

CD28/CD154 Blockade Prevents Autoimmune Diabetes by Inducing Nondeletional Tolerance After Effector T-Cell Inhibition and Regulatory T-Cell Expansion

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OBJECTIVE—Blocking T-cell signaling is an effective means to prevent autoimmunity and allograft rejection in many animal models, yet the clinical translation of many of these approaches has not resulted in the success witnessed in experimental systems. Improved understanding of these approaches may assist in developing safe and effective means to treat disorders such as autoimmune diabetes.

RESEARCH DESIGN AND METHODS—We studied the effect of anti-CD154 and CTLA4-Ig on diabetes development, and the requirements to induce tolerance in *nod.scid* mice after transfer of transgenic β -cell reactive BDC2.5.NOD T-cells.

RESULTS—*Nod.scid* recipients of diabetogenic BDC2.5.NOD cells were protected indefinitely from diabetes by a short course of combined costimulation blockade, despite the continued diabetogenic potential of their T-cells. The presence of pathogenic T-cells in the absence of disease indicates peripheral immune tolerance. T-cell maturation occurred in protected recipients, yet costimulation blockade temporarily blunted early T-cell proliferation in draining pancreatic nodes. Tolerance required preexisting regulatory T-cells (Tregs), and protected recipients had greater numbers of Tregs than diabetic recipients. Diabetes protection was successful in the presence of homeostatic expansion and high T-cell precursor frequency, both obstacles to tolerance induction in other models of antigen-specific immunity.

CONCLUSIONS—Immunotherapies that selectively suppress effector T-cells while permitting the development of natural regulatory mechanisms may have a unique role in establishing targeted long-standing immune protection and peripheral tolerance. Understanding the mechanism of these approaches may assist in the design and use of therapies for human conditions, such as type 1 diabetes. *Diabetes* 57:2672–2683, 2008

Reagents that bind T-cell surface molecules and interfere with T-cell activation and effector function hold the promise of being safe and effective therapies for organ transplantation and autoimmunity. Disruption of CD154, CD28, and LFA-1 pathways, either by biological agent or by genetic means, can prolong allograft survival and prevent autoimmune disease in

murine models and at times (re)establish immune tolerance (1–7). Type 1 diabetes is caused by the activation of peripheral β -cell autoreactive T-cells that have escaped central tolerance (8). In the NOD mouse model of type 1 diabetes, treating juvenile NOD mice with anti-CD154, CTLA4-Ig, or anti-B7.2 to interrupt the CD154:CD40 and CD28:B7 costimulatory pathways provides long-term disease protection (9,10). Although this is purported to result from preventing auto-aggressive T-cell activation, the full immunological mechanisms of how disease is prevented and protection is maintained are unclear (9–11). Studies involving CD154 knockout NOD mice (which are protected from diabetes) and B7-1/B7-2-deficient and CD28-deficient mice (which have exacerbated spontaneous diabetes due the lack of Tregs) provide evidence for the importance of T-cell regulatory-to-effector (Treg:Teff) balance in the development of autoimmune diabetes and the maintenance of self-tolerance (13–17).

The translation of agents that target T-cells for use in human conditions has been trying. In part, this is due to unforeseen side effects in preclinical and clinical testing, including thromboembolic events with some anti-CD154 antibodies and lethal cytokine storm with superagonist anti-CD28 antibodies (18,19). Yet many other biological agents that interfere with T-cell signaling, such as anti-CD3, anti-CD25, CTLA-4/LEA29Y, and anti-LFA-1, have impressive safety profiles in clinical trials in autoimmunity and/or transplantation (10–22).

One of our goals was to better understand how therapeutic strategies induce long-term immune protection of and immune tolerance to allo- and autoimmune targets. We studied the effect of costimulation blockade on diabetes development after the adoptive transfer BDC2.5.NOD CD4⁺ T-cells. BDC2.5 T-cells recognize a yet-unidentified β -cell antigen presented by IA^{g7}, a unique class II molecule found in NOD and derivative-strain mice (23–25). Although BDC2.5.NOD (NOD.BDC) mice harbor a monoclonal population of β -cell-reactive T-cells and possess a substantial peri-insulinitis, they rarely become diabetic because of inducible costimulatory molecule (ICOS)-, transforming growth factor- β (TGF- β)-, and programmed death (PD)-1 Treg-dependent mechanisms (26–28). Yet adoptively transferring their T-cells to major histocompatibility complex (MHC)-matched lymphopenic recipients results in rapid β -cell destruction and diabetes (24,29).

Herein, we show that diabetes resulting from adoptively transferred BDC.NOD T-cells can be prevented by a brief course of anti-CD154 and CTLA4-Ig. This therapy is successful in the presence of high T-cell precursor frequency and antigen-specific and homeostatic T-cell activation, known barriers to costimulation blockade-mediated tolerance. Long-term protected recipients harbor T-cells capable of causing diabetes, and immune tolerance is

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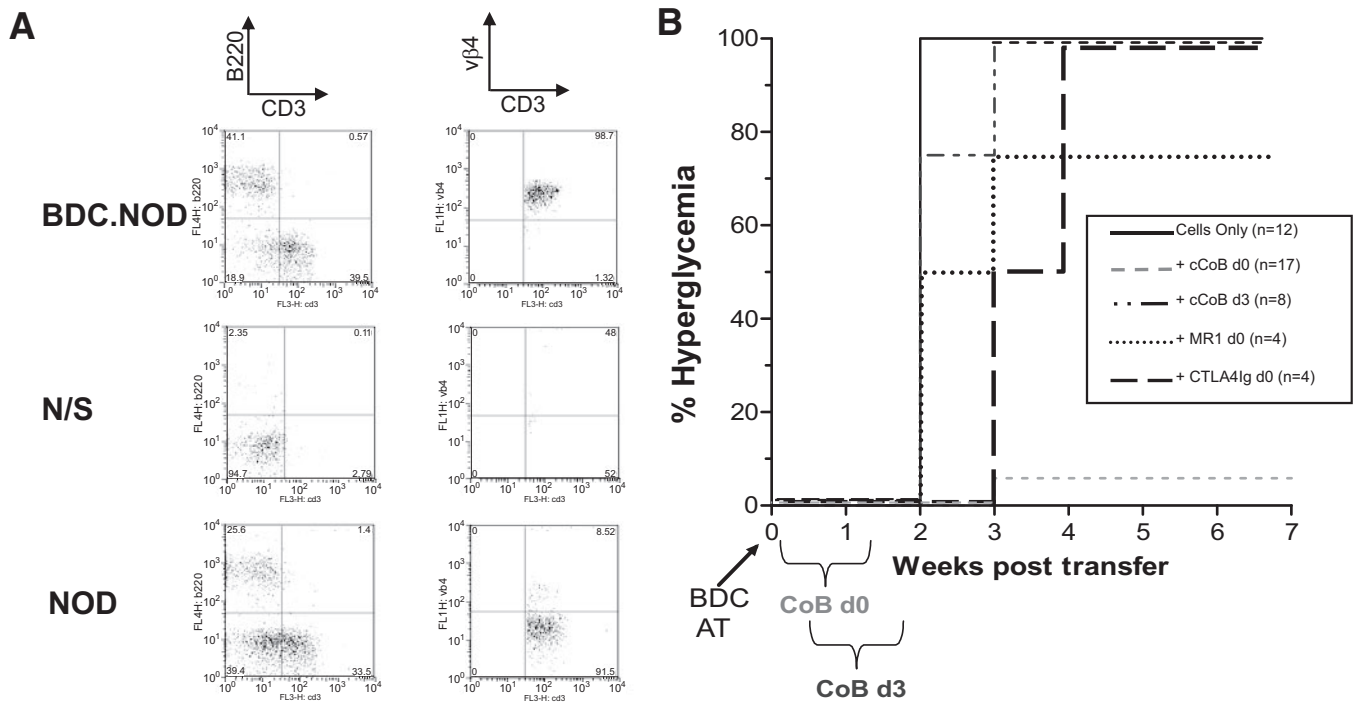


