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Inotuzumab Ozogamicin Murine Analog–Mediated B-Cell Depletion Reduces Anti-islet Allo- and Autoimmune Responses

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B cells participate in the priming of the allo- and autoimmune responses, and their depletion can thus be advantageous for islet transplantation. Herein, we provide an extensive study of the effect of B-cell depletion in murine models of islet transplantation. Islet transplantation was performed in hyperglycemic B-cell-deficient (μMT) mice, in a purely alloimmune setting (BALB/c into hyperglycemic C57BL/6), in a purely autoimmune setting (NOD,SCID into hyperglycemic NOD), and in a mixed allo-autoimmune setting (BALB/c into hyperglycemic NOD). Inotuzumab ozogamicin murine analog (anti-CD22 monoclonal antibody conjugated with calicheamicin [anti-CD22/call]) efficiently depleted B cells in all three models of islet transplantation examined. Islet graft survival was significantly prolonged in B-cell–depleted mice compared with control groups in transplants of islets from BALB/c into C57BL/6 (mean survival time [MST]: 16.5 vs. 12.0 days; P = 0.004), from NOD,SCID into NOD (MST: 23.5 vs. 14.0 days; P = 0.03), and from BALB/c into NOD (MST: 12.0 vs. 5.5 days; P = 0.003). In the BALB/c into B-cell–deficient mice model, islet survival was prolonged as well (MST: μMT = 32.5 vs. WT = 14 days; P = 0.002). Pathology revealed reduced CD3+ cell infiltration and confirmed the absence of B cells in treated mice. Mechanistically, effector T cells were reduced in number, concomitant with a peripheral Th2 profile skewing and ex vivo recipient hyporesponsiveness toward donor-derived antigen as well as islet autoantigens. Finally, an anti-CD22/call and CTLA4-Ig–based combination therapy displayed remarkable prolongation of graft survival in the stringent model of islet transplantation (BALB/c into NOD). Anti-CD22/call–mediated B-cell depletion promotes the reduction of the anti-islet immune response in various models of islet transplantation. Diabetes 61:155–163, 2012

RESEARCH DESIGN AND METHODS

Mice. Female C57BL/6, BALB/c, B6.C.H-2bm12 (bm12), NOD, and NOD,SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B-cell-deficient (μMT) mice on a C57BL/6 genetic background were purchased from

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B-cell depletion and B-cell depletion in both C57BL/6 and NOD mice (data not shown).

To evaluate the effect of islet transplantation on peripheral B-cell numbers, we performed islet transplantation in three different immune settings: 1) a purely allogeneic setting (BALB/c islets transplanted into C57BL/6 mice) labeled as allo, 2) a purely autoimmune setting (NOD.NOD SCID islets transplanted into NOD mice) labeled as auto, and 3) a mixed allo-and autoimmune setting (BALB/c islets transplanted into NOD mice) labeled as allo/auto. Anti-CD22/cal was administered in two injections (160 μg.i.p. at days 0 and 5), as was cal treatment, which was given at the same dosage and timing. A group of mice was left untreated. CTLA4-Ig was administered to NOD mice using the following protocol: 500 μg on day 0 and 250 μg on days 2, 4, 6, 8, and 10. NOD mice were transplanted after a minimum of 7 to a maximum of 14 days from the onset of hyperglycemia to avoid a possible confounding effect, since we demonstrated that diabetes may be reverted by B-cell depletion within 3 days from the onset of hyperglycemia (9). When required, streptozotocin-induced diabetic or spontaneously hyperglycemic NOD mice were kept alive by insulin pellet.

**ELISPot and Luminex analysis.** ELISPot (BD Biosciences, San Jose, CA) and Luminex assays (Millipore, Billerica, MA) were performed, as described, to measure cytokines (25).

**Statistical analysis.** Data are expressed as means ± SEM. Kaplan-Meier analysis was used for survival analysis. To compare groups, we used a two-sided unpaired Student t test (for parametric data) or Mann-Whitney U test (for nonparametric data) according to distribution. P < 0.05 (by two-tailed testing) was considered an indicator of statistical significance. Data were analyzed with an SPSS statistical package for Windows (SPSS, Chicago, IL).

