailed, unpaired t test P = NS). Surprisingly, animals that received the combination therapy showed an increased rate of CD4+CD45RBlow cells (75 ± 3.4%; P = 0.021 versus transplanted controls; Fig. 2A and B). These results suggest that in spontaneously diabetic NOD mice, unlike in B6 mice (11), administration of anti-CD45RB mAb alone is not sufficient to induce a significant shift in the CD45RB isoform expression on CD4+ T-cells and that the concomitant administration of anti-CD154 mAb allows for the occurrence of this phenomenon.

To test whether the age of the recipient plays a role in determining the efficacy of the anti-CD45RB mAb treatment in promoting CD45RB isoform shift, we performed additional experiments using 1-week-old NOD mice. Animals received the short-course treatment with the antibody directed to CD45RB either alone or in combination with anti-CD154 mAb, and phenotypic analysis was performed on spleen cells after 1 and 2 weeks from the last injection. In contrast to what was observed in adult diabetic NOD mice, when CD45RB mAb alone was administered, young prediabetic NOD mice showed a dramatic shift from CD45RBhi to CD45RBlow isoform expression that was comparable to that observed in adult animals that

**FIG. 1. Allogeneic islet graft survival in spontaneously diabetic NOD mice.** NOD mice that received a transplant were treated acutely (ac) with anti-CD154 or anti-CD45RB mAb on days −1, 0, and 5 (induction). Chronic (chr) treatment groups received, after induction, either weekly administration of anti-CD154 or three injections (same schedule as the induction) of the anti-CD45RB mAb semimonthly. A: Prolongation of graft survival was observed in all treated groups (Cox-Mantel test P = 0.002 versus controls). Administration of the combination of both antibodies acutely resulted advantageously when compared with test group (64.8 ± 15.3 days; n = 7; P = 0.67; Fig. 1B). Extending the administration of anti-CD45RB mAb alone, by repeating the course of administration (three injections) every 15 days, did not provide additional advantage over the peritransplant administration of the same mAb alone (19.2 ± 5.6 days; n = 5; P = NS; Fig. 1B).

When anti-CD154 mAb was given during the peritransplant period in association with chronic anti-CD45RB mAb therapy (until day 50), prolongation of the graft survival was observed (64.8 ± 31.5 days; n = 5), although it was not statistically different from that achieved using peritransplant combination therapy with anti-CD154 plus anti-CD45RB mAb (Fig. 1B).
were treated with the combination of both antibodies (not shown).

Phenotypic analysis of the CD45RB isoforms on CD8+ T-cells showed a pronounced increase of the CD45RBlow isoform expression that occurred both in animals that were treated with the anti-CD45RB mAb only and, to a higher degree, in those that were treated with the combination of anti-CD45RB and anti-CD154 mAb. In control mice, the expression of the CD45RBlow isoform on CD8+ T-cells was 20.9 ± 4.7% and rose to 37.3 ± 8.2% in anti-CD45RB–treated recipients and to 75 ± 3.5% in animals that received combination therapy (Fig. 2 A and B). Treatment with anti-CD45RB and anti-CD154 mAb effectively limits CD8+ T-cell invasion into the peri-islet infiltrate. Histological analysis of the grafted kidneys showed peri-islet mononuclear cell infiltration with preservation of the islet morphology in the long-term surviving grafts. Immunohistochemistry of the grafts obtained 10–12 days posttransplantation or from long-term accepted implants showed a reduction of the relative proportion of CD8+ T-cells within the infiltrate in the animals that received anti-CD45RB mAb in combination with anti-CD154 mAb, when compared with nontreated or anti-CD154 mAb–treated recipients (Fig. 3). This finding is consistent with what has been previously described in an allogeneic combination (11).