FIG. 1. Costimulation blockade prevents diabetes after adoptive transfer of BDC.NOD cells to nod.scid mice. **A:** The phenotype of cells from BDC.NOD donors, demonstrating the presence of T- and B-cells, with uniform population of $v\beta 4^+$ transgenic T-cells. nod.scid (N/S) mice, which lack T- or B-cells, and NOD mice are shown for comparison. **B:** After adoptive transfer of 2.5×10^6 cells from nondiabetic BDC.NOD mice, nod.scid recipients become diabetic usually within 2 weeks. Therapy with five doses of 200 μg MR1 and CTLA4-Ig beginning the day of adoptive transfer (day 0) protects most recipients from diabetes; whereas protection was not as complete or lasting with the individual reagents, and there was no impact of this therapy on diabetes if combined treatment was delayed to day +3 after adoptive transfer.

dependent on preexisting Tregs. Understanding how non-deletional peripheral tolerance can be generated by therapeutic means is likely an important step in developing safe and effective approaches to prevent and treat autoimmune conditions such as type 1 diabetes.

RESEARCH DESIGN AND METHODS

BDC2.5.NOD (BDC.NOD), nod.scid, and NOD mice from The Jackson Laboratories (Bar Harbor, ME) were bred and housed in sterile conditions at Emory University. BDC.NOD mice were defined via blood phenotype containing B220⁺ cells and CD3⁺ cells that were uniformly $v\beta 4^+$. Studies were conducted in accordance with the Emory University Institutional Animal Care and Use Committee guidelines.

Fluorochrome-conjugated monoclonal antibodies to CD3, CD4, $v\beta 4$, CD8, B220, CD62L, CD25, CD44, interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukin (IL)-2, IL-4, and IL-10 for flow cytometry were from BD Biosciences/Pharmingen. For immunohistochemistry, CD4, $v\beta 4$, and B220 monoclonal antibodies were obtained from BD Biosciences/Pharmingen, and anti-insulin was from Dako. Anti-FoxP3 staining kits were from eBiosciences. In vivo treatment antibodies, anti-CD154(MR1), CTLA4-Ig, anti-PD1(J43), anti-ICOS(17G9), anti-CD25(PCC-61.5.3), and anti-TGF- β (1D11.16.8) were from BioExpress (West Lebanon, NH).

Lymphocyte preparation and adoptive transfer. Lymphocytes were obtained from indicated mice after euthanasia. Pan-lymph nodes and spleens were harvested, made into cell suspensions, and washed (RPMI+5% FCS). For CD25 depletion, lymphocytes incubated with anti-CD25-coated magnetic beads (Miltenyi Biotec) were column collected (VarioMacs; Miltenyi-Biotec). For fluorescent-labeling, cells were incubated with 5 $\mu\text{mol/l}$ carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) at 37°C \times 10 min, quenched with cold RPMI+10% FCS, and washed. Of the indicated cells, 2.5×10^6 in 500 μl RPMI were injected via lateral tail vein of mice indicated.

Administration of costimulation blockade, other antibodies, and cyclophosphamide. Recipients received 500 μg anti-CD154 and/or CTLA4-Ig intraperitoneally every other day for five doses. Other antibody treatments were intraperitoneally administered: anti-PD-1, 500 μl once and then 250 μg every other day for five doses; anti-TGF β , 1 mg once and then 500 μg every 5 days for five doses; anti-ICOS, 500 μg on days 1 and 3; anti-CD25, 1 mg once. Cyclophosphamide (Mead Johnson) was intraperitoneally administered once at 200 mg/kg.

Assessment of diabetes and insulin treatment. Tail vein blood was analyzed using a Bayer Ascensia Elite Glucometer. Diabetes was defined as two consecutive readings of >250 mg/dl. When needed, one to two insulin pellets (LinBit, Linshin, Canada) were introduced subcutaneously to maintain diabetic mice.

Immunohistochemistry. Pancreata were frozen in OCT compound in liquid nitrogen. Cut sections (5 μm) were incubated with the indicated antibody using the LSAB+System HRP-Immunohistochemistry kit (Dako Cytomation) and visualized with light microscopy.

Flow cytometry. Lymphocytes (5×10^5) were incubated with fluorochrome-conjugated antibodies for 20 min, washed, and analyzed using a FACSCalibur (BD Biosciences) and FlowJo (Tree Star). For FoxP3 analysis, cells were first surface stained and then processed per the manufacturer's instructions.

Intracellular cytokine staining. Lymphocytes were incubated in RPMI plus 10% FCS at 37°C in the absence or presence of 10 $\mu\text{g/ml}$ BDC2.5 peptide (RTRPLWVRME [28]; Emory University Microchemical facility). After 5 h, brefeldin A (GolgiPlug, BD/Bioscience) was added for 1 h. Cells were then stained for surface molecules, washed, fixed, permeabilized (BD Cytotfix/Cytoperm kit; BD Biosciences), and incubated with anti-cytokine-conjugated antibodies for 30 min at 4°C.

Statistics. Where indicated, unpaired Student's *t* test was used. Significance was determined if the *P* value was ≤ 0.05 .

RESULTS

Combined therapy with anti-CD154 and CTLA4-Ig prevents adoptive transfer of diabetes by BDC.NOD T-cells. To dissect how costimulatory blockers prevent autoimmune diabetes, we studied the effect of anti-CD154 and CTLA4-Ig on diabetes induced in nod.scid recipients of BDC.NOD lymphocytes. Within 2 weeks, $>95\%$ of such recipients developed hyperglycemia. When recipients were also treated with five doses of MR1 anti-CD154 and CTLA4-Ig starting the same day as cell transfer, diabetes was prevented in $>90\%$ of recipients (Fig. 1). In mice treated with either anti-CD154 or CTLA4-Ig, protection was not as complete or long lasting. A delay of treatment to day