**RESULTS**

Inotuzumab ozogamicin murine analogue (anti-CD22/cal) depletes B cells in islet-transplanted mice. We first evaluated the effect of islet transplantation on peripheral B-cell numbers. In chemically (streptozotocin) induced hyperglycemic C57BL/6 mice receiving BALB/c islets, the percentage of splenic B220+ cells remained unchanged at days 7, 14, and 28 after islet transplantation (data not shown). To assess whether treatment with anti-CD22/cal was able to efficiently deplete B cells in murine models, we performed islet transplantation in three different immune settings: 1) a purely allogeneic setting (BALB/c islets transplanted into C57BL/6 mice) labeled as allo, 2) a purely autoimmune setting (NOD.NOD SCID islets transplanted into NOD mice) labeled as auto, and 3) a mixed allo- and autoimmune setting (BALB/c islets transplanted into NOD mice) labeled as allo/auto. The percentage of B cells in the spleens of mice treated with anti-CD22/cal (160 μg.i.p., at days 0 and 5) or cal (160 μg.i.p., at days 0 and 5) or untreated (160 μg.i.p., at days 0 and 5) or untreated was evaluated at different time points by fluorescence-activated cell sorter analysis (Fig. 1). In all three models of islet transplantation, anti-CD22/cal treatment depleted B cells up to 14 days after transplantation (Fig. 1). In allo-treated mice (in C57BL/6 but not NOD recipient mice), a small B-cell depletion was evident. Of note, a strain-dependent effect with regard to the efficacy of B-cell depletion was observed. Indeed, a less profound depletion was evident in anti-CD22/cal–treated C57BL/6 mice receiving BALB/c islets (Fig. 1A, C), compared with NOD mice receiving NOD.NOD SCID or BALB/c (Fig. 1B, C and 3). The full recovery of B cells after anti-CD22/cal treatment was observed at ~8 weeks after transplantation in both C57BL/6 and NOD mice (data not shown).

**B-cell depletion and beta-cell deficiency prolong islet graft survival without inducing tolerance.** We evaluated the effect of anti-CD22/cal–mediated B-cell depletion on islet allograft survival in the three aforementioned settings of the immune response (Fig. 2A–D). In allo, untreated and control antibody (cal)-treated mice invariably rejected allografts (mean survival time [MST]: untreated = 14.0 days; cal = 12.0 days; NS) (Fig. 2A), whereas mice that were B-cell depleted with anti-CD22/cal treatment displayed a slight prolongation in allograft survival compared with controls (MST: anti-CD22/cal = 16.5 days; anti-CD22/cal vs. all, P < 0.05) (Fig. 2A). In auto, mice treated with anti-CD22/cal showed a significant delay in syngeneic islet graft rejection compared with both cal-treated and untreated mice (MST: anti-CD22/cal = 23.5 days; untreated = 14.0 days; cal = 15.0 days; anti-CD22/cal vs. all, P < 0.05) (Fig. 2B). In allo/auto, in which both auto- and alloimmunity exert their effects, untreated and cal-treated mice rapidly rejected islet grafts within a few days after transplantation, whereas B-cell-depleted mice displayed significant prolongation of graft survival (MST: anti-CD22/cal = 12.0 days; untreated = 5.5; cal = 7.5 days; anti-CD22/cal vs. all, P < 0.05) (Fig. 2C). To study the effect of the absence of B cells on islet allograft survival, we transplanted BALB/c islets into genetically B-cell–deficient μMT mice (on a C57BL/6 background); in this setting, islet rejection was significantly delayed compared with wild-type (WT) control mice (MST: WT = 14.0 vs. μMT = 32.5 days; P = 0.002), but none of the islet-transplanted mice achieved graft tolerance (Fig. 2D).

**B-cell depletion preserves islet morphology.** Islet grafts were analyzed at different time points in the three aforementioned immune settings of islet transplantation to evaluate the effect of B-cell depletion on the quality of graft structure and islet infiltrate. Grafts from BALB/c into C57BL/6 and NOD.SCID into NOD transplants were collected 14 days after transplantation, whereas BALB/c into NOD transplant grafts were collected at day 7 (the average time of rejection of control mice was chosen) (Fig. 3). In allo, mice treated with anti-CD22/cal (Fig. 3A, 1–5) displayed preserved islet structure with a mild CD3+ cell infiltrate and no B220+ cells in the graft compared with control treated mice (Fig. 3B, 1–5). In auto, anti-CD22/cal–treated mice showed a clear preservation of islet structure with some CD3+ cells surrounding the islets, few B220+ and FoxP3+ cells, and a clear insulin-positive cell staining (Fig. 3C, 1–5). Cal-treated mice (Fig. 3D, 1–5) maintained preservation of islet structure with some insulin staining and abundant CD3+ and B220+ cells infiltrating the graft. In allo/auto at day 7, islet morphology was maintained in anti-CD22/cal–treated mice (Fig. 3E, 1–5), with clear insulin staining but several CD3+ cells (and some B220+ cells) infiltrating the graft. Conversely, cal-treated mice (Fig. 3F, 1–5) displayed an equal distribution of CD3+ and B220+ cells throughout the islet graft, resulting in a complete disruption of islet architecture and few remaining insulin-positive cells in the remnants of β-cells.

