Immunomodulation Followed by Antigen-Specific T_{reg} Infusion Controls Islet Autoimmunity

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Optimal immune-based therapies for type 1 diabetes (T1D) should restore self-tolerance without inducing chronic immunosuppression. $CD4^+Foxp3^+$ regulatory T cells (T_{regs}) are a key cell population capable of facilitating durable immune tolerance. However, clinical trials with expanded T_{regs} in T1D and solid-organ transplant recipients are limited by poor T_{reg} engraftment without host manipulation. We showed that T_{reg} engraftment and therapeutic benefit in nonautoimmune models required ablative host conditioning. Here, we evaluated T_{reg} engraftment and therapeutic efficacy in the nonobese diabetic (NOD) mouse model of autoimmune diabetes using nonablative, combinatorial regimens involving the anti-CD3 (αCD3), cyclophosphamide (CyP), and IAC (IL-2/JES6–1) antibody complex. We demonstrate that αCD3 alone induced substantial T-cell depletion, impacting both conventional T cells (T_{conv}) and T_{regs}, subsequently followed by more rapid rebound of T_{regs}. Despite robust depletion of host T_{conv} and host T_{regs}, donor T_{regs} failed to engraft even with interleukin-2 (IL-2) support. A single dose of CyP after αCD3 depleted rebounding host T_{regs} and resulted in a 43-fold increase in donor T_{reg} engraftment, yet polyclonal donor T_{regs} failed to reverse diabetes. However, infusion of autoantigen-specific T_{regs} after αCD3 alone resulted in robust T_{reg} engraftment within the islets and induced remission in all mice. This novel combinatorial therapy promotes engraftment of autoantigen-specific donor T_{regs} and controls islet autoimmunity without long-term immunosuppression.

The key role played by regulatory T cells (T_{regs}) in self-tolerance (1,2) and suppression of rejection (3–6) makes them attractive for tolerogenic cell-based therapies. Much effort is being devoted to developing T_{reg} therapy for recent-onset type 1 diabetes (T1D) and in transplant settings (7–10). Several groups have established in vitro T_{reg} expansion protocols (11–15); clinical trials with autologous, expanded T_{regs} are ongoing in T1D (9,10) using unselected, polyclonal T_{regs} (14,16). A phase 1 study revealed that in vitro expanded, autologous T_{regs} were safe and tolerable in children with recent-onset T1D, with evidence of improved fasting *C*-peptide and reduced insulin requirement 4 months posttreatment. Therapeutic effects correlated with increased T_{regs} post-infusion but only persisted for a short time. A subsequent trial confirmed the limited persistence of expanded T_{regs} even after a second infusion (9,10,17).

Data emerging from these trials highlight the limitations of protocols that rely solely on T_{reg} infusion without recipient manipulation, including immunomodulation and homeostatic support. In fact, our previous work identified critical requirements for infused T_{reg} engraftment and function: *1*) generation of peripheral space for engraftment, *2*) overcoming competition from host T_{regs}, *3*) sufficient interleukin 2 (IL-2), and *4*) antigen availability to select disease-relevant T_{regs} (18–20). Our observations are supported by other studies (for review see Cabello-Kindelan et al. [21]) and should be considered when designing T_{reg}-based clinical trials, whether these T_{regs} are expanded in vitro or infused after isolation for in vivo expansion. Our previous protocols involved ablative (radiation) conditioning of the host for T_{reg} engraftment and therapeutic efficacy. Although effective, ablative conditioning bears

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translational concerns for clinical application that in T1D would also involve children. Hence, we sought to develop effective but safer methods to create immunological space and enable engraftment of autologous T_{regs}. To this end, we explored novel, nonablating, clinically applicable immunomodulatory regimens that included combinations of a short course of intact αCD3, a single injection of cyclophosphamide (CyP), and the addition of IL-2 complex (IAC, IL-2/clone JES6-1), which progressively improved engraftment. However, the reversal of recent-onset diabetes in nonobese diabetic (NOD) mice was only observed when donor T_{regs} were autoantigen specific rather than polyclonal, and this setting immunomodulation with αCD3 alone resulted in 100% durable diabetes remission (>6 months), with robust T_{reg} engraftment within the islets. Thus, our results demonstrate that robust engraftment and disease-relevant, antigen-specific T_{regs} are key requirements for successful immunotherapy in a preclinical model of autoimmune diabetes.

RESEARCH DESIGN AND METHODS

Mice

NOD mice were from The Jackson Laboratory (Bar Harbor, ME). Breeder pairs of NOD.NON-Thy1^a/1LtJ and NOD.Cg-Tg(TcraBDC2.5,TcrbBDC2.5)1Doi/DoiJ were purchased from The Jackson Laboratory and colonies were maintained at the University of Miami. Animal studies were performed in accordance with protocol approval by the University of Miami Institutional Animal Care and Use Committee.

Cell Purifications

$CD4^+CD25^+$ T_{regs} were purified from spleen (SP) and lymph nodes (LNs) as previously described (22) from NOD or $Thy1.1^+$ NOD mice. T_{regs} on average were >92% pure. For remission studies, islet-specific $CD4^+TCRV\beta4^+CD25^+$ T_{regs} were purified from SP and LNs of NOD.Cg-Tg(TcraBDC2.5,TcrbBDC2.5)1Doi/DoiJ mice by cell sorting and were on average >98.5% pure. In brief, CD4 T cells were enriched by anti-CD4 MACS microbeads (Miltenyi Biotec, San Diego, CA), stained with Alexa Flour 700–conjugated CD4, phycoerythrin (PE)-conjugated Vβ4 antibody, and PE-CF594–conjugated CD25 (BD Biosciences, San Diego, CA) and cell sorted on Beckman Coulter Astrios. Purities were assessed by flow cytometry.

Adoptive T_{reg} Transfer

Freshly isolated T_{regs} (0.5×10^6), either polyclonal or islet-specific T_{regs}, were adoptively transferred by i.v. injections through tail vein to NOD mice that received immunomodulation in various combinations using intact αCD3 (50 μg, clone 2C11) (Leinco Technologies, St. Louis, MO) one time per day for five consecutive days, a single injection of CyP (200 mg/kg) (Sigma-Aldrich, St. Louis, MO), and IL-2 complex (IAC) given every other day for 1 week starting at the time of T_{reg} infusion, or mice were left untreated (Fig. 3*A*). IAC was prepared using recombinant murine IL-2 with anti–IL-2 monoclonal antibody (mAb; clone JES6-1A12) (eBioscience, San Diego, CA) and incubated at a molar ratio of 2 to 1 (1 μg IL-2 and 5 μg Ab) for 15 min at room temperature. The resulting IAC was intraperitoneally injected in 200 μL PBS as indicated in each illustration.