Intrgraft cytokine mRNA levels do not differ in experimental and control groups. For analyzing whether any of the immunomodulatory treatments had any sizable effect on the intrgraft cytokine expression, islet grafts of treated or untreated animals were obtained 10–12 days posttransplantation. Cytokine steady-level expression assessed by quantitative RT-PCR showed no difference in the levels of IL-10, IFN-γ, IL-2, and transforming growth factor-β between the different groups (Table 1).

In vitro lymphocyte proliferation reveals an increased response to IL-7 after CD3-stimulation in anti-CD45RB– and anti-CD154–treated animals. Proliferation of NOD recipient’s splenocytes (10–12 days posttransplantation) in response to IL-2, IL-4, or IL-7 in the

FIG. 2. FACS analysis of CD45RB isoform expression on T-cells. A: FACS profiles of CD45RB isoform expression on CD4+ and CD8+ T-cells of NOD mice treated with the indicated regimens (one representative experiment). B: CD45RB isoform expression on CD4+ and CD8+ cells expressed as mean ± SD of five individual experiments; significant shifts were observed in both CD4+ and CD8+ cell subsets in the group that received the anti-CD45RB and anti-CD154 mAb therapy and in CD8+ cells in animals that were treated with anti-CD45RB only. *P = 0.02; **P = 0.01; ***P = 0.000002.

FIG. 3. Histopathological analysis of islet grafts. Islet grafts were collected from control NOD mice 10 days posttransplantation (A–C) or from NOD mice that were treated with the anti-CD45RB and anti-CD154 mAb therapy 120 days after transplantation (D–F). Hematoxylin and eosin staining (H&E) was performed on paraflin-embedded sections (A and D). Immunohistochemistry for CD4+ (B and E) and CD8+ (C and F) T-cell subsets was performed on frozen sections. Control animals showed a classic pattern of rejection, with mononuclear cells infiltrating the graft, and loss of islet morphology. Conversely, recipients treated with anti-CD45RB and anti-CD154 mAb therapy showed only perisulular mononuclear infiltration, with preservation of islet structure. A remarkable reduction in CD8+ T-cell was also observed in this group, in grafts analyzed both at 10 days (not shown) and after 120 days posttransplantation. CD4+ cells were equally represented in both groups.
presence of PMA was not affected by any of the treatment protocols (Fig. 4A). Likewise, proliferation in response to anti-CD3 mAb in the presence of IL-2 or IL-4 revealed no differences between any of the treatment groups or untreated controls. However, an increased response to IL-7 after CD3 stimulation was observed in the splenocytes obtained from animals that received the double treatment (Fig. 4B). Mixed lymphocyte reaction (MLR) showed no difference among experimental groups in response to mitomycin-treated, donor-specific, third-party or syngeneic cells (not shown), indicating that the prolonged graft survival achieved was not associated with a generalized immunosuppressive effect or in vitro donor-specific hyporesponsiveness. Furthermore, these data indicate that the prolonged graft survival observed was not consequent to impaired proliferation potential and/or to reduced responsiveness to cytokines.

**Treatment with anti-CD45RB and anti-CD154 mAb induces enhanced IFN-γ production by CD3-stimulated lymphocytes.** Supernatants from anti-CD3 mAb-stimulated splenocytes obtained from transplanted NOD 10–12 days after transplantation mice showed no difference in IL-4 levels regardless of the treatment received. However, treatment with anti-CD45RB and anti-CD154 mAb led to a dramatic increase in IFN-γ production, when compared with untreated controls that received a transplant (unpaired, two-tailed t test \( P = 0.0022 \)), or to naïve NOD mice that did not receive a transplant (\( P = 0.013 \); Fig. 5A). Similarly, a substantial increase in IL-10 levels was observed in the group that received combination therapy, when compared with untreated controls, although it did not reach statistical significance (Fig. 5B).