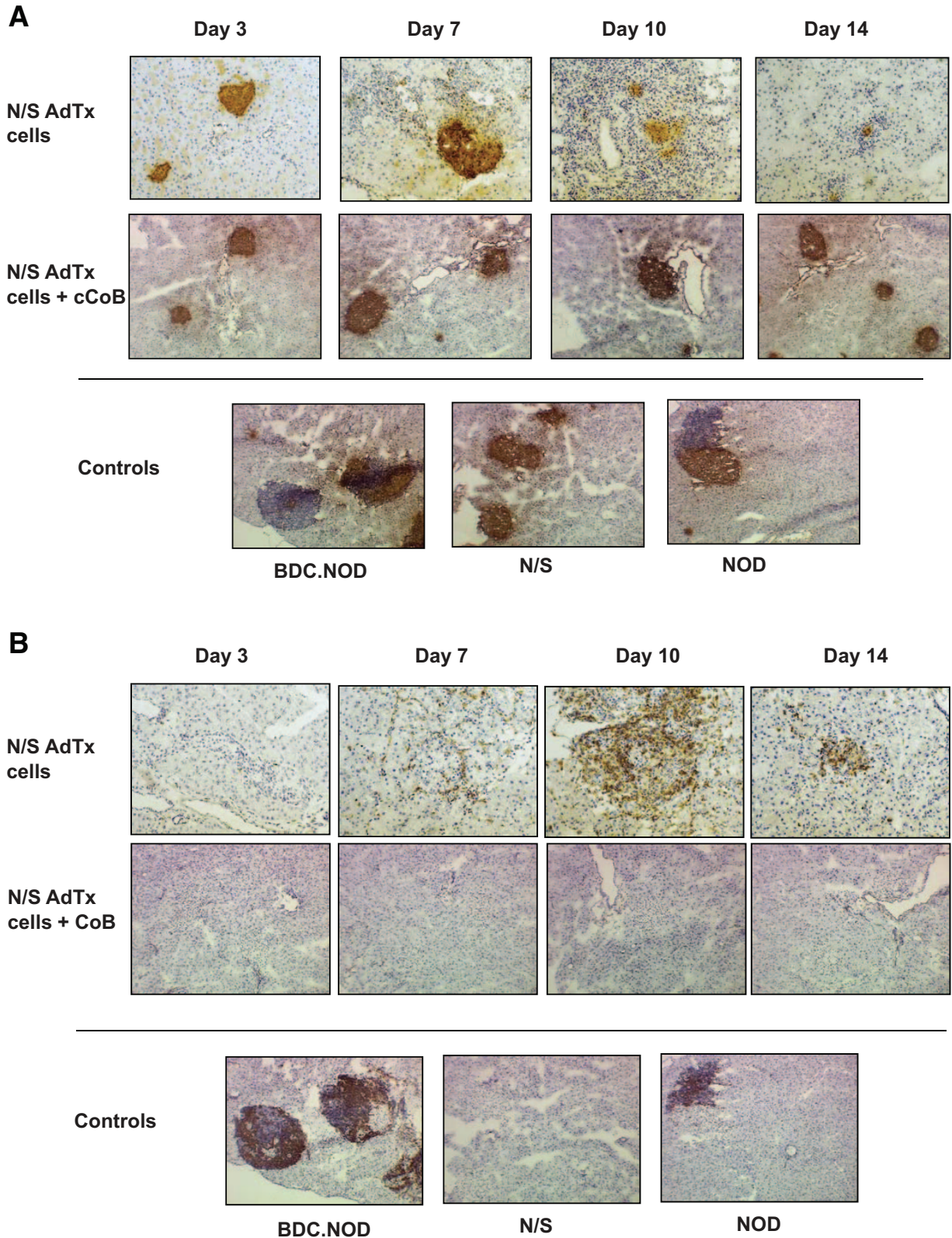


FIG. 2. Islet of recipients of diabetogenic cells and costimulation blockade lack inflammation. *nod.scid* mice were adoptively transferred without or with costimulation blockade as above. Recipients were killed at days 3, 7, 10, and 14 after adoptive transfer. Pancreata from killed mice were frozen in OCT compound in liquid nitrogen, cut, and examined using immunohistochemistry for insulin (A), CD4 (B), $v\beta$ 4 (C), and B220 (D). Recipients of cells only show progressive islet destruction after insulinitis within 2 weeks after adoptive transfer. Recipients of costimulation blockade remain free from peri-islet inflammation and retain islets. For comparison, stained sections of pancreata from nondiabetic NOD, BDC.NOD, and *nod.scid* mice are shown. Shown are representative experiments of at least three independent experiments. (Please see <http://dx.doi.org/10.2337/db07-1712> for a high-quality digital representation of this figure.)

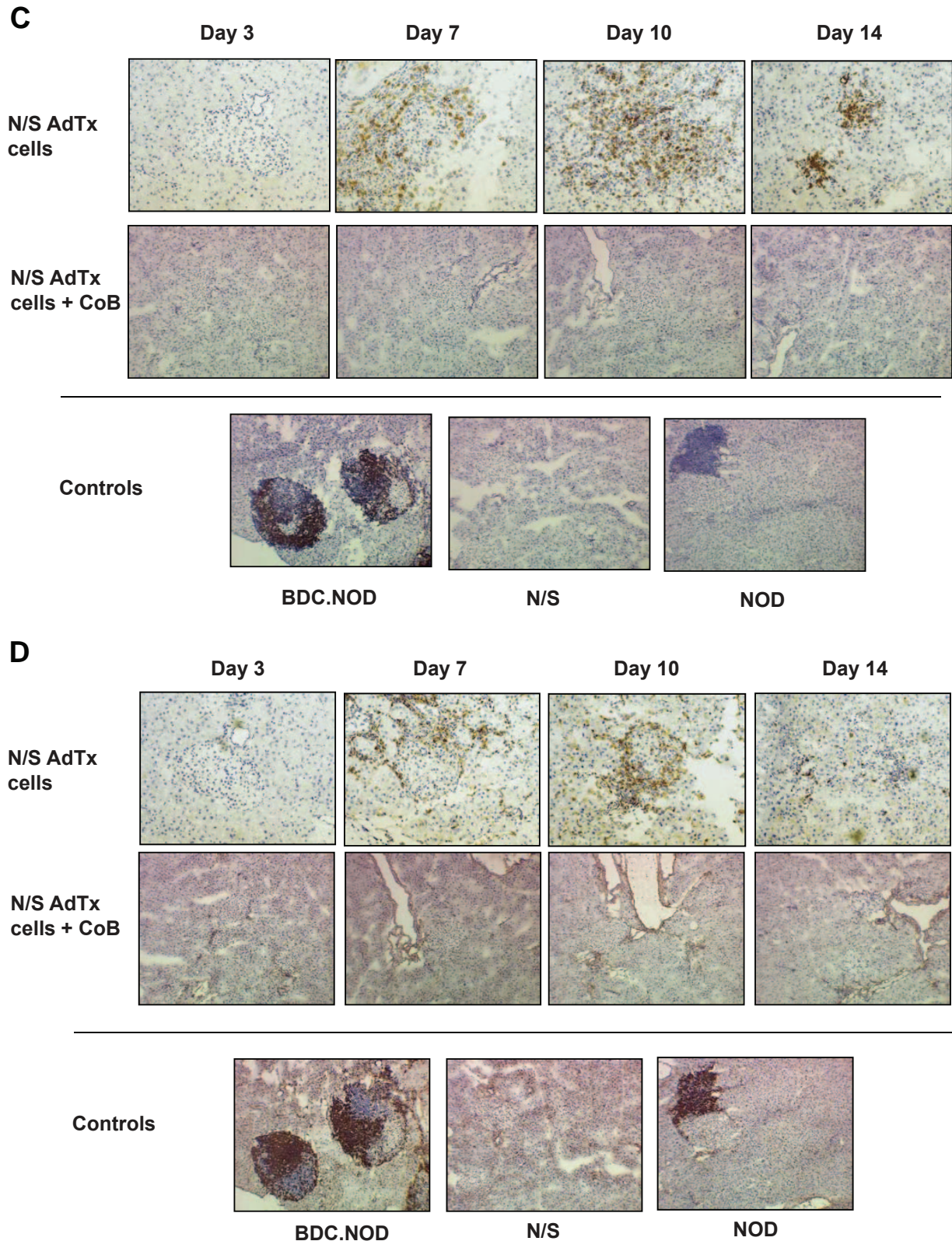


FIG. 2. Continued.

+3 after adoptive transfer failed to impact diabetes incidences or tempo (Fig. 1).

CD154/CD28 blockade prevents peri- and intra-islet inflammation and destructive insulinitis in recipients of BDC.NOD cells. Autoimmune diabetes is the result of a stepwise breakdown of self-tolerance to insulin-producing

β -cells (30). In NOD and BDC.NOD mice, β -cell autoreactive T-cells can circulate without pathogenic effects because of endogenous peripheral self-tolerance mechanisms (checkpoint 1). Naïve autoreactive T-cells can become partially activated and accumulate in a nondestructive manner around the islets of Langerhans (peri-