Remission Studies

Recent-onset diabetic NOD mice (>250 mg/dL for two consecutive blood readings) received a single insulin pellet subcutaneously to initially control blood sugars but no insulin thereafter. These mice received immunomodulation in various combinations using intact αCD3, CyP, IAC, and/or T_{reg} infusion (autologous, polyclonal NOD, or islet-specific NOD T_{regs}), or mice were left untreated as indicated in each illustration. Diabetes reoccurrence was monitored for 6 months. Mice were tested two to three times per week for weight and glycosuria. The absence of glycosuria and hyperglycemia indicates lack of diabetes relapse.

Skin Transplantation

Skin grafting was performed as a modification of the Billingham and Medawar technique (23). Anesthetized mice received full-thickness donor skin (prepared from dorsal tissue from ear) on separate graft beds on the back of NOD mice 6 months after αCD3+BDC2.5 T_{reg} infusion. Each recipient received two grafts, syngeneic NOD and allogeneic B6 grafts. A bandage was placed over these two grafts for 7 days. Grafts were monitored every other day and scored rejected when >75% of the original graft was lost or became necrotic as assessed by visual examination.

Antibodies and Flow Cytometry Analysis

SP and LN were made into single cell suspensions by mechanic disruption. Pancreatic LN (pLN) and pancreas were digested with collagenase D (2 mg/mL) (Roche) at 37°C for 30 min. Equal volumes of collected blood were used for peripheral blood mononuclear cells and purified from whole blood on a Ficoll-Paque PLUS gradient (GE Life Sciences). Red blood cells from tissues were lysed with ACK lysing buffer. Total cell counts were performed on tissues and blood using a hemocytometer.

Lymphocytes were stained with LIVE/DEAD Fixable Near-IR according to the manufacturer's instructions (Invitrogen/Thermo Fisher Scientific, Eugene, OR) and washed twice with PBS. Cells were then incubated with rat anti-CD16/32 (clone 24G2) to block nonspecific Ab binding followed by cell surface staining with fluorescence-conjugated Abs against mouse CD45, CD19, CD4, CD8, Thy1.2, CD25 (BD Biosciences), and Thy1.1 (BioLegend, San Diego, CA). Intracellular Foxp3 (clone FJK-16s) staining was performed according to the manufacturer's instructions (eBioscience) along with Ki67 staining (clone B56; BD Biosciences).