**B-cell depletion halts the expansion of effector T cells.** We studied the effect of B-cell depletion on the immune system and particularly on effector T cells (Teffs) and regulatory T cells (Tregs) 14 days after islet transplantation in allo and auto and at day 7 in allo/auto. Teffs and Tregs were quantified in the spleens of anti-CD22/cal–treated, cal-treated, or untreated mice. In allo, anti-CD22/cal–treated mice displayed a significant reduction in CD4+CD44hiCD62Llow (CD4+ effector) cell number compared with control mice (anti-CD22/cal = 4.68 × 10^5 ± 6.8 × 10^5; untreated = 11.6 × 10^5 ± 5.6 × 10^5; cal = 8.4 × 10^5 ± 5.2 × 10^5, absolute number of cells; anti-CD22/cal vs. all, P < 0.05) (Fig. 4A, 1), whereas cal-treated mice showed a partial reduction in CD4+ effector cell number (cal vs. untreated, P = 0.01) (Fig. 4A, 1). No differences in the number of peripheral CD8+CD44hiCD62Llow (CD8+ effector)
cells were evident among the three groups (Fig. 4A, 2). In this model, B-cell depletion is associated with an overall reduction of peripheral CD4+CD25+FoxP3+ (Tregs) both in anti-CD22/cal- and cal alone-treated mice compared with untreated mice (Fig. 4A, 2). Indeed, anti-CD22/cal treatment did not affect CD4+ and CD8+ T-eff number, nor did it affect T-reg number. In this model, B-cell depletion is associated with an overall reduction of peripheral CD4+CD25+FoxP3+ (Tregs) both in anti-CD22/cal- and cal alone-treated mice compared with untreated mice (Fig. 4A, 2). In auto, no significant differences in the absolute numbers of T-effs and Tregs were evident among the three groups of mice studied (Fig. 4B, 1–3). Indeed, anti-CD22/cal treatment did not affect CD4+ and CD8+ T-eff number, nor did it affect Treg number. In auto/auto, a reduction in the absolute number of T-effs was found in anti-CD22/cal-treated mice compared with cal-treated and untreated mice. Both CD4+ and CD8+ T-effs (Fig. 4C, 1–2) were reduced in anti-CD22/cal-treated mice, whereas no differences were evident in the absolute number of Tregs (Fig. 4C, 3).

B-cell depletion reshapes the peripheral cytokine profile by skewing Th1 to Th2. We then evaluated the effect of B-cell depletion on peripheral cytokine profile after islet transplantation using the Luminex assay. In allo 14 days after islet transplantation, peripheral levels of the Th1 cytokine γ-interferon (IFN-γ) were reduced in the anti-CD22/cal-treated group, whereas an increase in peripheral levels of the Th2 cytokines interleukin (IL)-4 and IL-10 was evident (Fig. 5A, 1). No differences in the proinflammatory cytokines IL-6, IL-2, and IL-15 peripheral levels were noted (Fig. 5A, 1). In auto, no polarization of the immune response was observed by serum analysis after transplantation (Fig. 5B, 1). In this model, peripheral levels of Th1, Th2 (IL-4 and IL-10), and proinflammatory cytokines (IL-6) were unaffected by B-cell depletion. In allo/auto 7 days after islet transplantation, an overall suppression of the Th1 response was evident, with B-cell–depleted mice showing lower peripheral levels of IFN-γ (anti-CD22/cal = 4.2 ± 2.1 pg/mL; untreated = 52.5 ± 11.1 pg/mL; cal = 24.9 ± 5.5 pg/mL; anti-CD22/cal vs. all, P < 0.0001) (Fig. 5C, 1). Moreover, B-cell–depleted mice showed an increase in peripheral levels of the Th2 cytokines IL-4 (anti-CD22/cal = 1.1 ± 0.1 pg/mL; untreated = 0.5 ± 0.1 pg/mL; cal = 1.0 ± 0.1 pg/mL; anti-CD22/cal vs. untreated, P < 0.0001) and IL-10 (anti-CD22/cal = 5.5 ± 2.3 pg/mL; untreated = 1.5 ± 0.3 pg/mL; anti-CD22/cal vs. untreated, P = 0.03), thus confirming the skewing of Th1 to Th2 immune response (Fig. 5C, 1). Finally, proinflammatory cytokines IL-2 (anti-CD22/cal = 1.6 ± 0.3 pg/mL; untreated = 1.2 ± 0.1 pg/mL; cal = 11.0 ± 2.9 pg/mL; anti-CD22/cal vs. cal, P = 0.009) and IL-6 (anti-CD22/cal = 11.7 ± 1.4 pg/mL; untreated = 24.4 ± 2.1