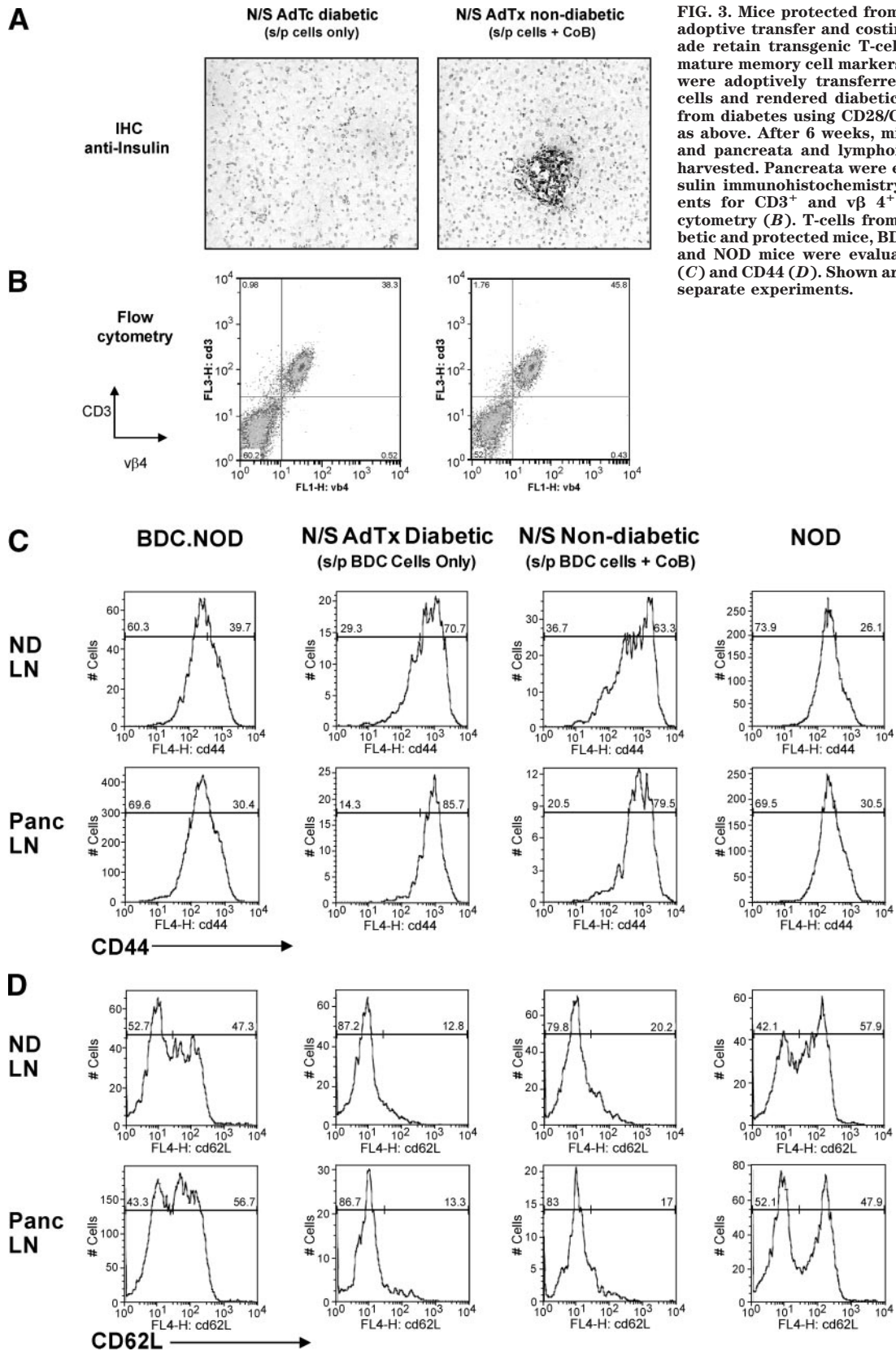


FIG. 3. Mice protected from diabetes after adoptive transfer and costimulation blockade retain transgenic T-cells that acquire mature memory cell markers. *nod.scid* mice were adoptively transferred with BDC2.5 cells and rendered diabetic and protected from diabetes using CD28/CD154 blockade as above. After 6 weeks, mice were killed, and pancreata and lymphoid organs were harvested. Pancreata were evaluated by insulin immunohistochemistry (A) in recipients for CD3⁺ and vβ 4⁺ cells via flow cytometry (B). T-cells from long-term diabetic and protected mice, BDC.NOD donors, and NOD mice were evaluated for CD62L (C) and CD44 (D). Shown are results of two separate experiments.

insulinitis). During this phase, β-cell-destructive T-cells and disease are held “in check” by local regulatory (Treg) mechanisms (checkpoint 2). To investigate where costimulation blockade arrests diabetes development in our

model, we evaluated pancreata of BDC.NOD cell recipients (Fig. 2). Within 3 days after transfer, recipients of BDC.NOD cells alone demonstrated T-cells and B-cells in and around islets. Infiltrate increased through day 7, and