**DISCUSSION**

Allograft transplantation in spontaneously diabetic NOD mice represents a stringent model for analysis of the concurrent effects of allore cognition and recurrence of autoimmunity on islet graft fate. It is common knowledge that strategies of immunosuppression that promote long-term survival of

![FIG. 4. Splenocyte responses to mitogens in vitro. Splenocytes obtained from NOD mice 10–12 days after transplantation were cultured in the presence of PMA and IL-2, IL-4, or IL-7. Thymidine incorporation (expressed as mean ± SE counts per minute [CPM] of three individual experiments) showed no difference in the proliferative response between the indicated groups (A). Proliferative response to IL-2 and IL-4 after anti-CD3 mAb stimulation also showed no difference among experimental groups, whereas an enhanced proliferation was observed in the anti-CD45RB and anti-CD154 mAb–treated group stimulated with IL-7 (mean ± SE CPM of three individual experiments B). \#\( P = 0.003 \).](image)
led to measurable prolongation of graft survival but did not allow for indefinite allograft acceptance in spontaneously diabetic NOD mice, suggesting an important contributing role for autoimmunity recurrence.

Extended administration of the anti-CD45RB mAb in combination with peritransplant anti-CD154 mAb showed a synergistic effect resulting in dramatic prolongation of graft survival and long-term graft acceptance in 40% of the recipients. Chronic administration of the anti-CD45RB mAb was critical for prolonging graft acceptance in the NOD mouse model, in contrast to what was observed in the allogeneic combination previously tested (11).

In B6 mice, the beneficial effect of the treatment with anti-CD45RB mAb was associated with CD45RB isoform shift (from high to low) on CD4+ T-cells (8–11). This altered isoform expression has been postulated as one of the mechanisms by which anti-CD45RB mAb therapy may alter T-cell activation signaling and promote immune tolerance (11). At variance, in the adult spontaneously diabetic NOD mice used as islet recipients, therapy with anti-CD45RB mAb alone induced only a small shift in CD45RB isoform expression on CD4+ T-cells, whereas a combination of mAb led to a significant shift toward the CD45RBlow phenotype. It is interesting that administration of anti-CD45RB mAb alone was able to induce a dramatic shift to the CD45RBlow expression in young prediabetic NOD mice. It might be speculated that after onset of autoimmune diabetes, the activation state of immune cells could be altered, resulting in a reduced sensitivity to the anti-CD45RB mAb–induced isoform shift in adult NOD mice.

Our data support an important role for this phenotypic change in the modulation of immune responsiveness, because it correlated with the treatment supporting longer graft survival. In addition, the group that received the combinatory treatment showed a dramatic shift to CD45RBlow expression in the CD8+ T-cell population that was reduced or absent in the other groups.

Although the mechanisms underlying the isoform shift are still not completely understood, the CD4+CD45RBlow population has been associated with downregulation of immune responses and with predominantly Th2 cytokine production (27–29). In our study, the beneficial effect of the treatment with anti-CD45RB and anti-CD154 mAb on graft survival was paralleled by an increased CD4+CD45RBlow cell population but was not associated with a classic skewing toward a Th2 response. In vitro assessment of cytokine production after CD3 stimulation revealed elevated levels of the Th2 cytokine IL-10 and of the predominantly Th1 cytokine IFN-γ by splenocytes obtained from animals that were treated with a combination of anti-CD45RB and anti-CD154 mAb, when compared with animals that received either mAb alone or were not treated. A similar cytokine production pattern, distinct from the generally accepted Th1, Th2, and Th0 classification, has been associated with a T-cell subset characterized by low proliferative potential, high production of IL-10 and IFN-γ, and immune regulatory properties both in vitro and in vivo (30). In particular, this cell subset was able to efficiently prevent CD4+CD45RBm-induced colitis in SCID mice in a manner comparable to that observed with CD4+CD45RBlow cells co-transfer (29–30).
Furthermore, in our study, the protective effect of the treatment was not related to a substantial deletion of selected T-cell subsets, because FACS analysis of splenocytes and peripheral blood lymphocytes showed no difference in the relative proportion of CD4+ or CD8+ cell populations. In addition, lymphocyte proliferative responses to IL-2 and IL-4, as well as to alloantigens, and to anti-CD3 mAb were unaffected by the treatments, suggesting a largely preserved immune responsiveness to relevant antigens. It is interesting that the combination therapy led to an increased response to IL-7 after anti-CD3 stimulation, suggesting an increased sensitivity of a T-cell subset(s) to this cytokine. IL-7 has been shown to induce T-cell proliferation, promoting CD8+ T-cell expansion and stimulating IFN-γ production (31). Also, IL-7 could promote expansion of thymus-derived regulatory cells, able to suppress diabetes occurrence when transferred, in combination with spleen cells obtained from spontaneously diabetic NOD mice (32). A more commonly used regulatory T-cell subset (CD4+CD25+) was unaltered by all treatments, suggesting that prolonged graft survival cannot be explained on the basis of a quantitative difference in this population.

