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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/cal]) 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 islet 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/cal 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/cal–mediated B-cell depletion promotes the reduction of the anti-islet immune response in various models of islet transplantation. *Diabetes* 61:155–165, 2012

Bcells traditionally have been investigated for their impact on hyperacute and chronic rejection (1–3) by virtue of their capacity to produce alloantibodies (4). In the past decade, investigators have focused on the antigen-presenting function of B cells in the priming of autoimmunity (5,6) and activation of allo- and autoreactive T cells (7–12). This notion is emphasized by two recent works from Yale University (11) and from our group (9), which demonstrate that B-cell–depletion strategies are capable of reversing established diabetes in NOD mice. B cells have been shown to be crucial players in the indirect allorecognition pathway as well by displaying a remarkable ability to capture alloantigens and activate alloreactive T cells (5,13). Indeed, Noorchashm et al. (5) demonstrated prolongation of heart allograft survival, disruption of antibody production, and a decrease in $CD4^+$ T-cell activation in a chimeric model of B-cell–restricted MHC class II–mediated antigen presentation deficiency. In the wake of these findings, authors have directed their attention to B-cell–depleting strategies to prolong graft survival (14–17). Among all transplant models, islet transplantation is of particular interest in applying a B-cell–based depleting strategy because islets endure attack from the allo- and autoimmune responses (18–20), but it is surprising that a systemic study of the role of B cells in murine models of islet transplantation is lacking. Indeed, treatment with anti-CD20 monoclonal antibody (mAb)—specifically, rituximab—in association with antithymocyte globulin induction, followed by a sirolimus-based immunosuppressive regimen, was found to improve long-term islet allograft survival in the nonautoimmune model of nonhuman primates (10), and a B-cell–depletion strategy is currently under investigation in a phase II clinical trial (NCT00468442). We have recently proposed the use of a B-cell–targeted cytotoxic immunoconjugate in autoimmune diabetes (anti-CD22 conjugated to calicheamicin, anti-CD22/cal or inotuzumab ozogamicin murine analog) capable of achieving complete depletion of B cells in peripheral blood, spleen, bone marrow, and lymph nodes (21). Given that CD22 also is expressed on $CD138^+$ plasma cells, this compound also has a potential effect on autoantibody production (9). Thus, we aimed to examine the effect of an inotuzumab ozogamicin murine analog–based B-cell–depletion strategy (anti-CD22/cal) in three different models of islet transplantation (BALB/c into C57BL/6, NOD.SCID into NOD, and BALB/c into NOD) in which allo- and autoimmune responses, separately or jointly, are responsible for the destruction of islet grafts (22,23). This approach will ultimately allow us to distinguish the beneficial effects of B-cell depletion on the different paths of the anti-islet immune response. Finally, we propose a novel and highly translational immunosuppressive strategy in islet transplantation with the goal of completely inhibiting indirect alloantigen presentation, based on the disruption of both primary aspects of antigen presentation (i.e., B-cell–mediated and dendritic cell [DC]-mediated T-cell activation).

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

The Jackson Laboratory as well. CD4$^+$ T-cell receptor transgenic mice specific for I-Abm12 expressed on bm12 mice (ABM) were available in our own laboratory (24).

Study design and treatment protocols. The inotuzumab ozogamicin murine analog compound (anti-CD22/cal mAb, provided by Pfizer) is a conjugate of an anti-mouse CD22 mAb and N-acetyl-calicheamicin dimethyl acid (9). Three murine models of islet transplantation were tested: *1*) a purely alloimmune setting (BALB/c islets transplanted into C57BL/6 mice) labeled as *allo*, *2*) a purely autoimmune setting (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. 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 analog (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 alloimmune setting (BALB/c islets into chemically induced hyperglycemic C57BL/6 mice) or allo, *2*) a purely autoimmune setting (NOD.SCID islets into spontaneously hyperglycemic NOD mice) or auto, and *3*) a mixed allo- and autoimmune setting (BALB/c islets into spontaneously hyperglycemic NOD mice) or 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 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 cal-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. 1*A*, *3*), compared with NOD mice receiving NOD.SCID or BALB/c (Fig. 1*B*, *3* and C, *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 B-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. 2*A*–*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. 2*A*), 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. 2*A*). 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. 2*B*). 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. 2*C*). 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. 2*D*).