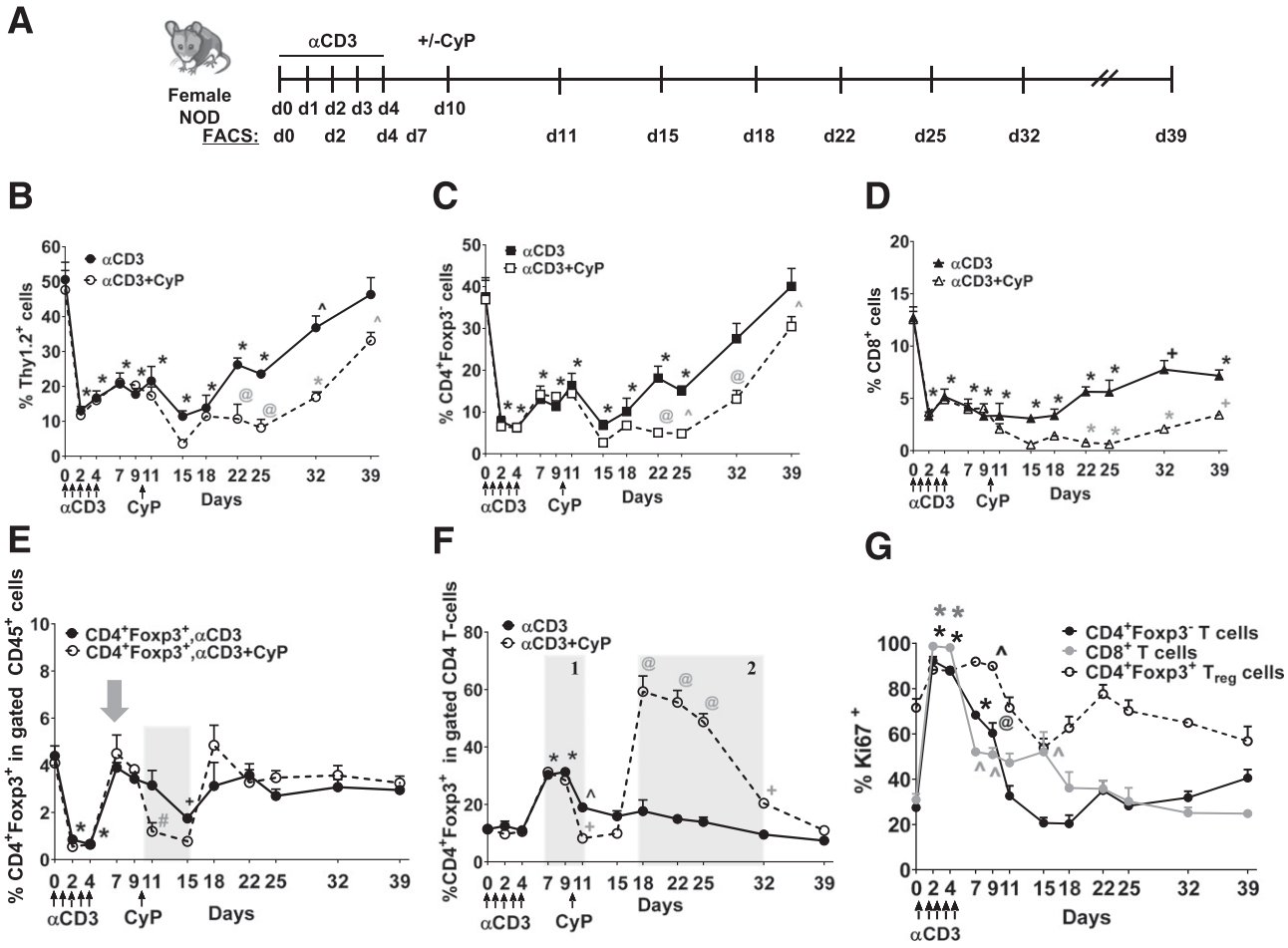

Figure 1—T-cell depletion and rebound after immunomodulation with αCD3, CyP, and IAC in the circulation. *A*: Experimental scheme. Percentage of Thy1.2 (*B*), CD4 (*C*), CD8 (*D*), and $CD4^+Foxp3^+$ (*E*) in the gated $CD45^+$ population and percentage of $CD4^+Foxp3^+$ in the gated CD4 T cells (*F*) (shaded area 1 and 2 indicate increase in the percentage of CD4+$Foxp3^+$ in the CD4 T cells) in prediabetic female NOD mice receiving αCD3 (solid lines) or αCD3+CyP (dashed lines). *G*: Percentage of $Ki67^+$ in the gated $CD4^+Foxp3^+$, $CD4^+Foxp3^-$, and CD8 T cells in the peripheral blood. Time points examined are 2, 4, 7, 11, 15, 18, 22, 25, 32, and 39 days. $n = 5$–6 mice per group. In *E*, the gray arrow indicates recovery in the T_{reg} compartment, and the shaded area indicates the decrease in $CD4^+Foxp3^+$ host T_{regs} following single CyP injection after αCD3 treatment. In *F*, shaded area 1 indicates the increase in $CD4^+Foxp3^+$ cells in the gated CD4 T cells after CD3 alone, and shaded area 2 indicates the increase in $CD4^+Foxp3^+$ cells in total CD4 T cells after CD3+CyP. *$P < 0.0001$; +$P < 0.001$; @$P < 0.01$; ^$P <$ 0.05. One-way ANOVA followed by Dunnett multiple comparison test compared with day 0. Two-way ANOVA followed by Sidak multiple comparison test; αCD3 compared with αCD3+CyP at each time point. #$P < 0.05$. d, day.

Tetramer staining was performed on islet lymphocytes. Murine islets were isolated as described previously (24) with minor modifications. Animals were killed under general anesthesia, and pancreas was exposed and injected with Hanks' balanced salt solution containing 0.5 mg/mL collagenase P (Sigma-Aldrich) via the main bile duct until distension was achieved. Digestion was performed at 37°C for 10–15 min with gentle agitation and terminated by the addition of cold buffer (RPMI containing 10% FCS and 2 mmol/L L-glutamine). The tissue was filtered through 70-μm mesh, placed on Euro-Ficoll (Mediatech) gradients by centrifugation at 2,000 rpm for 15 min, and washed twice with PBS. Single cell suspensions were incubated with rat anti-CD16/32 (clone 24G2) followed by staining with PE-conjugated BDC2.5 tetramer Ab [I-A(g7) BDC2.5 mimetope RTRPLWVRME; National Institutes of Health Tetramer Core] for 3 h at 37°C, and fluorescence-conjugated Abs against cell surface mouse CD45, Thy1.2, CD4, CD8, and CD25 were added to the last 30 min of incubation. Cells were then stained with LIVE/DEAD Fixable Near-IR followed by intracellular Foxp3 staining.

FACS analysis was performed using a BD Biosciences LSRII and analyzed with Diva or Kaluza software. The total number of events collected was between 50,000 and 200,000 cells, except for pancreas samples in which entire samples were collected, and $CD45^+$ cells were set as the storage gate.

Statistical Analysis

One-way ANOVA was followed by Dunnett multiple comparison test. Two-way ANOVA was followed by Sidak multiple comparison test. Unpaired Student *t* test was performed in which αCD3 was compared with αCD3+CyP at each time