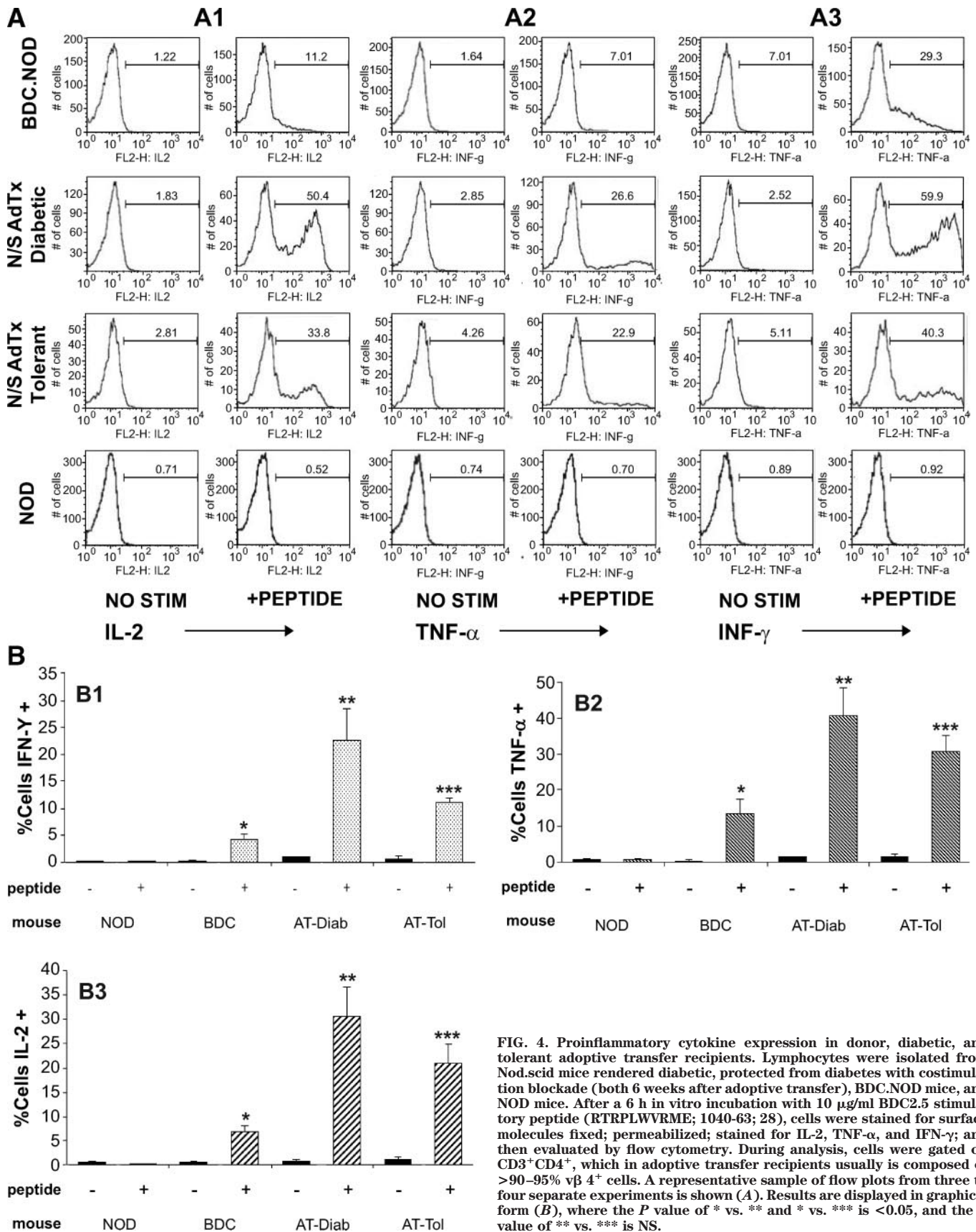


FIG. 4. Proinflammatory cytokine expression in donor, diabetic, and tolerant adoptive transfer recipients. Lymphocytes were isolated from Nod.scid mice rendered diabetic, protected from diabetes with costimulation blockade (both 6 weeks after adoptive transfer), BDC.NOD mice, and NOD mice. After a 6 h in vitro incubation with 10 μ g/ml BDC2.5 stimulatory peptide (RTRPLWVRME; 1040-63; 28), cells were stained for surface molecules fixed; permeabilized; stained for IL-2, TNF- α , and IFN- γ ; and then evaluated by flow cytometry. During analysis, cells were gated on CD3⁺CD4⁺, which in adoptive transfer recipients usually is composed of >90–95% $\nu\beta$ 4⁺ cells. A representative sample of flow plots from three to four separate experiments is shown (A). Results are displayed in graphical form (B), where the *P* value of * vs. ** and * vs. *** is <0.05, and the *P* value of ** vs. *** is NS.

by day 14 there was nearly complete islet obliteration, concurrent with clinical disease. In sharp contrast, pancreata of recipients treated with costimulation blockade

possessed only sparse inflammation in the days, weeks, and months after BDC.NOD cell transfer. This suggests that interrupting the CD154 and CD28 pathways prevents

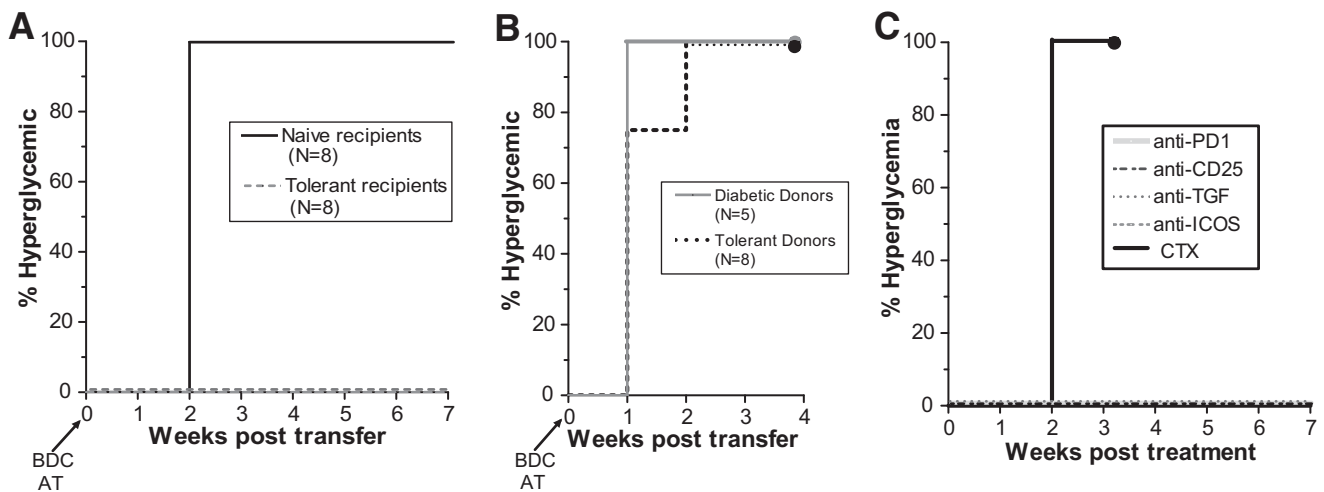


FIG. 5. Mice rendered tolerant to diabetes are further protected from new diabetogenic T-cells and harbor pathogenic T-cells. **A:** After 6 weeks, nod.scid recipients of BDC2.5 cells protected from diabetes with costimulation blockade were given a second adoptive transfer of BDC2.5 cells in the absence of any further treatment. Unmanipulated nod.scid mice were also treated with BDC2.5 cells only. **B:** In other experiments, mice were protected from diabetes and others were rendered diabetic and maintained on insulin for 6 weeks. Splenocytes and LN cells were isolated from such mice and 2.5×10^6 resultant cells were adoptively transferred into new nod.scid recipients. **C:** Lastly, mice that were rendered tolerant were treated with agents known to precipitate diabetes in other susceptible mouse models, including anti-CD25, anti-ICOS, anti-PD1, anti-TGF β , or cyclophosphamide.

the anti- β -cell inflammatory response early in the pathogenic process, at a time equivalent to Checkpoint 1, before the trafficking of lymphocytes to islets.

Transgenic T-cells are present in mice protected from diabetes by costimulation blockade and express an antigen-experienced phenotype. To ascertain the fate of transferred cells in diabetic and protected nod.scid mice, T-cells in recipients were isolated and evaluated. Both diabetic mice and protected recipients harbor CD4⁺ T-cells that are exclusively $\nu\beta 4^+$ (Fig. 3A). In protected recipients, BDC2.5 T-cells are found in the spleen and draining and nondraining nodes at the time when pancreata are devoid of inflammation. The presence of this transgenic T-cell population in the presence of target antigen without pathogenesis is consistent with a state of peripheral immune tolerance and additionally suggests against deletion or “central” tolerance as the primary tolerogenic mechanism (31,32).

Furthermore, analysis of cell surface markers suggests T-cells in both diabetic and tolerant recipients go through similar maturational changes. T-cells from donor BDC.NOD mice contain a preponderance of CD62L⁻, CD44⁺ (naïve) CD4⁺ T-cells, whereas T-cells from both diabetic and tolerant recipients express high levels of CD44 and low levels of CD62L (Fig. 3B). These similar surface marker changes suggest that transferred cells have similar immunological “experiences” and encounters regardless of disease state or anatomic location, and therefore suggest against immune ignorance in this model of β -cell protection (33).

Cytokine expression from T-cells from protected and diabetic recipients. T-cell functional and maturational status can often be inferred by the types of cytokines expressed and the tempo of production (34). T-cells in nondiabetic recipients of BDC.NOD cells are not pathogenic to the host, whereas cells in diabetic recipients are. One explanation could be that T-cells from protected mice have acquired a “less” proinflammatory profile than cells from protected mice. To investigate this, cytokine production after antigen encounter was evaluated. After *in vitro* antigen-specific stimulation, there is greater expression of

IFN- γ , TNF- α , and IL-2 from either tolerant or diabetic mice compared with nascent BDC.NOD mice (Fig. 4A). There was also a nonsignificant trend for greater expression of these proinflammatory cytokines in cells from diabetic compared with tolerant mice. (Fig. 4B). Few cells (i.e., <5%) from either tolerant or diabetic mice expressed significant IL-4 or IL-10 (data not shown). This suggests that T-cells from all recipients, whether diabetic or protected, have transitioned from functionally naïve to mature memory T-cells with a highly proinflammatory profile. Therefore β -cell protection does not appear to be due to an overwhelming shift from Th1-type T-cells to T-cells that express abundant regulatory cytokines (i.e., Th2 or regulatory cells).

Suppression of diabetogenic Teff in protected mice. We examined the robustness of engendered tolerance. Protected mice that were rechallenged with freshly isolated BDC.NOD cells remained normoglycemic (Fig. 5A). We also examined whether tolerant mice continued to harbor cells capable of β -cell destruction. Lymphocytes from tolerant and diabetic recipients were adoptively transferred into unmanipulated nod.scid mice. Regardless of the donor, recipients developed diabetes with a tempo similar to that of initial disease transfer (Fig. 5B). This suggests that pathogenic cells are contained in tolerant mice but under continued, active suppression. To investigate the mechanism of this protection, long-term nondiabetic recipients were treated with agents that interfere with endogenous regulatory processes and precipitate autoimmune diabetes in NOD and BDC.NOD mice. Protected mice remained normoglycemic after treatment with anti-CD25, anti-PD1, anti-ICOS, and anti-TGF β but rapidly developed diabetes after a single dose of cyclophosphamide (Fig. 5C).