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. 3*A*, *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. 3*B*, *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. 3*C*, *1*–*5*). Cal-treated mice (Fig. 3*D*, *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. 3*E*, *1*–*5*), with clear insulin staining but several CD3$^+$ cells (and some B220$^+$ cells) infiltrating the graft. Conversely, cal-treated mice (Fig. 3*F*, *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$^+$CD44^{hi}CD62L^{low} (CD4$^+$ effector) cell number compared with control mice (anti-CD22/cal = 4.68 × 10^7 ± 6.8 × 10^6; untreated = 11.6 × 10^7 ± 5.6 × 10^6; cal = 8.4 × 10^7 ± 5.2 × 10^6, absolute number of cells; anti-CD22/cal vs. all, $P < 0.05$) (Fig. 4*A*, *1*), whereas cal-treated mice showed a partial reduction in CD4$^+$ effector cell number (cal vs. untreated, $P = 0.01$) (Fig. 4*A*, *1*). No differences in the number of peripheral CD8$^+$CD44^{hi}CD62L^{low} (CD8$^+$ effector)
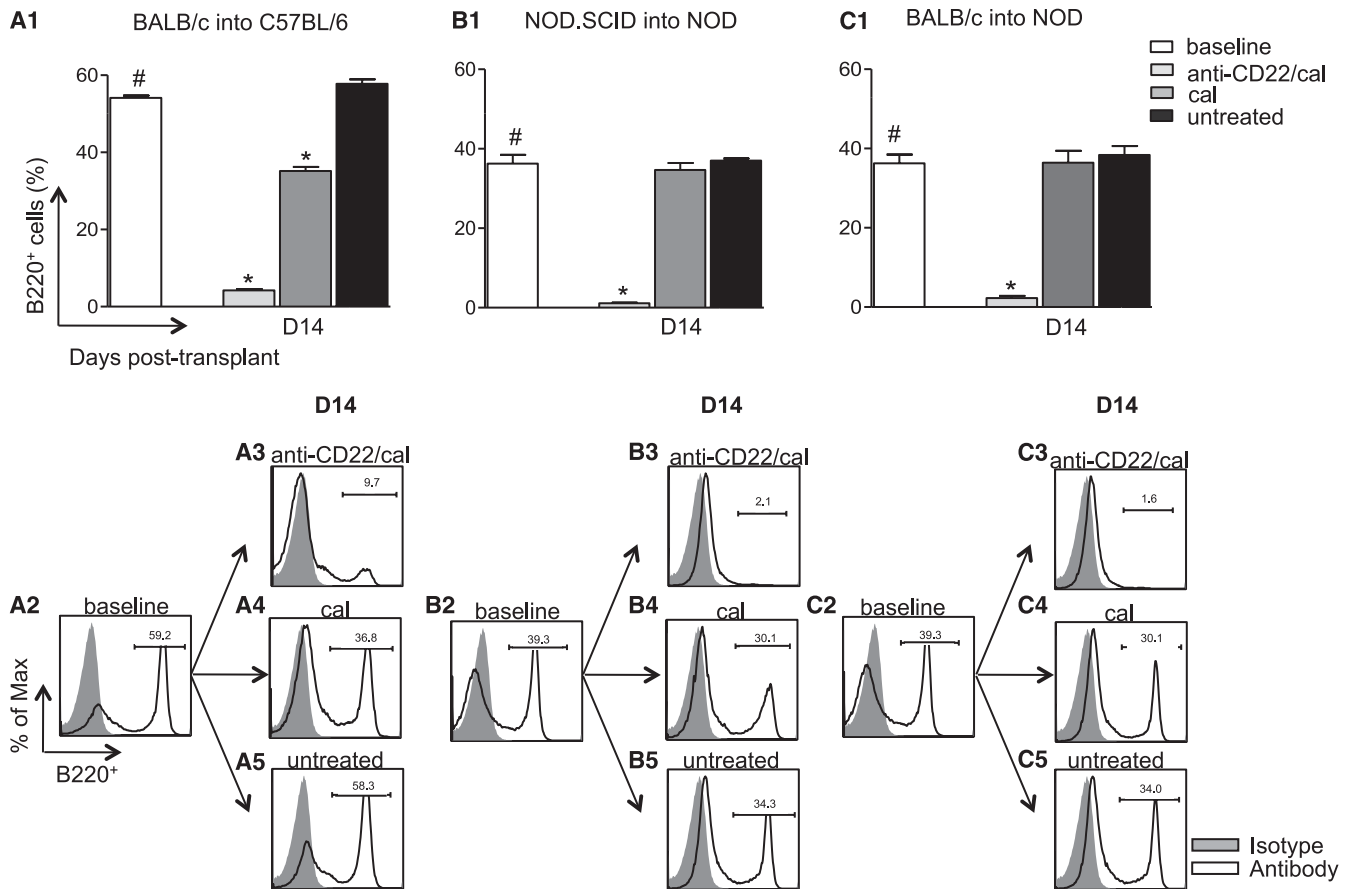
FIG. 1. Anti-CD22/cal–mediated B-cell depletion in three immune settings of islet transplantation (allo, auto, and allo/auto). The percentage of B220$^+$ cells was analyzed in the spleen of C57BL/6 and NOD mice at baseline and at 14 days after islet transplantation ($n = 4$ mice/condition). *1*) In allo, 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) (#$P < 0.0001$) and control antibody (cal)-treated mice (*$P < 0.0001$) (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 naive untransplanted (#$P < 0.0001$) and control-treated mice (*$P < 0.0001$) (B1–5), whereas no depleting effect was found when mice were treated with cal or were untreated (NS). *3*) 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 (#$P < 0.0001$), cal-treated (*$P < 0.0001$), and untreated mice (*$P < 0.0001$) (C1–5), whereas no differences in B-cell peripheral percentage were observed in mice treated with cal or untreated (NS) (C4–5).