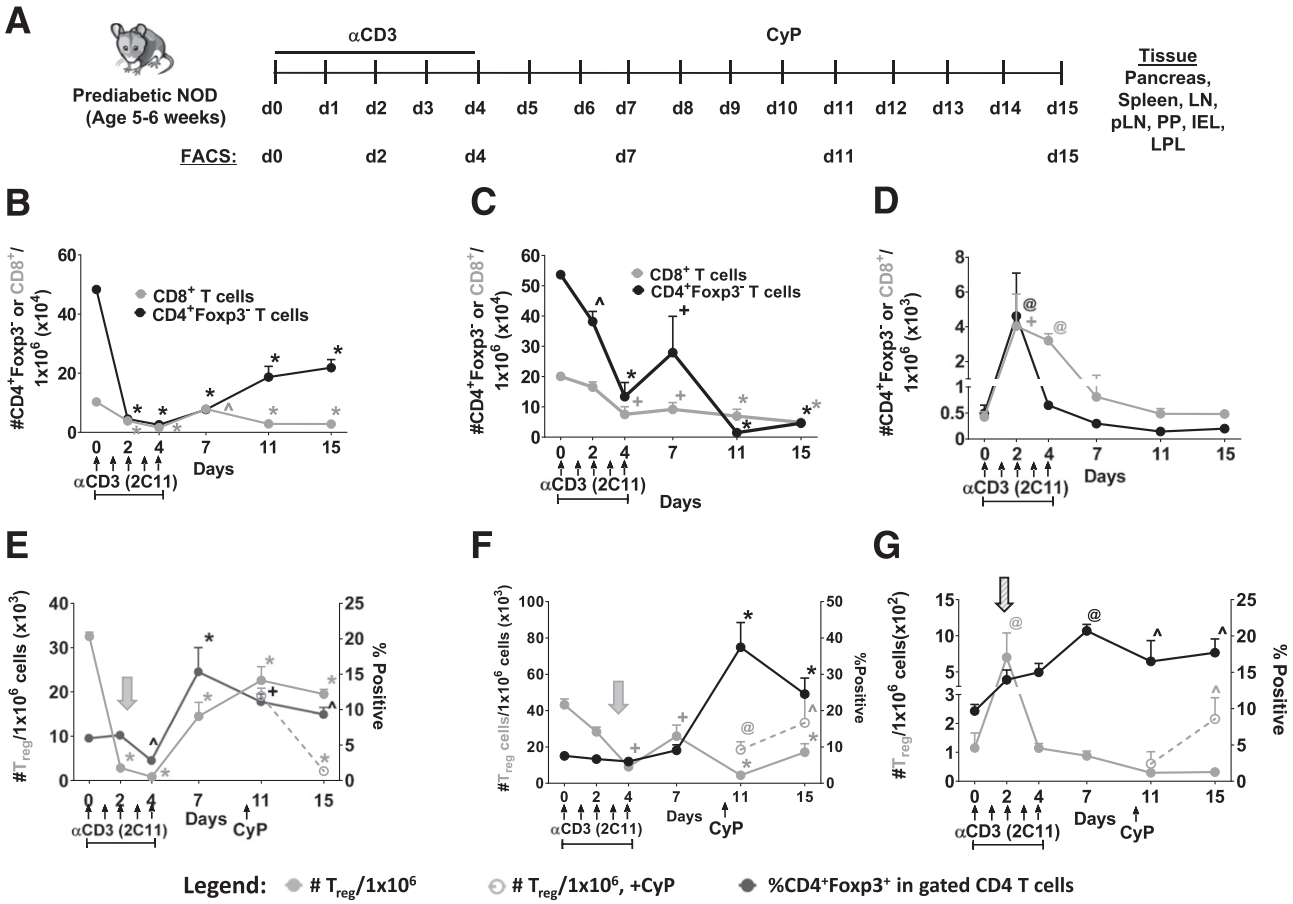

Figure 2—T_{reg} depletion and rebound in the tissues after αCD3 and CyP immunomodulation. *A*: Experimental scheme. Number of $CD8^+$ and $CD4^+Foxp3^-$ T cells (per 1×10^6 lymphocytes) in peripheral blood (*B*), pLNs (*C*), and pancreas (*D*). Number of $CD4^+Foxp3^+$ cells (per 1×10^6 $CD45^+$ lymphocytes) and percentage of $CD4^+Foxp3^+$ in the gated CD4 T cells in peripheral blood (*E*), pLNs (*F*), and pancreas (*G*) in the presence or absence of CyP of young, prediabetic NOD mice (aged 5–6 weeks). $n = 3$–6 mice per group. In *E* and *F*, gray arrows indicate an early decrease in the number of $CD4^+Foxp3^+$ cells after the start of αCD3 in the blood and pLNs. In *G*, hatched arrow indicates an initial increase in the number of $CD4^+Foxp3^+$ cells after the start of αCD3 in the pancreas. *$P < 0.0001$; +$P < 0.001$; @$P < 0.01$; ^$P < 0.05$. One-way ANOVA followed by Dunnett multiple comparison test compared with day 0. Unpaired Student *t* test, αCD3 compared with αCD3+CyP at each time point. $n = 3$–6 mice per group. d, day.

point. Comparisons yielding $P < 0.05$ were considered statistically significant. Survival curves were subjected to Mantel-Cox log-rank test. The *P* value is indicated on the graph.

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. All resources, including animal models and reagents, are commercially available.

RESULTS

A Combinatorial Regimen of αCD3 and CyP Creates Space in the Host T_{reg} Compartment in Female NOD Mice

In previous studies, intact αCD3 was more effective at depleting T cells than the $F(ab')_2$ form (25–27); while regimens varied in timing and duration of administration, depletion was dose dependent. Yang et al. (27) reported that a single injection of intact αCD3 led to transient T-cell depletion in both C57BL/6 and NOD mice, but NOD required a higher dose (50 μg) than C57BL/6 mice; depletion was age dependent in NOD mice. Moreover, in NOD mice, 50 μg of intact αCD3 more efficiently depleted CD4 T cells than 100 μg of $F(ab')_2$-αCD3 (27). Hence, we used intact αCD3 antibody (50-μg dose) as a depleting agent in our study. We investigated the effects of αCD3 therapy on conventional T-cell and T_{reg} compartments in the circulation of 5- to 6-week-old prediabetic NOD female mice. We used a 5-day course of αCD3 and followed the effects for 39 days (Fig. 1*A*). αCD3 readily decreased conventional T cells, including $CD4^+Foxp3^-$ and CD8 T cells (Fig. 1*B–D*, solid lines). $CD4^+Foxp3^-$ T cells returned to normal levels at around 39 days after the first αCD3 dose; CD8 T cells did not fully recover. Our assessments at earlier time points (as early as 2 days) revealed that T_{reg} depletion occurs very early post-αCD3 (Fig. 1*E*); in fact, T_{regs} returned to normal levels within 7 days of the first dose (Fig. 1*E*, solid lines, gray arrow). Thus, we demonstrated a previously unknown early T_{reg} depletion post-αCD3. We also showed that T_{regs} rebound faster than