FIG. 1. Anti-CD22/cal-mediated B-cell depletion in three immune settings of islet transplantation (allo, auto, and allo/auto). The percentage of B220+ cells was analyzed in the spleen of C57BL/6 and NOD mice at baseline and at 14 days after islet transplantation (n = 4 mice/condition). 1) In allo, BALB/c islets were transplanted into chemically induced C57BL/6 mice and treated with anti-CD22/cal, cal alone, or were left untreated ((A1–5)). Robust depletion of B220+ cells was evident 14 days after transplantation in mice treated with anti-CD22/cal compared with naïve untransplanted mice (baseline) (A1–5). 2) In auto, NOD SCID islets were transplanted under the kidney capsule of spontaneously hyperglycemic NOD mice (B1–5). Complete B-cell depletion was observed 14 days after transplantation in mice treated with anti-CD22/cal compared with naïve untransplanted mice (B1–5), whereas no depleting effect was found when mice were treated with cal or were untreated (NS, B3). In allo/auto, BALB/c islets were transplanted into spontaneously hyperglycemic NOD mice (C1–5). A complete B-cell depletion occurred 14 days after transplantation in mice treated with anti-CD22/cal compared with untransplanted (C1–5). No differences in B-cell peripheral percentage were observed in mice treated with cal or untreated (NS) (C1–5).
untreated, or cal-treated mice (Fig. 5A). In allo, a delay in graft rejection was evident in B-cell-depleted mice compared with control-treated and untreated mice (\(P = 0.004\)) (A). In auto, a prolongation of syngeneic graft survival was observed in B-cell-depleted mice compared with cal-treated (\(P = 0.006\)) and untreated mice (\(P = 0.003\)) (B). In allo/auto, delayed graft rejection was achieved in B-cell-depleted mice compared with cal-treated (\(P = 0.003\)) and untreated mice (\(P = 0.001\)) (C). When BALB/c islets were transplanted in chemically induced C57BL/6 B-cell-deficient mice, islet graft survival was significantly prolonged compared with WT C57BL/6 mice (\(P = 0.002\)) (D).

To provide clear evidence of the link between the absence of B cells and the skewing of the Th1 immune response, we performed an in vitro mixed-lymphocyte reaction where 0.5 \(\times 10^6\) splenocytes from transplanted mice 7 days after transplantation were rechallenged ex vivo with donor-derived antigen (i.e., BALB/c irradiated splenocytes) and islet-derived peptides. The ex vivo challenge with alloantigens confirmed that B-cell-depleted mice were donor hyporesponsive, with fewer IFN-\(\gamma\)-producing cells (anti-CD22/cal = 293.6 pg/mL; cal = 44.5 \(\pm\) 5.0 pg/mL; anti-CD22/cal vs. all, \(P < 0.01\)) were reduced compared with controls (Fig. 5C, 1).

**B-cell depletion promotes antidonor hyporesponsiveness.**

We also examined the effect of B-cell depletion on in vitro rechallenge with donor-derived alloantigens or islet-derived autoantigens using the ELISpot assay. In allo, splenocytes from transplanted mice were harvested 14 days after islet transplantation and rechallenged ex vivo with donor-derived antigen (i.e., BALB/c irradiated splenocytes) to stimulate an antidonor-specific alloimmunologic response. Anti-CD22/cal–treated mice displayed a reduction in global reactivity toward autoantigens with a skewing of the immune system from a Th1 to a Th2 phenotype (Fig. 5D). IFN-\(\gamma\)-producing cells were substantially reduced in B-cell–depleted mice (anti-CD22/cal = 84.6 \(\pm\) 10.3; untreated = 404.3 \(\pm\) 34.2; cal = 345.3 \(\pm\) 125.7, counted as number of IFN-\(\gamma\)-producing cells per 5 \(\times\) \(10^6\) total splenocytes; anti-CD22/cal vs. all, \(P < 0.05\)), with an increase in IL-4–producing cells (anti-CD22/cal = 203.6 \(\pm\) 24.5; untreated = 133.3 \(\pm\) 27.2; cal = 174.8 \(\pm\) 28.1, counted as number of IL-4–producing cells per 5 \(\times\) \(10^6\) total splenocytes; anti-CD22/cal vs. all, \(P < 0.05\)) (Fig. 5A, 2–3). In auto, the rechallenge of splenocytes 14 days after transplantation with islet autoantigens (i.e., BDC2.5 and islet-specific glucose-6-phosphatase catalytic subunit–related protein [IGRP]) did not show any differences in responsiveness toward autoantigens in anti-CD22/cal–treated, untreated, or cal-treated mice (Fig. 5B, 2). In allo/auto, B cells and the skewing of the immune system from a Th1 toward a Th2 response, we performed an in vitro mixed-lymphocyte reaction where 0.5 \(\times 10^6\) irradiated splenocytes of BALB/c mice were challenged with 0.5 \(\times 10^6\) splenocytes harvested from B-cell-deficient (\(\mu\)MT) or C57BL/6 WT mice...
in an ELISpot assay. The number of IFN-γ-producing cells was similar between the two groups (WT = 115 ± 28; B-cell deficient = 139 ± 28 number of IFN-γ-producing cells; NS), whereas the number of IL-4-producing cells increased in the absence of B cells (WT = 104 ± 20; B-cell deficient = 493 ± 48 number of IL-4-producing cells; P = 0.001) (Fig. 6A), with a lower IFN-γ-to-IL-4 ratio (WT = 1.2 ± 0.2; B-cell deficient = 0.2 ± 0.03 IFN-γ-to-IL-4 ratio; P = 0.008) (Fig. 6B).