Tregs expand in the absence and presence of costimulation blockade. Our data indicate that combined CD154/CD28-blockade arrests diabetogenesis before the peri-insulinitis stage. Tregs are involved in the induction and maintenance of immune tolerance in many models of allo- and autoimmunity. In NOD and BDC.NOD mice, Tregs

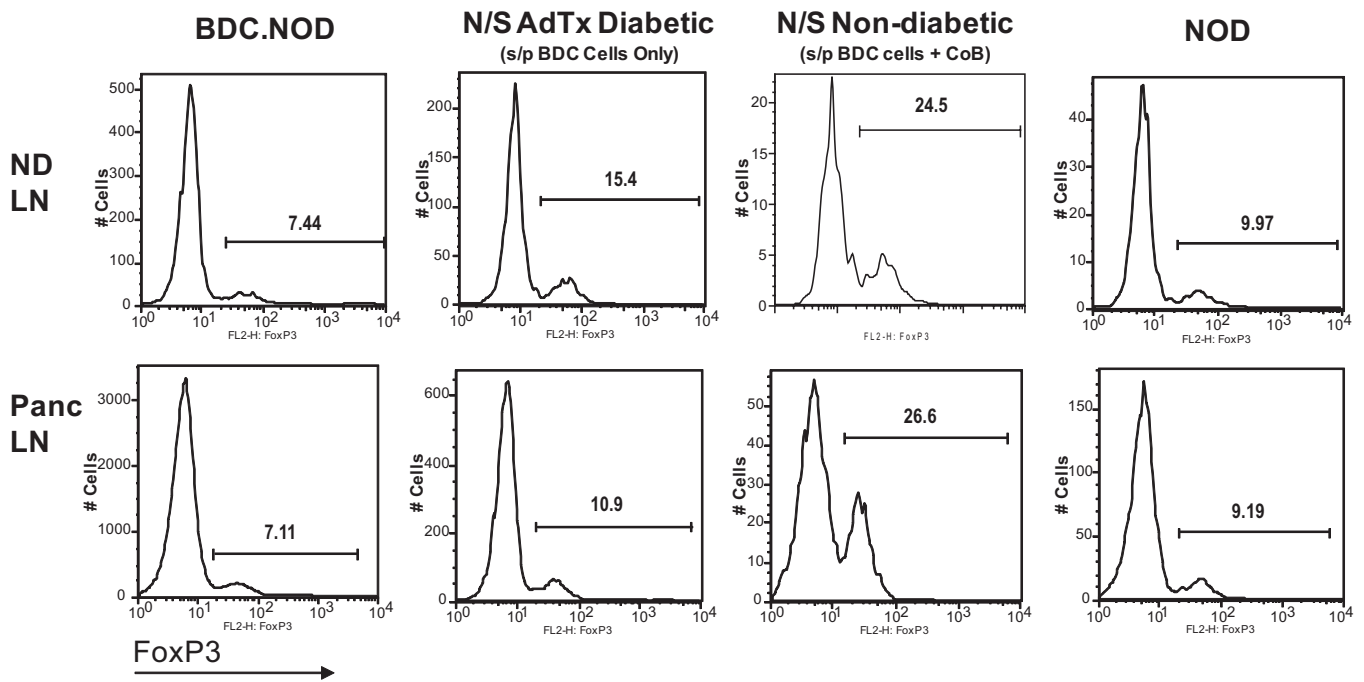


FIG. 6. More FoxP3⁺ T-cells are found in tolerant than diabetic or donor BDC.NOD mice. Lymphocytes were isolated from pancreatic (Panc LN) and mesenteric and cervical nondraining LNs (ND LN) from nod.scid mice rendered diabetic, protected from diabetes with costimulation blockade (both 6 weeks after adoptive transfer), from BDC.NOD mice, and from NOD mice. Cells were stained for surface molecules, fixed, permeabilized, stained for intracellular FoxP3, and then analyzed by flow cytometry. FoxP3 expression gated on CD4⁺CD3⁺ cells is shown and is representative of at least four independent experiments.

contribute to natural peripheral self-tolerance by interfering with the progression from peri-insulinitis to destructive insulinitis (checkpoint 2). To further elucidate the role of Tregs in this model, BDC.NOD donors and diabetic and tolerant recipients were evaluated for Tregs ($\nu\beta 4^+CD4^+FoxP3^+$) (Fig. 6). Protected mice contain a greater percentage of Tregs than diabetic recipients, and interestingly both recipient groups have higher Treg percentages than donor BDC.NOD mice (Fig. 6) (35). Although Tregs (regulatory) and Teffs (nonregulatory) expand in nod.scid mice treated with CD154/CD28 blockade, expanded Teffs do not cause β -cell destruction concurrent with an apparent preferential expansion of Tregs. **Tregs are required for costimulation blockade-mediated tolerance.** To better define the role of Tregs in tolerance generation with costimulation blockade, we depleted the donor cell inoculum of CD25-expressing cells, resulting in a >10- and >3- to 4-fold reduction in CD25⁺ and FoxP3⁺ T-cells, respectively (Fig. 7A). Recipients of Treg-reduced cells and costimulation blockade were not protected from diabetes, although diabetes onset was delayed from 2 to 4 weeks (Fig. 7B). From this, we conclude that a critical mass of preexisting Tregs appears requisite for the long-term immune protection rendered by CD154/CD28 blockade.

Costimulation blockade blunts early proliferation of antigen-specific T-cells. Although recipients of Treg-reduced cells and CD154/CD28 blockade acquire disease, they do so more slowly than in the absence of costimulation blockade, suggesting that there is a direct, but transient, suppressive effect of costimulation blockade on Teffs. To examine whether costimulation blockade disrupts the early steps in diabetogenic T-cell activation in vivo, CFSE-labeled BDC.NOD cells were transferred into nod.scid recipients in the absence or presence of costimulation blockade. Three days later, CFSE content in

$\nu\beta 4^+CD4^+$ cells from recipients was analyzed (Fig. 8). In recipients of cells only, the proliferative response was most robust in draining pancreatic lymph nodes versus nondraining nodes, consistent with antigen-specific activation (36,37). Yet there was some comparatively minimal division in nondraining sites consistent with homeostatic proliferation (38). In recipients of cells and costimulation blockade, there was a distinct retardation of early proliferation in draining nodes. Yet by day 10, CFSE signals in cells in draining and nondraining compartments were fully diluted. Therefore, in the absence of costimulation blockade, cells in the draining nodes have already been through several rounds of antigen-driven proliferation, which is a possible explanation of why CD154/CD28 blockade must be given early (before 3 days) after adoptive transfer to prevent diabetes (Fig. 9B and C).