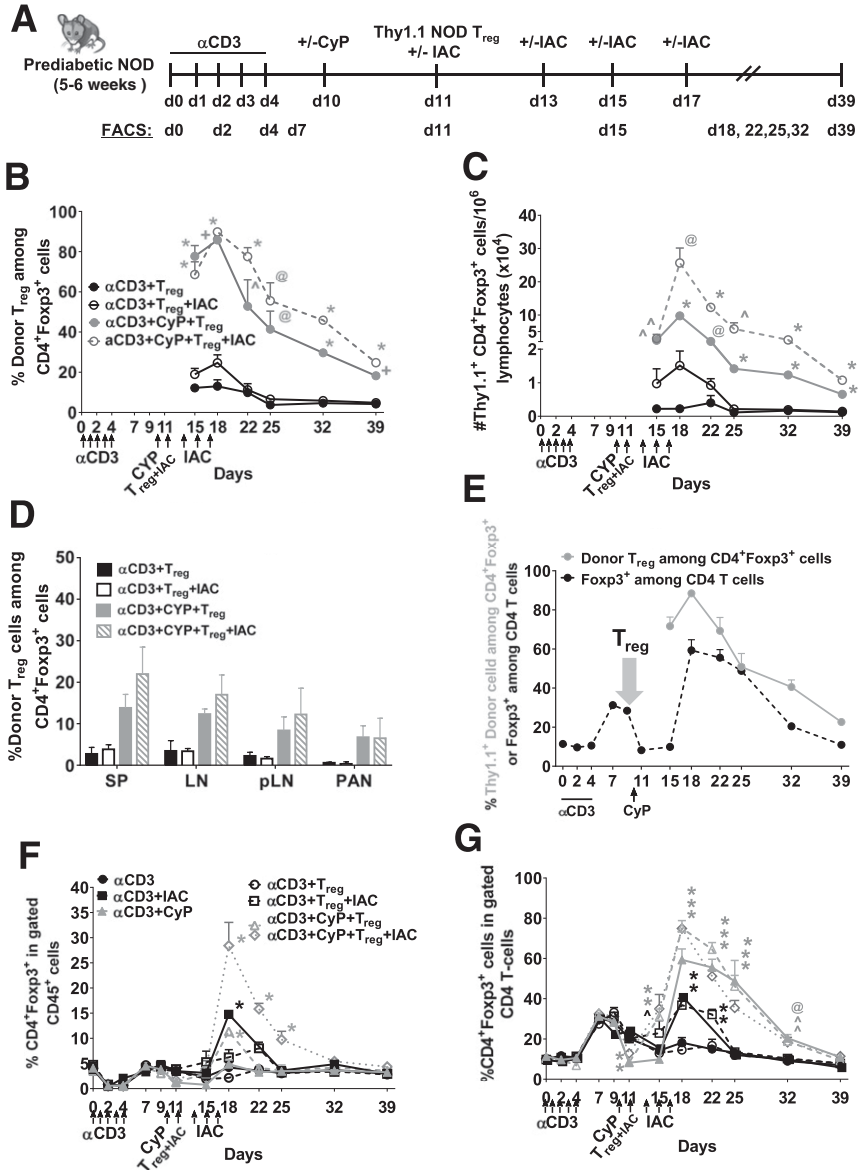

Figure 3—Adoptive transfer of T_{regs} leads to engraftment after immunomodulation in young, prediabetic NOD mice. *A*: Experimental scheme. *B*: Percentage of Thy1.1$^+$ donor T_{reg} engraftment among the total gated CD4$^+$Foxp3$^+$ T cells in peripheral blood. *C*: Number of donor T_{regs}/1×10^6 CD45$^+$ lymphocytes in peripheral blood. *D*: Percentage of Thy1.1$^+$ donor T_{reg} engraftment in the total gated CD4$^+$Foxp3$^+$ T cells 4 weeks post–T_{reg} infusion in SP, LN, pLN, and pancreas (PAN) of young, female prediabetic NOD mice receiving αCD3+T_{reg}, αCD3+T_{reg}+IAC, αCD3+CyP+T_{reg}, or αCD3+CyP+T_{reg}+IAC. IAC = anti–IL-2 (JES6)+rmIAC. *E*: Percentage of Thy1.1$^+$ donor T_{regs} in the gated CD4$^+$Foxp3$^+$ T cells in mice receiving the αCD3+CyP+T_{reg}+IAC regimen and CD4$^+$Foxp3$^+$ in the gated total CD4 T cells in mice receiving the αCD3 regimen in the peripheral blood. *F*: Percentage of CD4$^+$Foxp3$^+$ in the gated CD45$^+$ population. *G*: CD4$^+$Foxp3$^+$ in the gated CD4 T cells in the peripheral blood of young, prediabetic female NOD mice (aged 5–6 weeks) in NOD mice receiving αCD3, αCD3+IAC, αCD3+CyP, αCD3+T_{reg}, αCD3+T_{reg}+IAC, αCD3+CyP+T_{reg}, or αCD3+CyP+T_{reg}+IAC. n = 5–6 mice per group. *$P < 0.0001$; +$P < 0.001$; @$P < 0.01$; ^$P < 0.05$. *B* and *C*: Multiple Student t test compared with αCD3+T_{reg}. *F* and *G*: Two-way ANOVA followed by Dunnett multiple comparison test compared with αCD3. d, day; rmIAC, recombinant murine IAC.

conventional T cells, which recover around day 32–39, leading to an increased proportion of CD4$^+$Foxp3$^+$ cells in the CD4$^+$ T-cell compartment (Fig. 1*F*, solid line, shaded area 1). Penaranda et al. (28) noted that treatment with F(ab′)$_2$-αCD3 induced a transient increase in the proportion of Foxp3$^+$ cells in the CD4 T-cell compartment that was accompanied by a slight reduction in the total count of T_{regs}, but that the increased Foxp3$^+$ proportion was due to greater depletion of the CD4$^+$Foxp3$^-$ conventional T cells. In our study, most T cells were initially proliferating after depletion with intact αCD3 (Fig. 1*G*); however, only T_{regs} maintained robust proliferation, including a much higher Ki67 mean fluorescence intensity, over the 39-day follow-up (Supplementary Fig. 1), which may contribute to the increased proportion of Foxp3$^+$ cells in the CD4$^+$ T-cell compartment after αCD3 treatment together with the slow recovery of the CD4$^+$Foxp3$^-$ T cells.

We next examined T-cell compartments after αCD3 treatment up to 15 days in pancreas, SP, LN, pLN, and Peyer's patches (PP), lamina propria lymphocytes (LPLs), and intraepithelial lymphocytes (IELs) in the small intestine (Fig. 2*A*). Similar to blood, αCD3 therapy rapidly depleted $CD4^+Foxp3^-$ and $CD8^+$ T cells in LN and pLN, with <30% recovery from baseline at the end of the 15-day follow-up (Fig. 2*B* and *C* and Supplementary Fig. 2). In contrast, there was an initial $CD8^+$ T-cell increase in SP, PP, IEL, and LPL followed by a decrease beginning on day 4, while $CD4^+Foxp3^-$ T cells were rapidly decreased after αCD3 treatment (Supplementary Figs. 2 and 3). There was a slow recovery in the small intestine, with <35% of $CD4^+Foxp3^-$ T cells recovering from baseline, excluding T cells in SP with ~36–58% recovery (Supplementary Figs. 2 and 3). In pancreas, there was an early increase followed by decrease of $CD4^+Foxp3^-$ T cells beginning on day 4, with no recovery by the end of the follow-up period. $CD8^+$ T cells had a more persistent increase before returning to baseline, leading to an inversion of the CD4:CD8 T-cell ratio after αCD3 treatment (Fig. 2*D*).

Resembling effects in blood (Fig. 2*E*, left panel, gray arrow), αCD3 treatment was immediately followed by a decrease of $CD4^+Foxp3^+$ T_{regs} in LN and pLN, with 44–60% recovery by day 15 (Fig. 2*E* and *F* and Supplementary Fig. 2, gray arrows). The slower recovery of $CD4^+Foxp3^-$ T cells in these tissues compared with T_{regs} led to increased proportions of $CD4^+Foxp3^+$ in the $CD4^+$ compartment (Fig. 2*E* and *F* and Supplementary Fig. 2). There was delayed T_{reg} depletion in SP (Supplementary Fig. 2, white arrow) compared with blood and LNs, but recovery was still around 50% at the end of follow-up (Fig. 2 and Supplementary Fig. 2). In contrast, αCD3 treatment resulted in an initial T_{reg} increase in the pancreas (Fig. 2*G*, hatched arrow) followed by delayed depletion starting at day 11, with no recovery of T_{regs} accompanied by an increase in the proportion of $CD4^+Foxp3^+$ cells in the $CD4^+$ T-cell compartment. A similar pattern was seen in LPL, IEL, and PP, with an initial T_{reg} increase, but T_{regs} did begin to recover (Supplementary Fig. 3). T_{reg} changes post-αCD3 did not appear to be solely due to redistribution of circulating T_{regs} to tissues. By linear regression, we found a link between T_{reg} rebound and proliferation in pLN (Supplementary Fig. 4*A*), pancreas, LPL, and peripheral blood (not shown). However, there was no correlation observed in LN (Supplementary Fig. 4*B*), SP, IEL, and PP (not shown), suggesting that T_{reg} rebound may only occur in certain compartments, and may be partly explained by T_{reg} redistribution. Moreover, consistent with earlier reports (25,29), lymphocytes isolated on day 15 from the SP or pancreas of treated, prediabetic mice exhibited inflammatory cytokine production with 18.7 ± 1.9% IFNγ- and 23.4 ± 7.5% TNFα-producing cells from the pancreas and 19.1 ± 2.5% IFNγ- producing cells from the SP (Supplementary Fig. 5). Circulating cells of αCD3-treated mice showed little production of these cytokines. Lymphocytes from untreated mice showed very little production of these cytokines from any of the tissues examined. Overall, this extended longitudinal evaluation in tissues reveals the complex effects of αCD3 treatment on T_{regs}, which vary in different tissues and include depletion, rebound, and redistribution. Moreover, αCD3 treatment does not appear to open the T_{reg} compartment for extensive periods of time in the pancreas or lymphoid tissues, suggesting that this treatment alone may not promote robust polyclonal T_{reg} engraftment.