Combinational targeting of indirect antigen presentation with B-cell depletion and CTLA4-Ig prolongs islet allograft survival in NOD mice. We sought to find a clinically relevant B-cell depletion–based immunosuppressive strategy to fully target indirect allorecognition in NOD mice. We combined anti-CD22/cal treatment (to inhibit B-cell–mediated T-cell activation) and CTLA4-Ig, a fusion protein that prevents the interaction between B7.1 and CD28 (to inhibit DC-mediated T-cell activation) (25). CTLA4-Ig treatment alone significantly prolonged islet graft survival with similar kinetic as anti-CD22/cal compared with untreated mice (MST: CTLA4-Ig = 13.0 days; anti-CD22/cal = 12.0 days; untreated = 5.5 days; untreated vs. all, P < 0.01) (Fig. 6C) and anti-CD22/cal+CTLA4-Ig treatment was able to further prolong islet allograft survival compared with monotherapeutic anti-CD22/cal or CTLA4-Ig (MST: anti-CD22/cal+CTLA4-Ig = 33.5 days; anti-CD22/cal+CTLA4-Ig vs. anti-CD22/cal, P < 0.001) (Fig. 6D). The relevance of this strategy was confirmed in the chemically induced hyperglycemic B-cell–deficient (μMT) mice transplanted with BALB/c islet, which treated with CTLA4-Ig showed indefinite islet allograft survival (data not shown). It is interesting that anti-CD22/cal+CTLA4-Ig–treated mice displayed a conspicuous number of peripheral reemerging B cells at the time of rejection (Fig. 6D). Histological analysis of the grafts of these anti-CD22/cal+CTLA4-Ig–treated mice revealed preserved islet structure and insulin staining with severe CD3+ and B220+ cell infiltration (Fig. 6E, I–5).