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, *3*). In auto, no significant differences in the absolute numbers of Teffs 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$^+$ Teff number, nor did it affect Treg number. In allo/auto, a reduction in the absolute number of Teffs was found in anti-CD22/cal–treated mice compared with cal-treated and untreated mice. Both CD4$^+$ and CD8$^+$ Teffs (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, $P < 0.01$) (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; cal = 1.0 ± 0.0 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
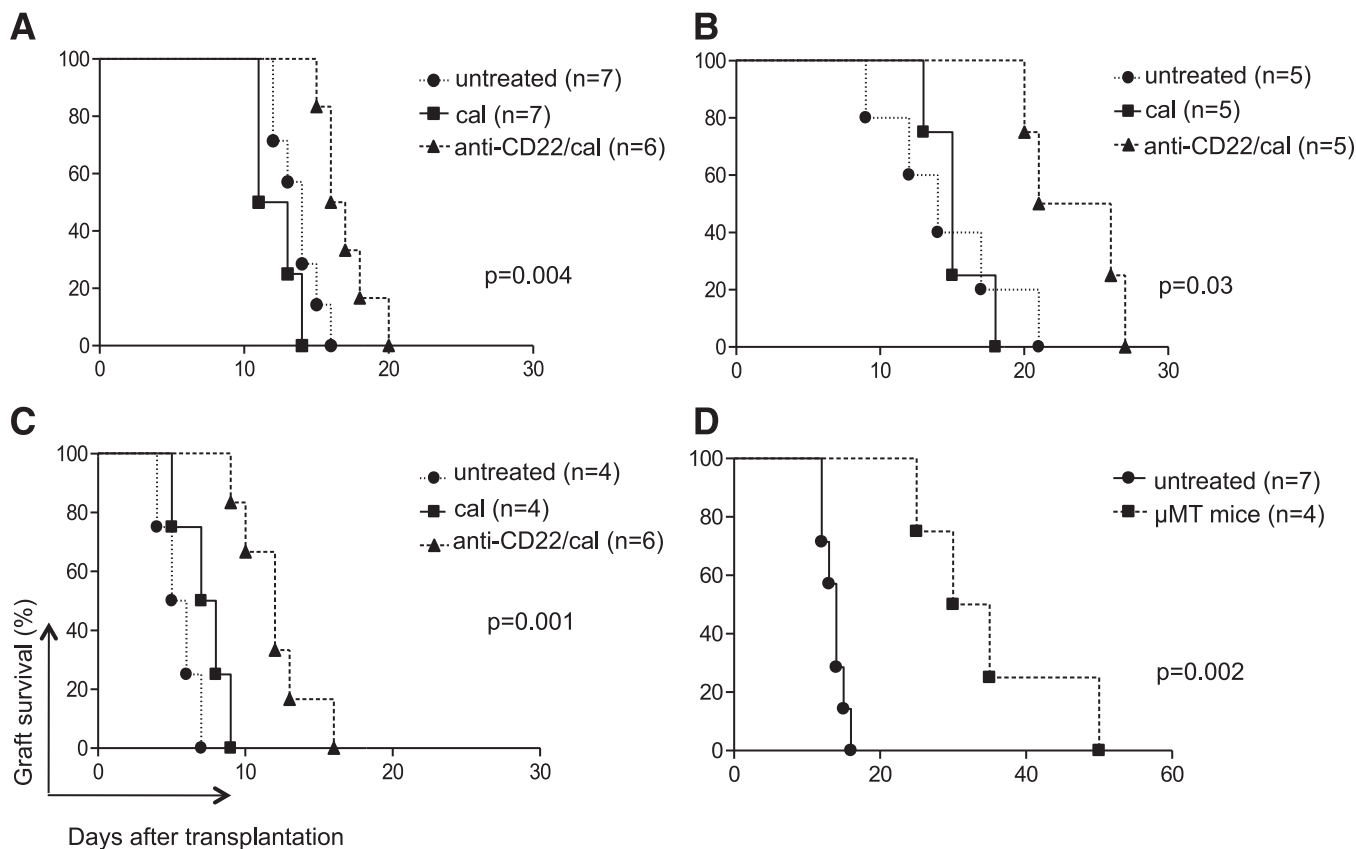
FIG. 2. Islet graft survival in B-cell–deficient (μMT) and B-cell–depleted (anti-CD22/cal) mice in the three immune settings of islet transplantation (allo, auto, and allo/auto). *1*) In allo, a delay in graft rejection was evident in B-cell–depleted mice compared with control-treated and untreated mice (*P* = 0.004) (*A*). *2*) 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* = 0.03) (*B*). *3*) 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*). *4*) 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*).

pg/mL; cal = 44.5 ± 5.0 pg/mL; anti-CD22/cal vs. all, $P <$ 0.01) were reduced compared with controls (Fig. 5*C*, *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 alloimmune response. Anti-CD22/cal–treated mice displayed a reduction in global responsiveness toward alloantigens with a skewing of the Th1 response toward a Th2 profile. IFN-γ–producing cells were substantially reduced in B-cell–depleted mice (anti-CD22/cal = 84.6 ± 10.3; untreated = 404.3 ± 34.2; cal = 345.3 ± 125.7, counted as number of IFN-γ–producing cells per 5×10^5 total splenocytes; anti-CD22/cal vs. all, $P < 0.05$), with an increase in IL-4–producing cells (anti-CD22/cal = 293.6 ± 24.5; untreated = 133.3 ± 27.2; cal = 174.8 ± 28.1, counted as number of IL-4–producing cells per 5×10^5 total splenocytes; anti-CD22/cal vs. all, $P < 0.05$) (Fig. 5*A*, *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. 5*B*, *2*). In allo/auto,