A Single Dose of CyP to αCD3 Therapy Depletes Rebounding Host T_{regs}

We have previously shown that robust donor T_{reg} engraftment requires not only creating peripheral space but also lessening competition from rebounding host T_{regs} (20). Because αCD3 treatment induces only an early, temporary T_{reg} depletion that is followed by rebound, achieving optimal engraftment of autologous donor T_{regs} may require further manipulation to control rebounding populations. CyP is known to eliminate proliferating cells in response to alloantigen but also depletes T_{regs} because of their higher proliferation rates compared with naïve T cells (30,31). To determine whether CyP could effectively deplete rapidly rebounding host T_{regs} post-αCD3, a single CyP injection was given at day 10 (Fig. 1*A*). CyP after αCD3 significantly decreased host T_{regs} by 62% (day 11, two-way ANOVA, $P = 0.0215$) for ~5 days post-CyP in blood (Fig. 1*E*, dashed lines), thus creating a more supportive T_{reg} environment (Fig. 1*E*, shaded area). CyP also depleted $CD4^+$ and $CD8^+$ T cells, which remained lower compared with αCD3 alone 39 days after the first αCD3 dose (Fig. 1*B–D*, dashed lines). After CyP, a substantial increase in the proportion of $CD4^+Foxp3^+$ cells in the $CD4^+$ T-cell compartment was observed during days 18–32 (Fig. 1*F*, dashed line, shaded area 2). Moreover, CyP after αCD3 decreased rebounding host T_{regs} by 21–82% in SP, LN, PP, and IEL (Supplementary Figs. 2 and 3). In contrast, host T_{regs} showed a modest increase in pancreas and pLN after CyP (Fig. 2*F* and *G*). Overall, these findings highlight that a single dose of CyP after αCD3 can effectively deplete rebounding host T_{regs}, which are expected to compete with infused donor T_{regs}, and can be used to synergize with αCD3 therapy to open the T_{reg} compartment to promote robust T_{reg} engraftment.

A Combinatorial Regimen of αCD3 and CyP Promotes Robust Engraftment of Polyclonal, Autologous Donor T_{regs} in NOD Mice

We tested whether these combinatorial therapies (Fig. 3*A*) supported engraftment of polyclonal, autologous donor T_{regs} in 5- to 6-week-old female NOD mice. Despite robust T-cell depletion with αCD3 alone, donor congenic $Thy1.1^+$ NOD T_{regs} were minimally detected 1–4 weeks post-infusion in blood (Fig. 3*B* and *C* and Supplementary Table 1, αCD3+T_{reg}), SP, LN, pLN, and pancreas (Fig. 3*D*). The addition of IL-2 support with a short course of IAC treatment after αCD3 enhanced engraftment during