DISCUSSION

We used the BDC2.5.NOD cell transfer model of diabetes to better understand how costimulatory agents prevent autoimmunity and establish immune tolerance. Herein, we show that blocking CD154 and CD28 signaling can engender robust peripheral tolerance in the face of high antigen-specific T-cell precursor frequency and lymphopenia, two factors shown to be significant obstacles to costimulation blockade-rendered immune protection (39,40). In our model, the donor cell inoculum contains a relatively pure precursor frequency of β -cell-specific T-cells. Recent studies in allograft models, including work from our own group, suggest that high antigen-specific T-cell precursor frequency can impair the toleragenic effects of costimulation blockade and can be responsible for costimulation blockade-resistant allograft rejection (40). Current collaborative studies are underway to investigate the contributions of precursor frequency versus absolute numbers of

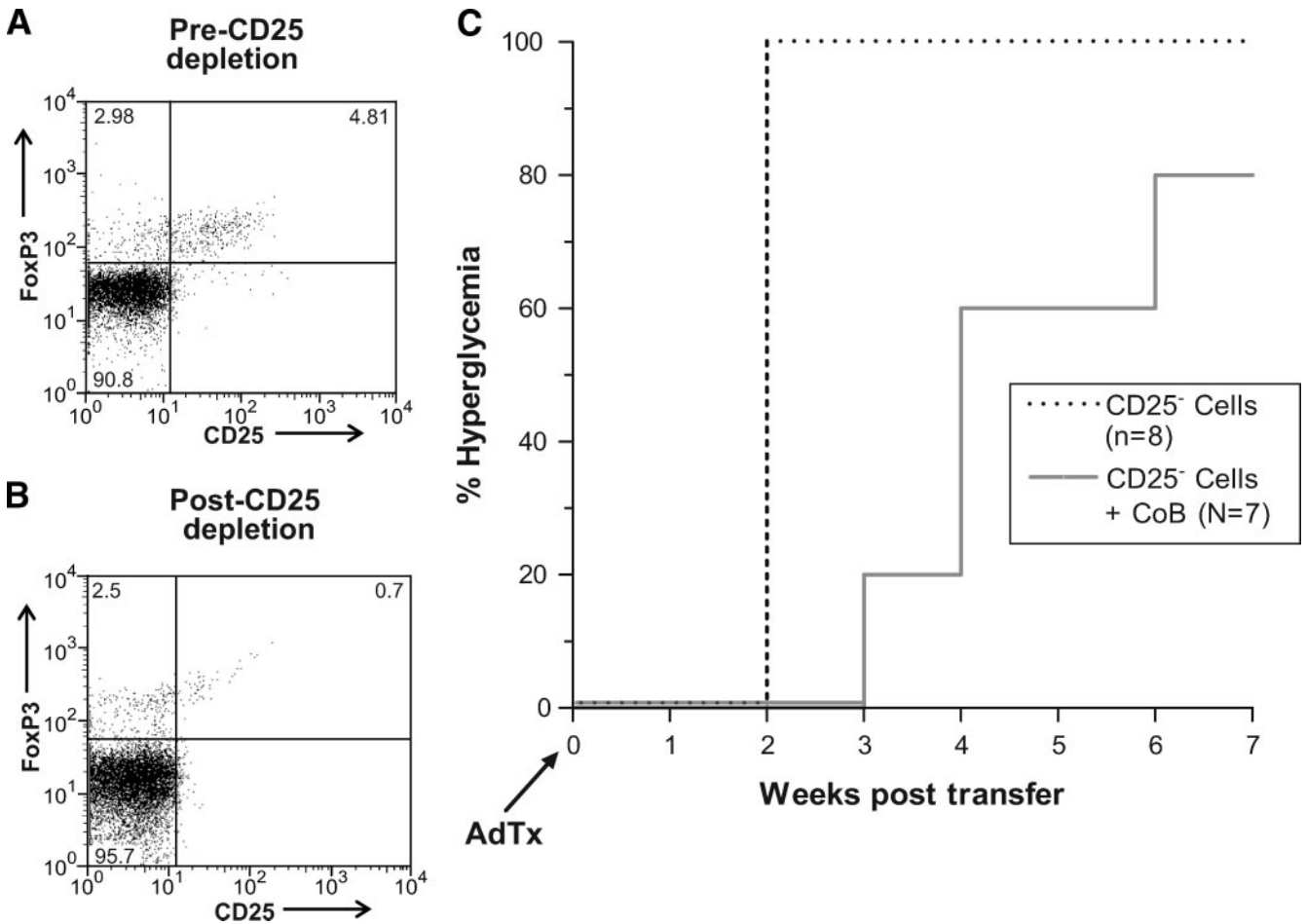


FIG. 7. Tregs are required for costimulation blockade-mediated peripheral tolerance. Donor BDC2.5 cells were depleted of CD25⁺ cells using a negative selection antibody column. Shown are representative plots of FoxP3⁺CD25⁺ expression on CD4 T-cells before (A) and after (B) depletion. C: In the absence or presence of 5 days of combined costimulation blockade therapy, 2.5×10^6 of the CD25-depleted cells were adoptively transferred into nod.scid mice, and hyperglycemia was assessed.

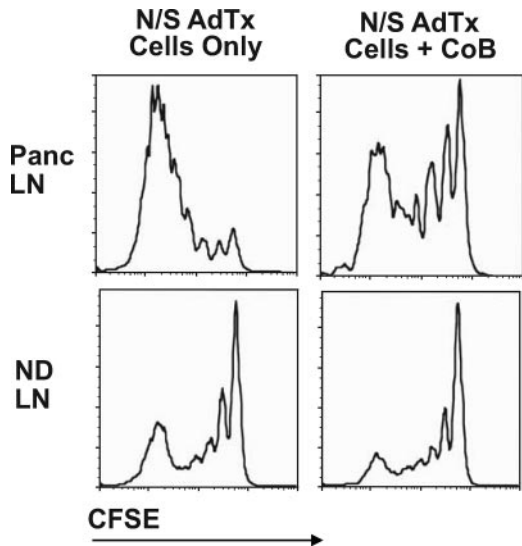


FIG. 8. Anti-CD154 and CTLA4-Ig blunts early T-cell proliferation in draining pancreatic lymph nodes. BDC2.5 cells were labeled with CFSE and adoptively transferred into nod.scid mice. Some recipients were otherwise untreated, whereas others were given combined costimulation blockade on the day of adoptive transfer (day 0) and on day +2. On day +3, nondraining LNs (ND LN) and draining pancreatic LN (Panc LN) were harvested. Shown are representative flow plots of CFSE content on CD4⁺ vβ4⁺ cells. The data are representative of three independent experiments.

antigen-specific Teffs and Tregs in the susceptibility versus resistance to costimulation blockade-mediated tolerance.

In this model of immune-mediated diabetes, pathogenic

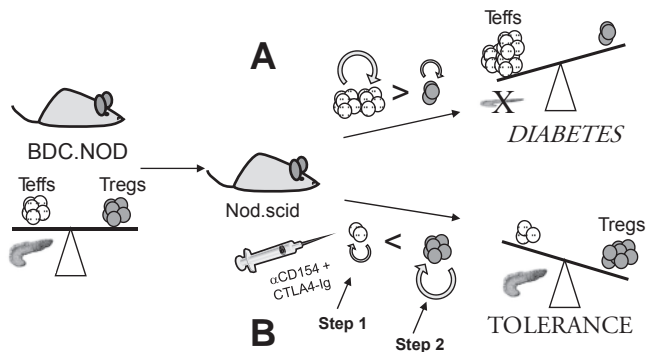


FIG. 9. Costimulation blockade induced peripheral immune tolerance by combining therapeutic and endogenous immunomodulatory mechanisms. Using these data in this report, we have developed a working model to explain our findings: After adoptive transfer of a T-cell inoculum containing Teffs and Tregs from nondiabetic BDC.NOD mice (A) into nod.scid mice, diabetogenic T-cells become activated and proliferate and destroy β-cells, which results in diabetes (A). Implicit in this model is the preferential expansion of diabetogenic Teffs over Tregs (B). In recipients treated with CD154/CD28 blockade, the expansion of diabetogenic Teffs is blunted directly by this therapy (step 1) with concomitant expansion of protective Tregs (step 2) to protect β-cells. The immunomodulatory effects of both steps 1 and 2 are required for generating and maintaining long-term peripheral tolerance by costimulation blockade.