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-γ–producing cells (anti-CD22/cal = 224.5 ± 49.7; untreated = 426.5 ± 66.9; cal = 352.0 ± 24.5, counted as number of IFN-γ–producing cells per 5×10^5 total splenocytes; anti-CD22/cal vs. all, $P < 0.05$) and more IL-4–producing cells (anti-CD22/cal = 402.2 ± 91.2; untreated = 227.0 ± 13.9; cal = 199.1 ± 22.0, counted as number of IL-4–producing cells per 5×10^5 total splenocytes; anti-CD22/cal vs. all, $P < 0.05$) (Fig. 5*C*, *2* and *4*). These results were confirmed by ex vivo challenge of splenocytes harvested from islet-transplanted mice with CD4^{+} and CD8^{+} T-cell–restricted islet peptides (BDC2.5 and IGRP, respectively), demonstrating hyporesponsiveness toward autoantigens as well as a clear shift from a Th1 to a Th2 immune response in B-cell–depleted mice compared with both untreated and cal-treated controls (Fig. 5*C*, *3* and *5*).
B-cell absence induces Th1 to Th2 skewing in vitro. To provide clear evidence of the link between the absence of B cells and the skewing of the immune system from a Th1 toward a Th2 response, we performed an in vitro mixed-lymphocytes reaction where 0.5×10^6 irradiated splenocytes of BALB/c mice were challenged with 0.5×10^6 splenocytes harvested from B-cell–deficient (μMT) or C57BL/6 WT mice
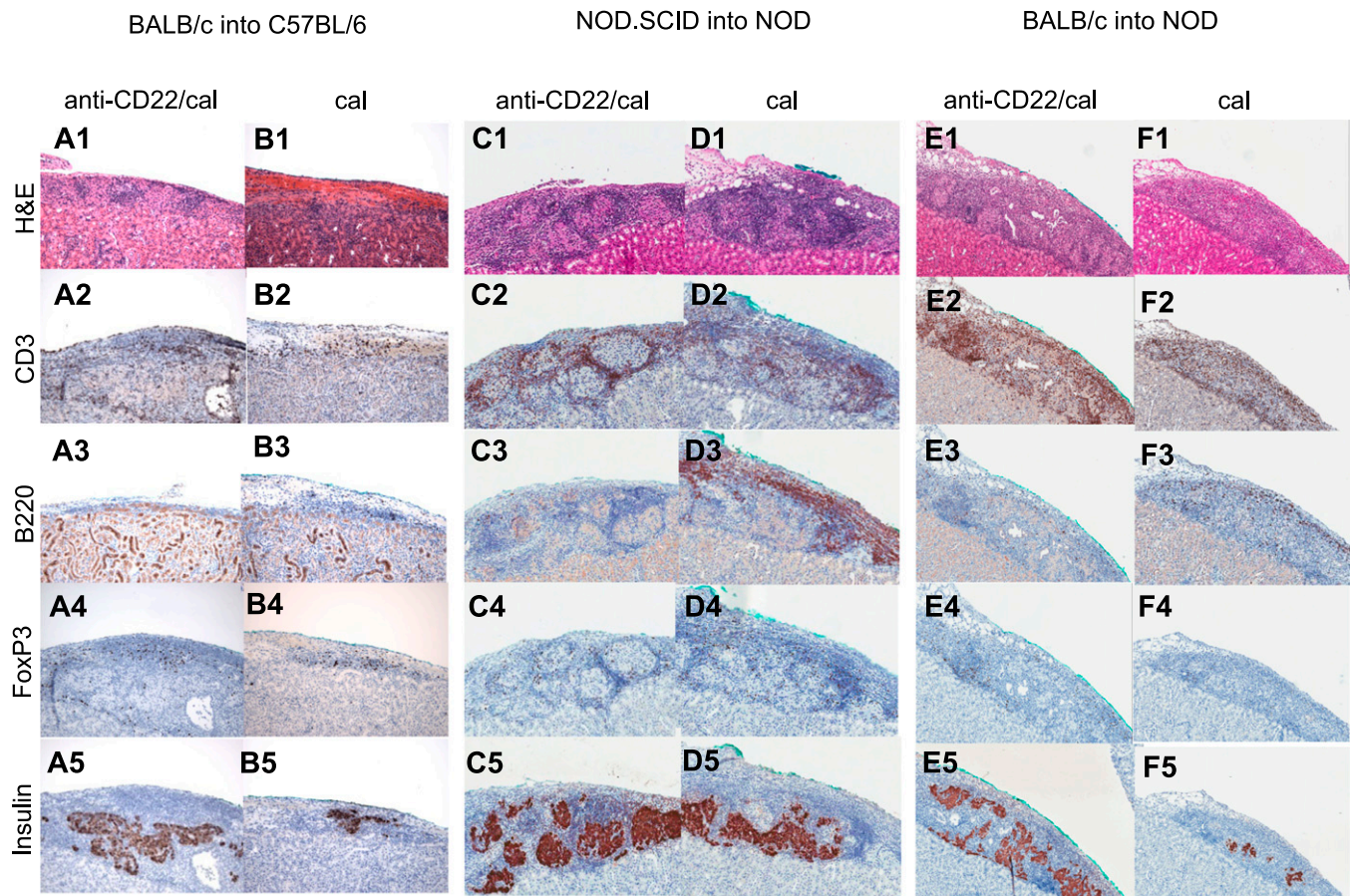
FIG. 3. Islet pathology in the three immune settings of islet transplantation (allo, auto, and allo/auto). Islets from BALB/c or NOD.SCID mice were transplanted under the kidney capsule of chemically induced hyperglycemic C57BL/6 or spontaneously hyperglycemic NOD mice treated with anti-CD22/cal or cal 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.)

