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

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

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Abstract

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 autoimmune diseases. 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 identify a yet-identified β-cell antigen presented by IA67, 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-insulitis, 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 maintained (13–17).
dependent on preexisting Tregs. Understanding how non-deleitional 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 Laboratory (Bar Harbor, ME) were bred and housed in sterile conditions at Emory University. BDC.NOD mice were defined via blood phenotype containing B220<sup>+</sup> cells and CD3<sup>+</sup> cells that were uniformly v<sub>B</sub><sup>+</sup><sup>+</sup>. Studies were conducted in accordance with the Emory University Institutional Animal Care and Use Committee guidelines.

Fluorochrome-conjugated monoclonal antibodies to CD3, CD4, v<sub>B</sub>, 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<sub>B</sub>, and B220 monoclonal antibodies were obtained from BD Biosciences/Pharmingen, and anti-insulin was from Dako. Anti-FoxP3 staining kits were from eBiosciences.

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 plus 5% FCS). For CD25 depletion, lymphocytes incubated with anti-CD25– coated magnetic beads (Miltenyi Biotec) were column collected (VarioMacs; Miltenyi-Biotec). CD25 depletion, lymphocytes incubated with anti-CD25– coated magnetic beads (Miltenyi Biotec) were column collected (VarioMacs; Miltenyi-Biotec). Anti-ICOS(17G9), anti-CD25(PCC-61.5.3), and anti-TGF-β (1D11.16.8) were obtained from BioExpress (West Lebanon, NH).

Lymphocytes (5×10<sup>6</sup>) 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.

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 lasting. A delay of treatment to day 3 after adoptive transfer.

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.

Intracellular cytokine staining. Lymphocytes were incubated in RPMI plus 10% FCS at 37°C in the absence or presence of 10 μg/ml BDC2.5 peptide (RTRPLWRMRE [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 Cytofix/Cytoperm kit; BD Biosciences), and incubated with anti-cytokine–conjugated antibodies for 30 min at 4°C.
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), vβ 4 (C), and B220 (D). Recipients of cells only show progressive islet destruction after insulitis within 2 weeks after adoptive transfer. Recipients of BDC2.5 cells and 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.)
+3 after adoptive transfer failed to impact diabetes incidences or tempo (Fig. 1).

**CD154/CD28 blockade prevents peri- and intra-islet inflammation and destructive insulitis 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 nondestuctive manner around the islets of Langerhans (peri-
insulitis). During this phase, β-cell–destructive T-cells and disease are held “in check” by local regulatory (Treg) mechanisms (checkpoint 2). To investigate where co-stimulation 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
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. 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 μg/ml BDC2.5 stimulatory peptide (RTRPLWYRME; 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+ 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.
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 vβ4+ (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 maturation changes. T-cells from donor BDC-NOD mice contain a preponderance of CD62L+CD44+ (naive) 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 maturation 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-TGFβ 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-insulitis 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...
CD25-depleted the donor cell inoculum of CD25-expressing tolerance generation with costimulation blockade, we Tregs are required for costimulation blockade–mediated tolerance. To further elucidate the role of Tregs in this model, BDC.NOD donors and diabetic and tolerant recipients were evaluated for Tregs (vβ4+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 vβ4+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 tolerogenic 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. 6. More FoxP3+ T-cells are found in tolerant than diabetic or donor BDC.NOD mice.](image-url)
antigen-specific Teffs and Tregs in the susceptibility versus resistance to costimulation blockade-mediated tolerance.

In this model of immune-mediated diabetes, pathogenic

**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, $2.5 \times 10^6$ 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 adoptive 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 CSFE content on CD4+ cells. The data are representative of three independent experiments.

**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.
responses, yet do not initiate /H9252

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proliferate and transition to “mature” T-cells (CD44
also show that in a lymphopenic environment, in the
independently from CD28, CD154, or 4–1BB signaling. We
showed that homeostatic expansion of T-cells occurs
accelerated T-cell proliferation in draining nodes but ap-
pears to have little effect on underlying (homeostatic)
destruction in a lymphopenic environment contrasts those
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 “tolerogenic”
doses of anti-CD154 and CTLA-4Ig. As a result, the authors
raised the concern of the utility of costimulation blockade
rejected in T-cell– depleted mice treated with “toleragenic”
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
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 costimu-
lation 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 iatrog-
ogenic 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 genera-
tion 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 path-
ways. This work may have clinically important transla-
tional 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 protec-
tion is unclear (17,35,45,46). Our work suggests that Tregs
can also be vitally important for immune tolerance in-
duced via therapeutic interventions. We challenged pro-
tected 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 addi-
tional 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 in-
duced 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 mecha-
nisms may identify additional processes that can be ex-
ploded to therapeutically render peripheral tolerance.

Not only do Treg-mediated mechanisms appear to be
critically involved in the maintenance of immune toler-
ance, but a particularly exciting finding of this report is
that Tregs also appear integral in establishing therapeuti-
cally induced immune tolerance. Recipients of Treg-de-
pleted 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, “sup-
presses” 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 recipi-
ents have higher Treg-to-Teff ratios than in diabetic recipi-
ents. 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 uni-
formly develop diabetes, indicating that CD28 signals are
more important in Treg than Teff development (13). It is
conceivable that the CD28-signaling requirements of pe-
ripheral Teffs and Tregs differ as well. On the other hand,
CD154 KO NOD mice are protected from disease, appar-
ently 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
courage further search for unique survival or activation
pathways in Teffs and Tregs and reagents that can selec-
tively target them.

Based on the recognition that the expression of autoim-
une diabetes is determined, in part, by Treg-to-Teff balance, we developed a working model of how nondele-
tional 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-insulitis 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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REFERENCES