T-cells likely expand by both antigen-specific and homeostatic factors (31,38,39). Many pathways, including those involving IL-15, IL-7, IL-21, and weak TcR:MHC signaling, work to nonspecifically activate and expand lymphocytes to “fill the empty space” in lymphopenic recipients (38,41). Chemotherapy, immunosuppressive medicines, and viral infections can result in lymphopenia, and questions have been raised regarding the utility of costimulation blockade in allo- and autoimmunity in these settings (38,39). Specifically, both human and experimental autoimmune diabetes have been associated with infections, T-cell lymphopenia, and homeostatic activation (8,38,42,43). In our report, we show that T-cells in nondraining nodes proliferated, likely because of homeostatic factors; yet in draining pancreatic nodes, T-cells proliferate more vigorously, suggesting that specific antigen encounter provides more potent activating signals to T-cells than homeostatic factors. Our finding that costimulation blockade can prevent T-cell-mediated destruction in a lymphopenic environment contrasts those by Wu et al. (39), who found that cardiac allografts were rejected in T-cell-depleted mice treated with “toleragenic” doses of anti-CD154 and CTLA-4Ig. As a result, the authors raised the concern of the utility of costimulation blockade in conditions with concomitant lymphopenia. In our work, costimulation blockade is able to temporarily dampen the accelerated T-cell proliferation in draining nodes but appears to have little effect on underlying (homeostatic) proliferation. In D011.10 transgenic mice, Prlic et al. (44) showed that homeostatic expansion of T-cells occurs independently from CD28, CD154, or 4-1BB signaling. We also show that in a lymphopenic environment, in the presence of CD154/CD28 blockade, immature T-cells can proliferate and transition to “mature” T-cells (CD44⁺ and CD62L⁻) with vigorous proinflammatory cytokine recall responses, yet do not initiate β -cell destruction. This suggests that Teff proliferation and maturation can be uncoupled from pathogenic activities and that such “placid” activation of Teffs may play a role in immune tolerance. Because our results demonstrate that costimulation blockers can prevent the pathogenic activities of Teffs in lymphopenic recipients, such agents should not be unilaterally dismissed from consideration in treating auto- and alloimmunity during times of spontaneous or iatrogenic lymphocyte depletion.

Our data show that diabetes protection clearly requires early blockade of the CD154:CD40 and CD28:B7 pathways. These pathways are integral in the initial steps in generation of Teffs from quiescent T-cells. Diabetes tempo and severity is not impacted with “delayed” costimulation blockade, even if given at times when recipient pancreata are devoid of inflammation (i.e., 3 days after transfer). Teffs continue to traffic to islets and orchestrate β -cell killing during delayed CD154/CD28 blockade, suggesting that many of the “pathogenic” functions of Teffs do not appear to require ongoing signaling through these pathways. This work may have clinically important translational implications. For example, if such therapeutic agents are only efficacious in disease prevention if given before the onset of diabetes pathogenesis, then that would strongly support efforts for the early identification and treatment of pre-diabetic individuals before or at the brink of developing diabetes. This also stresses the need to develop immunotherapies that arrest diabetogenic T-cells after initial activation but before tissue destruction.

It is well established that Tregs are one of the primary mechanisms to maintain natural self-tolerance in vivo, yet

the precise mechanism of how these cells provide protection is unclear (17,35,45,46). Our work suggests that Tregs can also be vitally important for immune tolerance induced via therapeutic interventions. We challenged protected recipients with agents that induce diabetes in NOD and/or BDC2.5.NOD mice. Specifically, we used antibodies to ICOS, PD-1, TGF- β , and CD25 to target Treg-mediated cellular and soluble processes that maintain self-tolerance and diabetes prevention in NOD and BDC2.5.NOD mice (26,47,48). These therapies did not precipitate diabetes in our protected adoptive transfer recipients. Although additional specific approaches can be used to attempt to break tolerance to further investigate the mechanisms involved in maintaining immune tolerance, the current studies suggest that processes integral to maintaining natural β -cell self-tolerance are not vital in therapeutically induced tolerance. Protected recipients do develop diabetes after cyclophosphamide, a treatment that nonspecifically impairs Treg function and/or survival, and lymphocytes from protected mice readily transfer disease (49). Taken together, this shows that diabetogenic Teffs are not only present in protected mice but under active regulation. Because the mechanism of this induced tolerance appears to be different from those involved with endogenous self-tolerance, further studies to elucidate these mechanisms may identify additional processes that can be exploited to therapeutically render peripheral tolerance.

Not only do Treg-mediated mechanisms appear to be critically involved in the maintenance of immune tolerance, but a particularly exciting finding of this report is that Tregs also appear integral in establishing therapeutically induced immune tolerance. Recipients of Treg-depleted cells and costimulation blockade develop diabetes, albeit at a slower tempo than cell recipients that do not receive costimulation blockade. In the absence of Tregs, CD154/CD28 blockade likely directly, but transiently, “suppresses” Teff activation and pathogenicity, resulting in delayed diabetes. It is at this junction that we hypothesize that Tregs expand, populate, and become established to protect β -cells. This would imply that Tregs can expand and function in the presence of costimulation blockade. Consistent with this premise, long-term protected recipients have higher Treg-to-Teff ratios than in diabetic recipients. Knockout studies in NOD mice indicate that the Teff and Tregs have different costimulatory requirements at different ontological stages. CD28-knockout NOD mice lack Tregs yet contain highly pathogenic Teffs and uniformly develop diabetes, indicating that CD28 signals are more important in Treg than Teff development (13). It is conceivable that the CD28-signaling requirements of peripheral Teffs and Tregs differ as well. On the other hand, CD154 KO NOD mice are protected from disease, apparently because of the specific need for CD154 signaling in the pathogenic transformation of peripheral Teffs (16). These knockout studies can serve as the foundation to encourage further search for unique survival or activation pathways in Teffs and Tregs and reagents that can selectively target them.

Based on the recognition that the expression of autoimmune diabetes is determined, in part, by Treg-to-Teff balance, we developed a working model of how nondeletional peripheral tolerance is induced with therapeutic costimulation blockade and maintained by natural (Treg) regulatory mechanisms (13,14,50) (Fig. 9). We hypothesize a two-step process for long-term β -cell protection from diabetogenic T-cells after costimulation blockade (Fig.

9B). Step 1 is the direct suppression of the early antigen- and homeostatic-driven pathogenic transformation of circulating diabetogenic T-cells by CD28/CD154 blockade. Yet, during this time, T-cells can proliferate and “mature” in the absence of β -cell destruction. Assuming that Tregs are less reliant on CD28 and CD154 signaling, during costimulation blockade, Tregs expand and mature due to antigen- and nonantigen-specific interaction. Eventually, the direct suppressive effect of costimulation blockade wanes. β -Cells remain protected because of step 2, which is the continued prevention of Teff pathogenicity by the Tregs that have expanded and become “entrenched.” Although Tregs are involved in this continued protection, they appear to use different mechanisms than those used to halt naturally occurring diabetes at the peri-insulinitis stage (checkpoint 2). In this way, exogenous and endogenous immunomodulatory mechanisms coordinate and cooperate in the short and long term to maintain a nonpathological Treg-to-Teff balance and preservation of β -cells.

We believe that this work demonstrates how approaches that promote the cooperation of therapeutic and endogenous immunomodulatory mechanisms can be used to establish a sustained state of antigen-specific immune protection. This approach may be of particular relevance to clinical translation as we find immune tolerance can be achieved in the presence of high antigen-specific T-cell precursor frequency and lymphopenia, both situations purported to be present near the onset of type 1 diabetes and present in other autoimmune and alloimmunity states. Understanding how peripheral immune tolerance can be induced and maintained in vivo using therapeutic agents will no doubt reveal sophisticated coordination of highly complex immune networks and interactions. Depending on immune state, antigenic target, and condition, different reagents and approaches may be required to render effective, long-lasting immune protection. The study of different models of immune dysregulation may find unique immunoprotective mechanisms translatable to a variety of auto- and alloimmune conditions. We believe that T-cell-selective agents that impair effector T-cells while encouraging the (re)establishment of natural immune regulatory mechanisms may provide a powerful, yet safe, approach to treat T-cell-mediated diseases in humans, including type 1 diabetes.

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