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 = 0.009) (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. all, $P < 0.01$) (Fig. 6C). 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, 1–5)
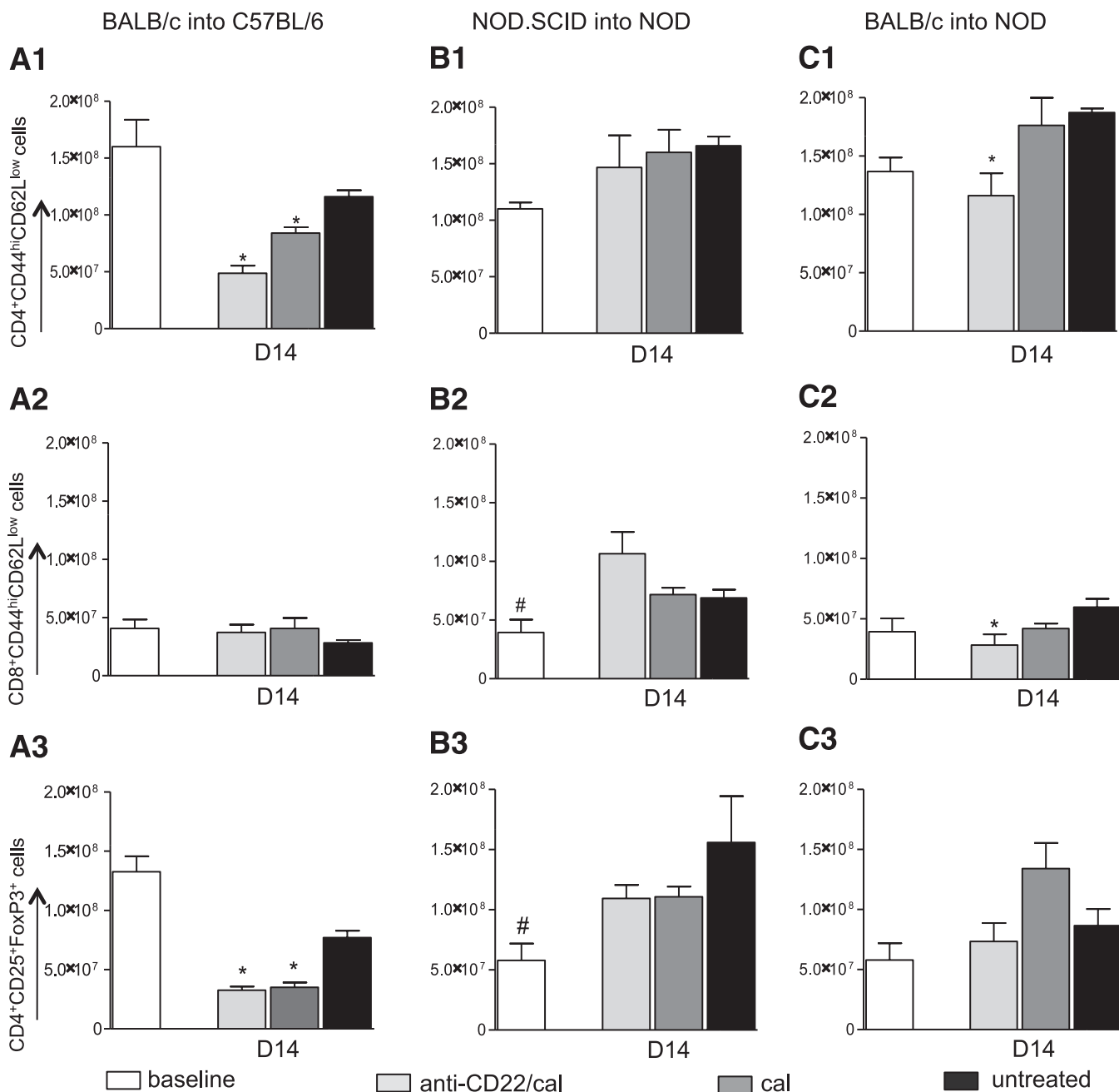
FIG. 4. Effect of B-cell depletion on the immune system in the three immune settings of islet transplantation (allo, auto, and allo/auto). Sple-nocytes were analyzed in the three groups of islet-transplanted mice at day 14 in the BALB/c into C57BL/6 and NOD.SCID into NOD groups and at day 7 in the BALB/c into NOD group ($n = 3$ mice/experiment). *1*) In allo, anti-CD22/cal treatment induced a decrease in the absolute number of CD4$^+$CD44^{hi}CD62L^{low} cells compared with untreated (*$P = 0.0002$) and cal-treated (*$P = 0.01$) mice (*A1*). Cal-treated mice, which experience a partial B-cell depletion, displayed a significant reduction in CD4$^+$CD44^{hi}CD62L^{low} cell (Teff) numbers as well (*$P = 0.01$) (*A1*). No differences in the absolute numbers of CD8$^+$CD44^{hi}CD62L^{low} cells (Teffs) were observed in this model (*A2*). In the same immune setting, a reduction in CD4$^+$CD25$^+$FoxP3$^+$ cell numbers was evident in the anti-CD22/cal–treated (*$P < 0.0001$) and in cal-treated (*$P = 0.0006$) groups compared with the untreated group (*A3*). *2*) In auto, no differences were evident with regard to either CD4$^+$CD44^{hi}CD62L^{low} or CD8$^+$CD44^{hi}CD62L^{low} cell numbers as well as in the peripheral numbers of CD4$^+$CD25$^+$FoxP3$^+$ cells in the B-cell–depleted group compared with both untreated and cal-treated mice (*B1–3*). Naïve untransplanted mice displayed reduced numbers of CD8$^+$CD44^{hi}CD62L^{low} T cells compared with anti-CD22/cal–treated mice (#$P = 0.03$) (*B2*), as well as reduced numbers of CD4$^+$CD25$^+$FoxP3$^+$ cells compared with both anti-CD22/cal–treated (#$P = 0.04$) and cal-treated mice (#$P = 0.03$) (*B3*). *3*) In allo/auto, anti-CD22/cal–treated mice displayed a significant reduction in CD4$^+$CD44^{hi}CD62L^{low} (*$P = 0.02$) and CD8$^+$CD44^{hi}CD62L^{low} (*$P = 0.04$) cell numbers (*C1–2*), with no significant differences in CD4$^+$CD25$^+$FoxP3$^+$ cell numbers (*C3*).