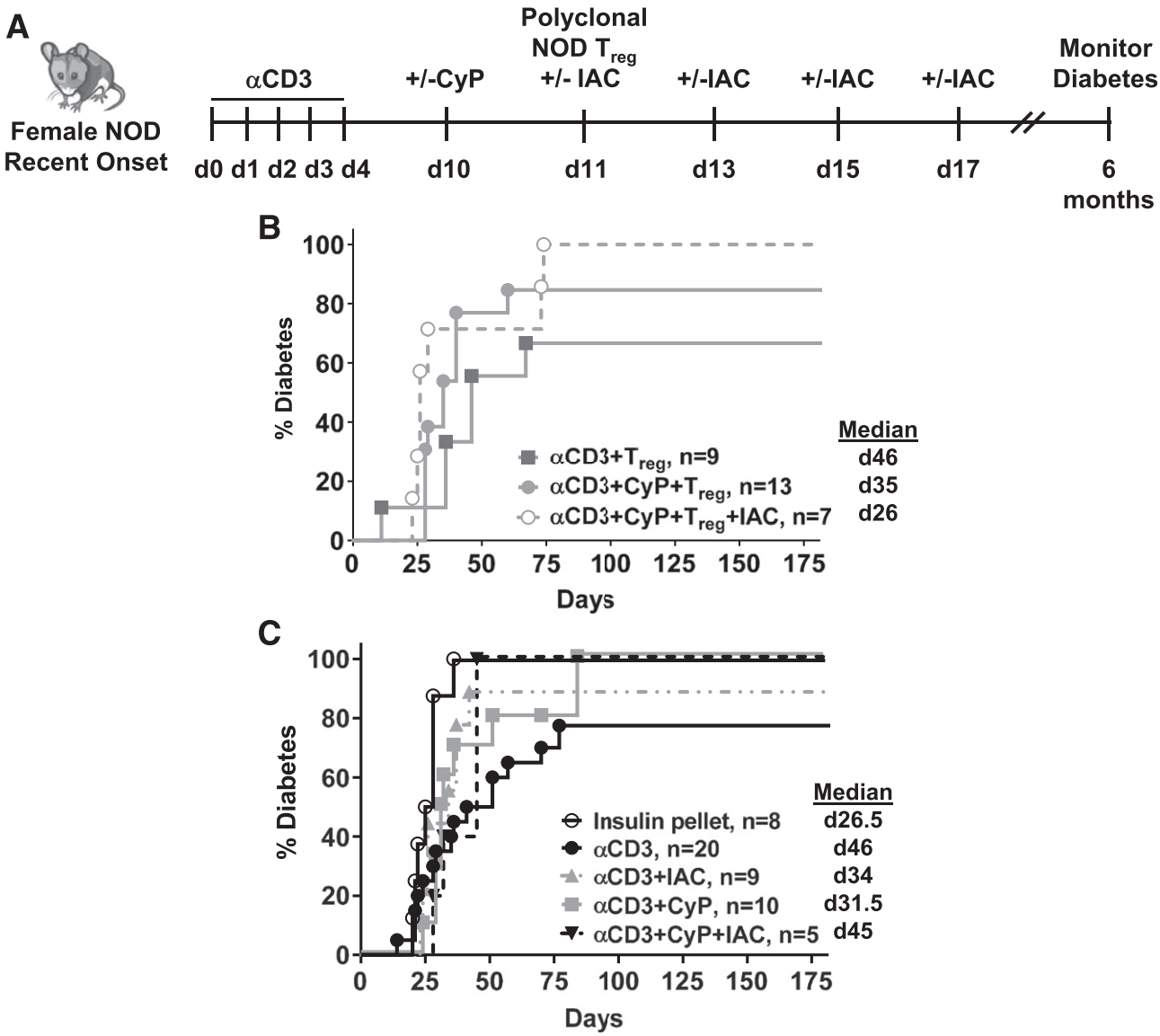

Figure 4—Diabetes remission after immunomodulation and infusion of polyclonal T_{regs} in recently diabetic NOD mice. *A*: Experimental scheme. *B*: Percentage of diabetic mice after αCD3, CyP, and/or IAC immunomodulation and adoptive transfer of polyclonal T_{regs} isolated from Thy1.1 congenic NOD mice into recently diabetic NOD mice. *C*: Percentage of diabetic mice after αCD3, CyP, and/or IAC. Diabetic mice were initially given a single insulin pellet at the start of αCD3 treatment to control diabetes. Urine and blood glucose levels were monitored for 6 months after the start of αCD3 treatment. Median values are indicated to the right of the legends. d, day.

treatment (αCD3+T_{reg}+IAC), but by the end of follow-up, engraftment was no better than αCD3 alone. However, a single dose of CyP after αCD3 (αCD3+CyP+T_{reg}), which depletes rebounding host T_{regs} (Fig. 1*E*, dashed line, shaded area), resulted in a 43-fold increase in donor T_{reg} engraftment compared with αCD3; the addition of IAC further enhanced engraftment by several fold (αCD3+CyP+T_{reg}+IAC) (Fig. 3*B* and *C* and Supplementary Table 1). The significant increase in the proportion of host $CD4^+Foxp3^+$ cells in the $CD4^+$ T-cell compartment during the rebound phase after αCD3+CyP (Fig. 1*F*, shaded area 2) did not inhibit donor T_{reg} engraftment, which was robust (Fig. 3*D*). Thus, key to robust engraftment is to create space that minimizes host T_{reg} competition at the time of T_{reg} infusion. The addition of CyP and IAC resulted in the greatest increase of the total $CD4^+Foxp3^+$ compartment compared with all other regimens tested (Fig. 3*F*), and was accompanied by an increased proportion of $CD4^+Foxp3^+$ cells in the $CD4^+$ T-cell compartment (Fig. 3*G*). While the total T_{reg} compartment returned to pretreatment levels at the end of the 39-day follow-up (Fig. 3*F* and *G*), donor T_{regs} comprised 8–25% of the T_{reg} compartment in mice given αCD3 and CyP with or without IAC (Fig. 3*B* and *D*). Importantly, congenic $Thy1.1^+$ donor T_{regs} maintained a phenotype similar to host T_{regs} in all treatments with comparable Foxp3/CD25 expression in treated mice (Supplementary Fig. 6*A*).

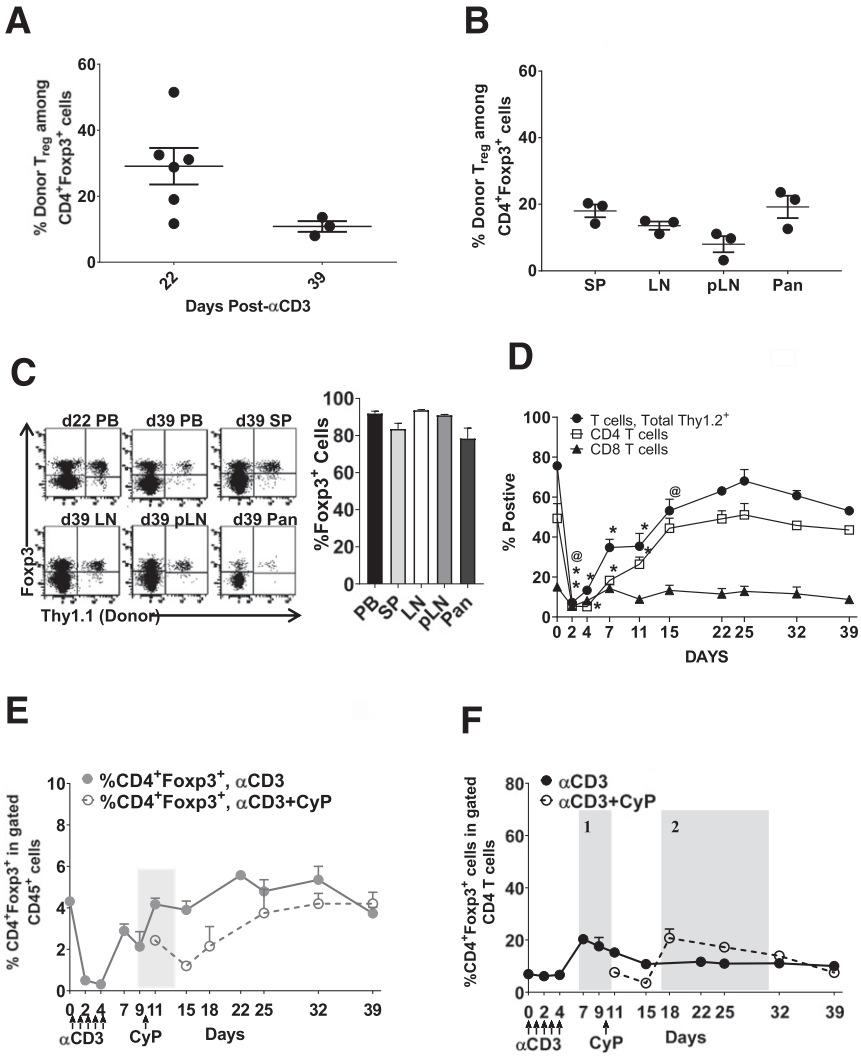

Figure 5—Donor T_{reg} engraftment after immunomodulation in recent diabetic NOD mice. *A*: Percentage of Thy1.1$^+$ donor NOD T_{reg} in CD4$^+$Foxp3$^+$ in peripheral blood (PB). *B*: 4–8 weeks in the SP, LN, pLN, and pancreas (Pan). *C*: Foxp3 and Thy1.1 staining in gated CD4 T cells day 22–39 after T_{reg} infusion in recent-onset diabetic NOD mice receiving αCD3+CyP+T_{reg}+IAC. *D*: Percent positive Thy1.2$^+$ total T cells, CD4$^+$Foxp3$^-$ T cells, or CD8 T cells in the peripheral blood of recent-onset NOD mice receiving αCD3 treatment. *$P < 0.0001$; @$P < 0.01$, one-way ANOVA followed by Dunnett multiple comparison test compared with day 0. *E*: Percentage of CD4$^+$Foxp3$^+$ in gated CD45$^+$ population. *F*: Percentage of CD4$^+$Foxp3$^+$ in the gated CD4 T cells with αCD3 (solid) or αCD3+CyP (dashed). Diabetic NOD mice were maintained with insulin pellets to control blood glucose. $n = 2$–6 mice per group. In *E*, the shaded area indicates the decrease in CD4$^+$Foxp3$^+$ host T_{regs} after single CyP injection after αCD3 treatment. In *F*, shaded area 1 indicates an increase in CD4$^+$Foxp3$^+$ cells among total in the gated CD4 T cells after CD3 alone, and shaded area 2 indicates an increase in CD4$^+$Foxp3$^+$ cells in the gated CD4 T cells after CD3+CyP. d, day.