**FIG. 3.** Islet pathology in the three immune settings of islet transplantation (allo, auto, and allo/auto). Islets from BALB/c or NOD.SCID mice were transplanted under the kidney capsule of chemically induced hyperglycemic C57BL/6 or spontaneously hyperglycemic NOD mice treated with anti-CD22/cal or cal and were retrieved for histological analysis at different time points (n = 3 mice/group). Grafts from both BALB/c into C57BL/6– and NOD.SCID into NOD–transplanted mice were collected 14 days after transplantation, whereas BALB/c into NOD grafts were collected at day 7 at the time of graft rejection of control mice. 1) In allo at day 14, anti-CD22/cal–treated mice displayed preserved islet architecture (A1) with mild CD3+ cell infiltrate (A2), no B220– cells (A3), and few FoxP3+ cells (A4). Insulin staining was clearly preserved (A5). Cal–treated and untreated mice showed severe cellular infiltration with a complete disruption of islet architecture (B1) and several CD3+ (B2) and B220+ cells (B3); some CD3+ cells also coexpressed FoxP3+ (B4). Very few cells were positive for insulin (B5). 2) In auto at day 14, anti-CD22/cal–treated mice displayed clear preservation of islet structure (C1) with several CD3+ cells in the infiltrate (C2) surrounding the islets, along with few B220– (C3) and FoxP3+ cells (C4) as well as abundant insulin-positive cells (C5). Cal–treated mice maintained preservation of islet structure (D1) with insulin-positive cells (D5) but with a massive CD3+ (D2) and B220+ (D3) cell infiltrate and with some FoxP3+ (D4) cells evident in the islet mass. 3) In allo/auto at day 7, islet architecture appeared preserved in anti-CD22/cal–treated mice (E1) with clear insulin staining (E5) but with severe CD3+ cell (E2) infiltrate and few FoxP3+ cells (E4) surrounding the islets, whereas B cells were absent (E3). Conversely, cal–treated mice displayed equally distributed CD3+ (F2) and B220+ (F3) cell infiltration, resulting in complete disruption of islet architecture (F1) and scant insulin staining (F5). Almost no infiltrating FoxP3+ cells were evident (F4). H&E, hematoxylin-eosin. (A high-quality digital representation of this figure is available in the online issue.)
compared with untreated mice, which displayed no insulin staining with lesser CD3+ and B220+ cell infiltration (Fig. 6F, 1–5). We then analyzed the frequency of Teffs and Tregs in the spleens of transplanted mice at the time of rejection, and a reduction in the number of CD8+ effector cells was observed in anti-CD22/cal+CTLA4-Ig–treated mice compared with untreated control mice (Fig. 6H), whereas no difference was seen in the percentages of CD4+ effector cells (Fig. 6G) or Tregs (Fig. 6I) at the time point examined. Moreover, splenocytes from transplanted mice were rechallenged in vitro with donor-derived antigen (BALB/c irradiated splenocytes), and the donor-specific
immune response was evaluated using the ELISpot assay. We observed a significant reduction in IFN-γ-producing cells in anti-CD22/cal+CTLA4-Ig–treated mice compared with untreated mice at the time of rejection (Fig. 6J). Moreover, the status of the autoimmune response in treated mice was evaluated by restimulating splenocytes from transplanted mice ex vivo with BDC2.5 (to activate the CD4+ autoreactive T-cell response) and IGRP (to activate the CD8+ autoreactive T-cell response) peptides (Fig. 6K). We confirmed that the immune response toward autoantigen was suppressed in anti-CD22/cal+CTLA4-Ig–treated mice compared with untreated mice at the time of rejection (BDC2.5-stimulated IFN-γ-producing cells: anti-CD22/cal+CTLA4-Ig = 84.4 ± 5.9; untreated = 129.8 ± 14.5, counted as number of IFN-γ–producing cells per 1 × 10^6 total splenocytes; P = 0.02; IGRP-stimulated IFN-γ–producing cells: anti-CD22/cal+CTLA4-Ig = 22.5 ± 4.0; untreated = 48.5 ± 10.5, counted as number of IFN-γ–producing cells per 1 × 10^6 total splenocytes; P = 0.04). We observed that anti-CD22/cal+CTLA4-Ig treatment inhibited, in a dose-dependent manner, the number of IFN-γ–producing cells induced by DCs (no drug = 332 ± 23; CTLA4-Ig 1 μg/mL = 127 ± 29; CTLA4-Ig 10 μg/mL = 31 ± 5; CTLA4-Ig 100 μg/mL = 15 ± 3, counted as number of IFN-γ–producing