compared with untreated mice, which displayed no insulin staining with lesser CD3$^+$ and B220$^+$ cell infiltration (Fig. 6*F*, *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. 6*H*), whereas no difference was seen in the percentages of CD4$^+$ effector cells (Fig. 6*G*) or Tregs (Fig. 6*I*) 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
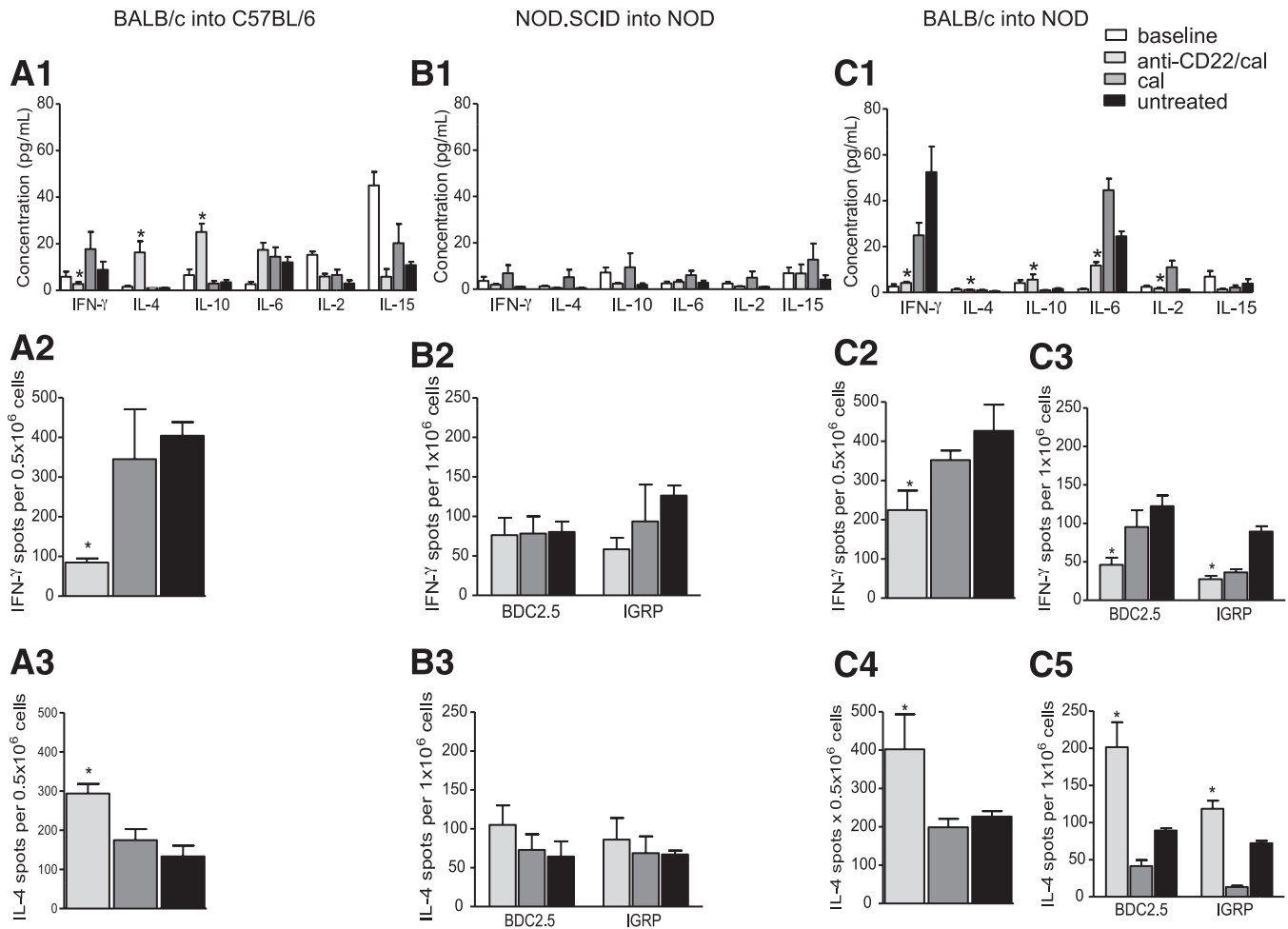
FIG. 5. Th1 to Th2 skewing of the immune response in the three immune settings of islet transplantation (allo, auto, and allo/auto) after B-cell depletion. Serum samples (n = 3 samples/experiment) and splenocytes (n = 3 mice/experiment) were analyzed in the three groups of islet-transplanted mice at day 14 in the BALB/c into C57BL/6 and NOD.SCID into NOD groups and at day 7 in the BALB/c into NOD group. Peripheral cytokine profiles and polarization of the auto- and alloimmune response were examined. 1) In allo, a decrease in the Th1 cytokine IFN-γ (*P < 0.05) and an increase in the Th2-relevant cytokines IL-4 (*P < 0.01) and IL-10 (*P < 0.001) were evident in B-cell–depleted mice compared with cal-treated and untreated mice (A1). These data were confirmed by the ELISpot assay in which donor-derived antigens (irradiated BALB/c splenocytes) were plated with splenocytes harvested from transplant recipients undergoing the different treatments. We found that anti-CD22/cal–treated mice exhibit a reduced number of IFN-γ–secreting cells compared with cal-treated and untreated mice (*P < 0.05) (A2), whereas IL-4–secreting cell numbers were significantly increased (*P < 0.05) (A3). 2) In auto, no change in peripheral cytokine levels was evident in the three groups of mice studied (B1), and similar numbers of IFN-γ– and IL-4–producing cells (B2 and B3, respectively) were observed after ex vivo restimulation with β-cell peptides (BDC2.5 and IGRP). 3) In allo/auto, examination of the cytokine profile (C1) revealed a systemic suppression of the inflammatory response (IL-6, *P < 0.01) with increased levels of the Th2-relevant cytokines IL-4 (*P < 0.01) and IL-10 (*P < 0.05) and a complete suppression in the Th1 immune response (IFN-γ, *P < 0.01; IL-2, *P < 0.01). In this animal model, both the allo- and autoimmune responses were examined (C2–5). Donor-derived antigen (BALB/c splenocytes) and islet peptides (BDC2.5 and IGRP) were plated with splenocytes harvested from the three groups of mice studied at day 7 after transplantation. B-cell–depleted mice displayed reduced numbers of IFN-γ–producing cells (*P < 0.05) and increased numbers of IL-4–producing cells (*P < 0.05) among both donor-specific T cells (stimulated using BALB/c splenocytes) (C2 and 4) and CD4⁺ (BDC2.5-stimulated, *P < 0.05) and CD8⁺ (IGRP-stimulated, *P < 0.05) (C3 and 5) autoreactive T cells.