Histological analysis of the grafts showed preservation of islet morphology in the presence of an intense peri-islet mononuclear cell infiltrate in animals that received the combination treatment. This observation is compatible with the known pattern of “benign peri-insulitis” and is in contrast with the “malignant insulitis” observed in rejecting animals in which the islet structure was completely lost to intra-islet infiltrating mononuclear cells (33). Assessment of graft-infiltrating cell subsets revealed a marked reduction of CD8+ T-cells, whereas no gross differences in CD4+ T-cell subset infiltration were observed. A reduction of CD8+ T-cells infiltrating islet allografts was previously reported in animals that were treated with anti-CD45RB mAb alone or in combination with anti-CD154 mAb in nonautoimmune diabetic mice (11).

In our study, anti-CD45RB mAb treatment was associated with a sizable shift to CD45RB-low isofrom of CD8+ T-cells, and the shift was enhanced by the combination with anti-CD154 mAb. This is at variance with the isofrom shift behavior of CD4+ cells, in which the administration of CD45RB mAb alone did not result in any significant isofrom shift in adult animals. This observation reveals different signaling requirements for CD4+ and CD8+ subsets and might contribute to explaining the differential behavior of the two subsets in terms of graft infiltrating efficacy. In this regard, a role for CD45 in regulating integrin-mediated adhesion of macrophages and T-cells has been described (11,34,35), as well as a partial depletion of CD8+ cells in lymph nodes after anti-CD45RB mAb treatment in rodents (11). It is therefore conceivable that these mechanisms might interfere with CD8+ T-cell migration to the graft site.

The NOD mouse has been indicated as characterized by a generalized defect in tolerance induction susceptibility (20,21), and indeed our data are indirectly in agreement with this observation, showing the requirement for additional immunomodulatory treatment to promote immunological alterations that are obtained with milder treatment in nonautoimmune diabetic mice. The reasons for the observed differences are not yet clear, but it is conceivable that the numerous immunological abnormalities peculiar to the NOD mouse might account, at least in part, for them (36).

Nonetheless, we have now demonstrated that prolonged islet allograft survival can be achieved in spontaneously diabetic NOD recipients without harsh preconditioning regimens and bone marrow transplantation. The long-term survival of islets that we observed in 40% of the recipients is paralleled by a remarkably preserved immune responsiveness to donor-specific and third-party allostimulation. It is interesting that these results demonstrate that agents that are able to induce tolerance in certain circumstances may still be useful in producing prolonged immunosuppression in resistant hosts. In this regard, it may not be surprising that the changes in the host immune system previously associated with anti-CD45RB-mediated tolerance were either minimized or absent in this setting. We have at this time no explanation for the observed variability of graft survival (in which 40% survive long-term, whereas the rest only show prolongation), a phenomenon that has been often reported and might be due entirely to normal biological interindividual variability, even within an inbred strain.

Because there are no described diabetogenic effects characterizing the reagents used in our study, we believe that exploiting these strategies of T-cell signaling modulation and co-stimulatory blockade might prove a viable strategy for the treatment of islet transplantation recipients.

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