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FIG. 6. In vitro Th1 to Th2 skewing in B-cell-deficient mice and combinational immunosuppressive strategy in NOD mice. BALB/c splenocytes were challenged with B-cell-deficient or C57BL/6 splenocytes. IL-4-producing cells were increased in B-cell-deficient mice ($n = 3$) ($^*P = 0.001$) (A) with a reduction of IFN-$\gamma$–to–IL-4 ratio ($^*P = 0.009$) in the absence of B cells (B). Spontaneously hyperglycemic NOD mice were transplanted with BALB/c islets and treated with anti-CD22/cal alone or in combination with CTLA4-Ig; control treatments were anti-CD22/cal and CTLA4-Ig alone (C). Mice treated with anti-CD22/cal+CTLA4-Ig displayed prolonged islet graft survival compared with CTLA4-Ig ($^*P = 0.006$) or anti-CD22/cal ($^*P = 0.006$) treatment alone (C). At almost 40 days after transplantation, B220$^+$ cells reemerged in the spleens of anti-CD22/cal+CTLA4-Ig NOD mice (D). At the time of rejection, islet structure appeared well preserved in anti-CD22/cal+CTLA4-Ig–treated mice ($^*P < 0.05$) (E1). As expected, severe infiltration of CD3$^+$ cells (E2) with some FoxP3$^+$ (E4) expression was evident in the graft area in anti-CD22/cal+CTLA4-Ig–treated mice. Few B220$^+$ cells infiltrated the graft (E3), and insulin staining remained clearly preserved despite the complete loss of graft function (E5). At the day of rejection, untreated mice displayed a complete disruption of islet architecture (F1), an equally distributed CD3$^+$ (F2) and B220$^+$ (F3) cell infiltrate, few FoxP3$^+$ cells (F4), and scant insulin staining (F5). Splenocytes harvested from islet-transplanted NOD mice treated with anti-CD22/cal+CTLA4-Ig or from untreated mice were analyzed at the time of rejection ($n = 3$ mice/experiment). The percentages of CD4$^+$CD44hiCD62Llow (G) and CD4$^+$CD25$^+$FoxP3$^+$ cells (I) were similar in the two groups of mice, whereas a reduction in CD8$^+$CD44hiCD62Llow cells ($^*P < 0.05$). Splenocytes harvested from anti-CD22/cal+CTLA4-Ig–treated NOD mice were stimulated with donor-derived antigen (BALB/c) or islet-specific peptides (BD2C.5 and IGRP) and compared with untreated mice at the time of rejection ($n = 3$ mice/experiment) (J and K). Reduced numbers of IFN-$\gamma$–producing cells were evident in mice treated with the combinational strategy upon stimulation of the alloantigen immune response ($^*P = 0.003$) (J). In a similar manner, CD4$^+$ and CD8$^+$ autoreactivity to BD2C.5 ($^*P = 0.02$) and IGRP ($^*P = 0.04$), respectively (K), was significantly reduced in NOD mice treated with anti-CD22/cal+CTLA4-Ig compared with untreated NOD mice. bm12-derived B220$^+$ cells were challenged with ABM-derived CD4$^+$ cells with serial concentrations of CTLA4-Ig (1, 10, and 100 ng/mL). IFN-$\gamma$–producing cells, 96 h after stimulation, showed a dose-dependent reduction in the presence of CTLA4-Ig ($n = 3$) (no drug vs. all, $^*P < 0.001$) (L). H&E, hematoxylin-eosin. (A high-quality digital representation of this figure is available in the online issue.)
cells per $1 \times 10^8$ total splenocytes; no drug vs. all, $P < 0.01$ (data not shown) and B cells (no drug = 510 ± 37; CTLA4-Ig 1 µg/mL = 119 ± 29; CTLA4-Ig 10 µg/mL = 14 ± 4; CTLA4-Ig 100 µg/mL = 2 ± 0, counted as number of IFN-γ–producing cells per $1 \times 10^8$ total splenocytes; no drug vs. all, $P < 0.001$) (Fig. 6L).

**DISCUSSION**

Islet transplantation represents a transplant model in which the alloimmune response and recurrence of autoimmunity coexist, jeopardizing long-term islet function and ultimately leading to graft rejection (9,18,26). B cells have been shown to have an essential role as antigen-presenting cells (APCs) in the priming of T cells during the allo- and autoimmune responses (6,8,27), thus constituting a suitable target to modulate the immune response in islet transplantation (28). It is interesting that although clinical trials based on B-cell depletion in islet transplantation are ongoing (28), the literature is lacking in murine studies examining the effect of B-cell depletion in islet transplantation. Herein, we delineate the effect of B-cell depletion in three different models of murine islet transplantation: 1) in a purely alloimmune setting (BALB/c into chemically induced hyperglycemic C57BL/6 mice), 2) in a purely autoimmune setting (NOD.SCID into spontaneously hyperglycemic NOD mice), and 3) in a mixed allo- and autoimmune setting (BALB/c into spontaneously hyperglycemic NOD mice). We used the novel B-cell-depleting agent inotuzumab ozogamicin murine analog (anti-CD22/cal), which targets the CD22 receptor and, thus, depletes mature B cells from lymphoid organs (9,21), and cal alone, as control antibody. We observed a nearly complete depletion of B cells in the lymphoid organs (9,21), and cal alone, as control antibody. We used the novel B-cell-depleting agent inotuzumab ozogamicin murine analog (anti-CD22/cal), which targets the CD22 receptor and, thus, depletes mature B cells from lymphoid organs (9,21), and cal alone, as control antibody. We observed a nearly complete depletion of B cells in the lymphoid organs (9,21), and cal alone, as control antibody. We used the novel B-cell-depleting agent inotuzumab ozogamicin murine analog (anti-CD22/cal), which targets the CD22 receptor and, thus, depletes mature B cells from lymphoid organs (9,21), and cal alone, as control antibody. We observed a nearly complete depletion of B cells in the lymphoid organs (9,21), and cal alone, as control antibody. We used the novel B-cell-depleting agent inotuzumab ozogamicin murine analog (anti-CD22/cal), which targets the CD22 receptor and, thus, depletes mature B cells from lymphoid organs (9,21), and cal alone, as control antibody. We observed a nearly complete depletion of B cells in the lymphoid organs (9,21), and cal alone, as control antibody.