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 × 10⁶ 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 × 10⁶ total splenocytes; P = 0.04) (Fig. 6K). We evaluated whether CTLA4-Ig is endowed with an immunologic effect on B/T-cell interaction. Thus, we took advantage of T-cell receptor transgenic mice and we plated 0.1 × 10⁶ CD11c⁺ or 0.2 × 10⁶ B220⁺ cells harvested from bm12 mice with 0.4 × 10⁶ CD4⁺ cells harvested from ABM mice in an ELISpot assay adding serial concentrations of CTLA4-Ig (1, 10, and 100 ng/mL). We found that CTLA4-Ig 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
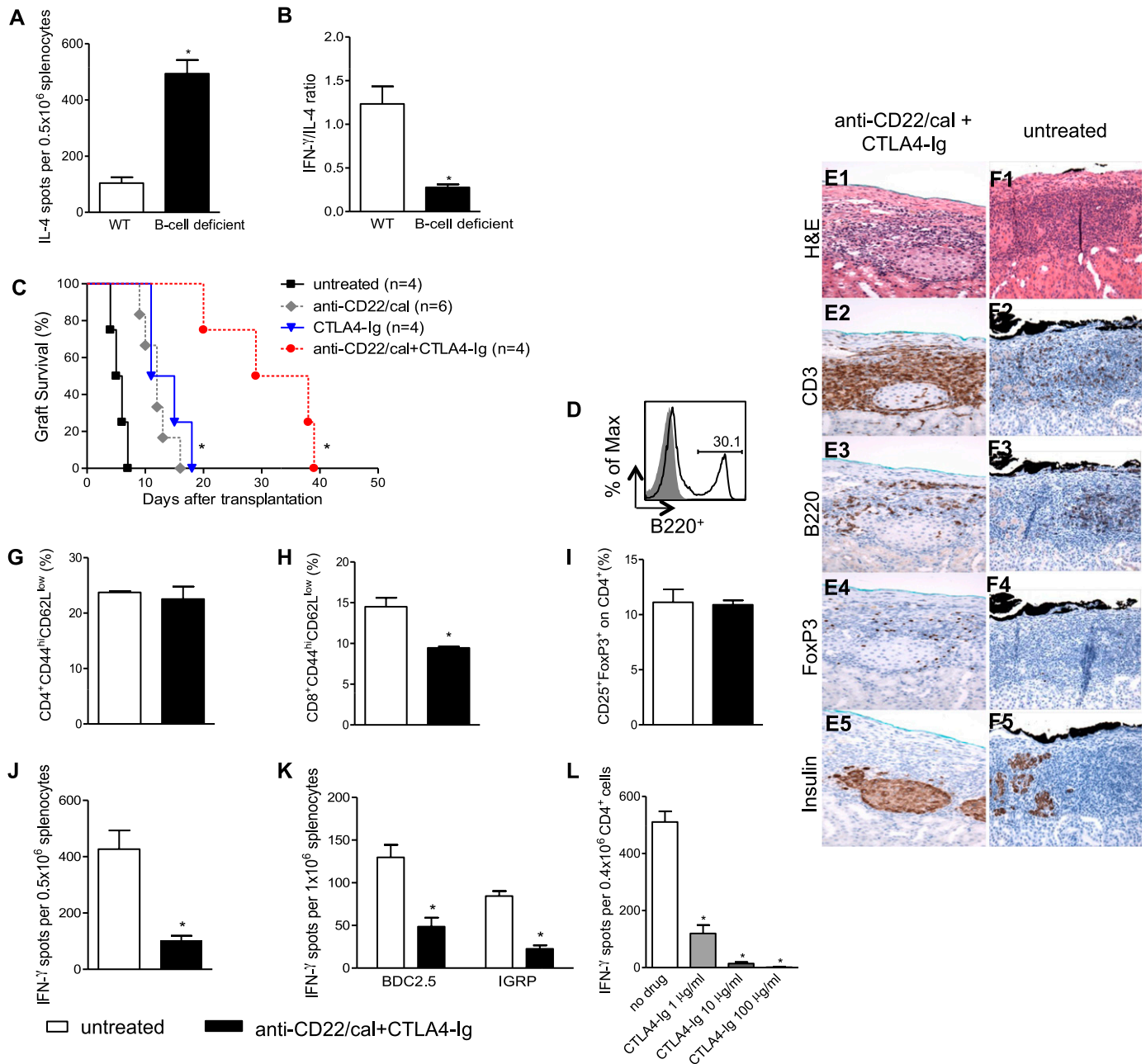
FIG. 6. In vitro Th1 to Th2 skewing in B-cell–deficient mice and combinational immunosuppressive strategy in NOD mice. BALB/c splenocytes were challenged with B-cell–deficient or C57BL/6 splenocytes. IL-4–producing cells were increased in B-cell–deficient mice ($n = 3$) (*$P = 0.001$) (A) with a reduction of IFN-γ–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 (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 (H) was evident in anti-CD22/cal+CTLA4-Ig–treated NOD mice (*$P < 0.05$). Splenocytes harvested from anti-CD22/cal+CTLA4-Ig–treated NOD mice were stimulated with donor-derived antigen (BALB/c) or islet-specific peptides (BDC2.5 and IGRP) and compared with untreated mice at the time of rejection ($n = 3$ mice/experiment) (J and K). Reduced numbers of IFN-γ–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 BDC2.5 (*$P = 0.02$) 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-γ–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 1×10^6 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 1×10^6 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 spleen 14 days after transplantation (depletion was more robust in NOD mice compared with the C57BL/6 strain), with a full recovery 8 weeks after treatment. When anti-CD22/cal was used as B-cell–depleting agent, a significant delay in islet graft rejection was found in all three models tested; however, B-cell depletion per se cannot induce tolerance, which was confirmed in B-cell–deficient µMT knockout mice. Graft pathology confirmed the complete absence of B cells in the graft infiltrate of the three models tested, with a prominent CD3^{+} cell infiltrate. Mechanistic studies revealed that mice treated with anti-CD22/cal experienced prolongation of graft survival, with preserved insulin staining in the graft, an overall reduction in peripheral Teff numbers in allo and allo/auto, and hyporesponsiveness to donor alloantigens (in allo) and to islet-derived autoantigens (in allo/auto), with a reshaping of the peripheral cytokine profile and an increase in IL-4 and IL-10 peripheral levels (Table 1). Of interest, we found that the absence of B cells leads to a polarization of allo- and autoimmune responses toward a Th2 response, with an inversion of IFN-γ/IL-4 production in the periphery, in the ex vivo challenge of T cells with donor-derived antigen and islet autoantigens and the in vitro mixed-lymphocytes reaction. 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 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