Unfortunately, the control antibody (cal), being a DNA-damaging cytotoxic agent, showed some depleting activity on B and T cells, but since there was no effect on islet allograft survival, we believe that this drug may be suitable as a negative control antibody for anti-CD22/cal. Our data, for the first time, elucidate the effect of B-cell depletion on the immune system in three settings of islet-transplanted mice and demonstrate that the absence of B cells (using depletion strategies or via genetic deletion) is not sufficient to induce islet graft tolerance. We speculate that B cells may be replaced by DCs as APCs or that the maintenance of some regulatory B cells (28–30) may be required to achieve graft tolerance (31,32). Indeed, our group previously demonstrated that B cells are required to obtain anti-CD45RB–mediated allograft tolerance and to inhibit alloreactive T-cell activation (33). The lack of efficacy of B-cell depletion in prolonging islet allograft survival may be related to the fact that B cells play a more relevant role in initial expansion of pathogenic T cells in NOD mice, but as soon as autoreactive T cells have become memory cells, B-cell depletion resulted in a limited effect. This can also explain why depleting B cells is slightly more effective in an alloimmune setting, in which T cells are naive at the time of transplantation. Our data revealed that B-cell depletion has a mild effect in prolonging islet graft survival, in reducing Teffs and skewing the immune response to Th2 profile in the auto setting, whereas autoimmune response was severely reduced in the mixed allo/auto setting. This may suggest that the reduced anti-islet response in the latter recipient group could be secondary to the suppressed alloimmune response. In summary, we demonstrate that B-cell depletion is effective in prolonging islet graft survival but is unable to induce allograft tolerance. Inotuzumab ozogamicin murine analog (anti-CD22/cal) is available for clinical use (34,35), thus representing a translational therapeutic approach (36,37). It is likely that depleting strategies (e.g., anti-T/B cells) are not fully effective when used as a monotherapy because of the redundancy of the immune response (38,39). Therefore, to completely halt T-cell activation and block residual APC function, we used an immunosuppressive regimen based on the combination of B-cell depletion and CTLA4-Ig treatment in the benchmark model of islet transplantation: the NOD mouse (Fig. 7). CTLA4-Ig is a clinically approved compound that prevents the interaction between CD80/CD86 (B7.1/B7.2) and CD28, leading to a reduction in clonal expansion, to T-cell anergy, and to inhibition of T-cell activation (40–42). In this study, we confirm that CTLA4-Ig inhibits DC/T-cell interaction in vitro, but for the first time, we show that B-cell–mediated T-cell activation is strongly inhibited by CTLA4-Ig as well. Our combination strategy

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<th>Outcome</th>
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<tr>
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<td>CD4+CD44hiCD62Llow (CD4+ Teffs)</td>
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<tr>
<td>CD4+CD25+FoxP3+ (Tregs)</td>
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<tr>
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<td>Proinflammatory cytokines (IL-6 and IL-2)</td>
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**TABLE 1**

The effect of B-cell depletion using inotuzumab ozogamicin murine analog (anti-CD22/cal) treatment on the anti-islet immune response in three murine models of islet transplantation. The B-cell-depletion effect is compared with control treatment in the three immune settings. Anti-CD22/cal treatment resulted in prolongation of islet graft survival, with preserved insulin-positive cells in the graft (in the three immune settings), a reduction in the absolute numbers of peripheral CD4+ and CD8+ effector cells (in allo and allo/auto), hyporesponsiveness to donor-derived and islet-derived antigens with a skewing from a Th1 to a Th2 immune response (in allo and allo/auto), and a reduction of proinflammatory cytokine levels (in allo/auto).

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prolonged allograft survival in a stringent model of islet transplantation, in which both allo- and autoimmune responses take part in the rejection process. A repeated, extended administration of a B-cell-depleting agent may be tested in clinical trials to inhibit reemerging B-cell-mediated chronic activation of the immune system, but because we show that islet allograft rejection occurs before B-cell recovery, we believe that B cells are partially responsible for triggering allograft rejection.

In conclusion, B-cell depletion significantly affects allo- and autoimmune responses, albeit to a lower extent in the latter process. B-cell depletion in monotherapy cannot be considered to be a tolerogenic strategy per se and must be accompanied by complementary or synergistic immunological strategies (e.g., costimulatory blockade).

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M.Ca. and A.P. performed research, analyzed data, and wrote the manuscript. A.V., K.M.L., S.T., and M.Ch. performed research. E.O., C.S., A.S., M.H.S., and J.F.M. designed research. K.D.-J. contributed vital new reagents. P.F. designed research, edited the manuscript, and is the guarantor for the article.

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