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

Outcome	Immune setting		
	Allo	Auto	Allo/Auto
Graft survival	↑	↑	↑↑
Insulin staining/islet morphology	↑↑	↑	↑↑
CD4^{+}CD44^{hi}CD62L^{low} (CD4^{+} Teffs)	↓↓	↔	↓
CD8^{+}CD44^{hi}CD62L^{low} (CD8^{+} Teffs)	↓↓	↔	↓
CD4^{+}CD25^{+}FoxP3^{+} (Tregs)	↓	↔	↔
Th1 cytokine (IFN-γ)	↓↓	↔	↓↓
Th2 cytokines (IL-4 and IL-10)	↑↑	↔	↑
Proinflammatory cytokines (IL-6 and IL-2)	↔	↔	↓↓

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).

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 naïve 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

B-cell depletion + CTLA4-Ig effect on indirect allorecognition
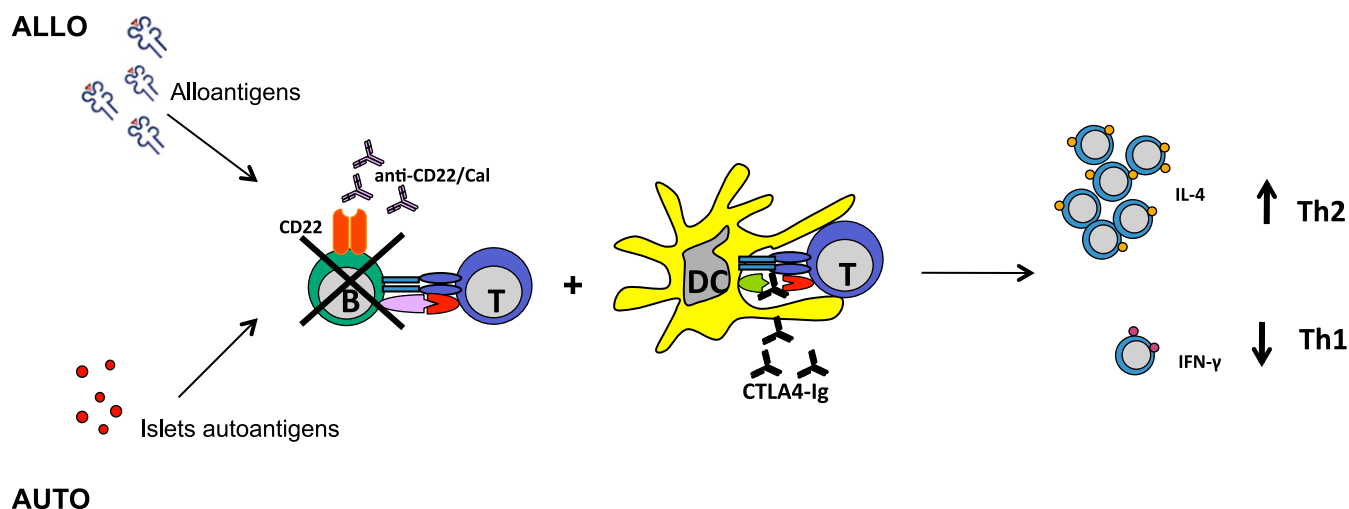

FIG. 7. Mechanism of action of the novel immunomodulatory regimen tested in islet transplantation in NOD mice. T-cell activation is mediated by APCs made up of B cells and DCs, as depicted. Our strategy is based on the complete blockade of T-cell activation in response to allo- and autoantigens through combining a B-cell–depleting agent (anti-CD22/cal) and CTLA4-Ig, which by blocking the CD28-B7.1 interaction, inhibits DC-mediated T-cell activation. This approach prolongs islet graft survival, induces hyporesponsiveness toward allo- and autoantigens, and tips the balance toward a Th2 anti-inflammatory phenotype of the immune response. (A high-quality color representation of this figure is available in the online issue.)

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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