We then treated late, prediabetic NOD mice aged 16–22 weeks with our various immunomodulation strategies followed by infusion of congenic, autologous Thy1.1$^+$ NOD T_{regs} and examined T_{reg} engraftment. Similar to the young, prediabetic NOD mice, substantial engraftment occurred in mice that received αCD3 and CyP with or without IAC in the peripheral blood and SP, LN, pLN, and pancreas (Supplementary Fig. 7*A*, *B*, and *E*). Again, optimal T_{reg} engraftment was observed in the αCD3+CyP+T_{reg}+IAC group, with a 53-fold increase in engraftment compared with αCD3 alone (day 18 after the start of αCD3), and led to the greatest increase in the total CD4$^+$Foxp3$^+$ population (Supplementary Fig. 7*A*–*C*). Similarly, we observed a decline in engraftment, but donor T_{regs} comprised 25–30% of the T_{reg} compartment at the end of follow-up in mice given αCD3/CyP (Supplementary Fig. 7*A*). Collectively, our results demonstrate a new therapeutic synergy between αCD3 and CyP in promoting engraftment of polyclonal, autologous T_{regs}.

A Combinational Regimen of CD3, CyP, and Polyclonal, Autologous Donor T_{regs} Fails to Induce Diabetes Remission in Female NOD Mice

We then examined whether diabetes remission could be induced by combinational regimens themselves or in combination with infusion of polyclonal, autologous T_{regs} (Fig.

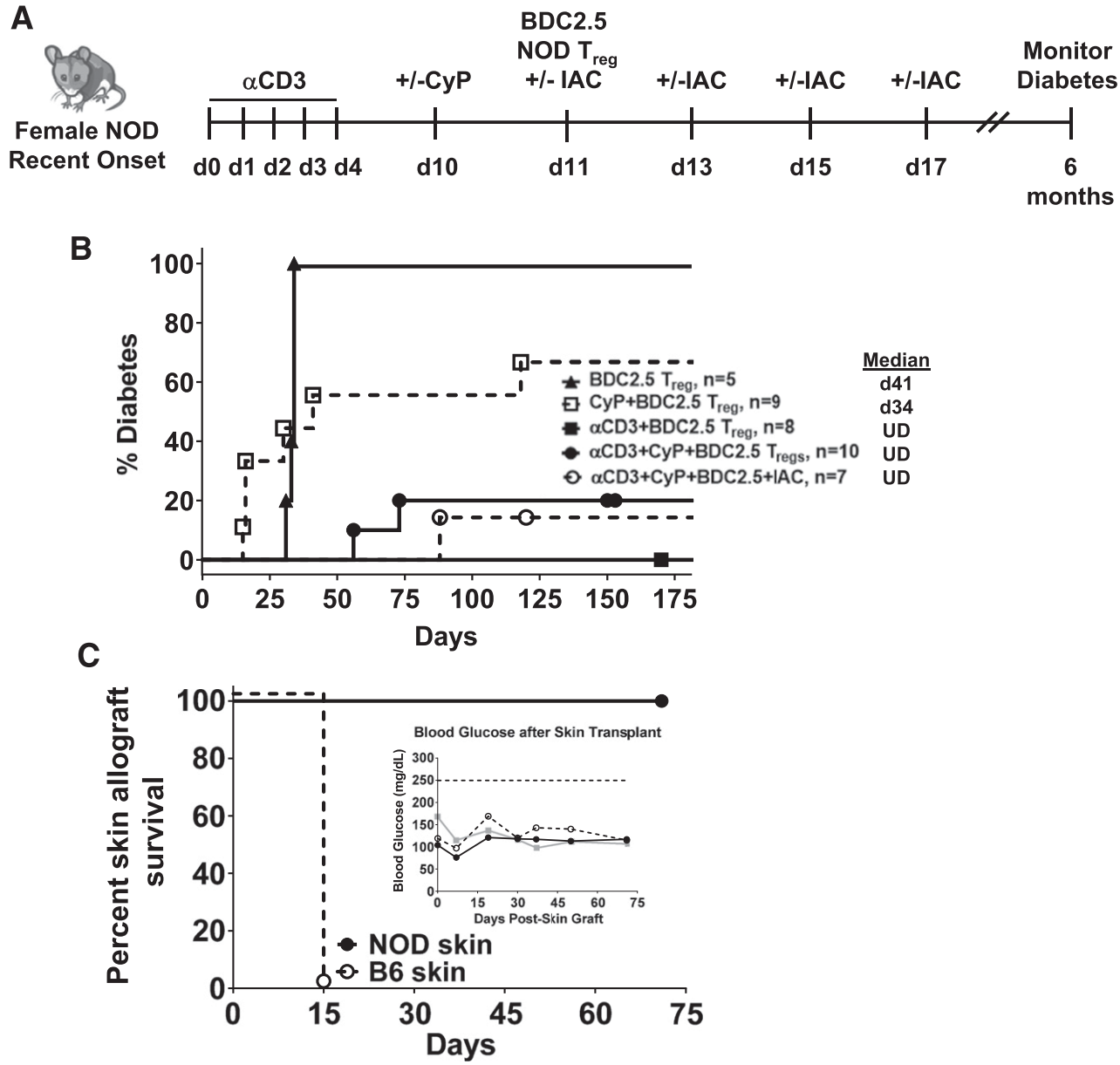

Figure 6—Diabetes remission after immunomodulation and infusion of antigen-specific T_{regs} in recently diabetic NOD mice. *A*: Experimental scheme. *B*: Percentage of diabetic mice after αCD3, CyP, and/or IAC immunomodulation and adoptive transfer of antigen-specific T_{regs} isolated from BDC2.5 TCR NOD mice into recently diabetic NOD mice. Diabetic mice were initially given a single insulin pellet at the start of αCD3 treatment to control diabetes. Urine and blood glucose levels were monitored for 6 months after the start of αCD3 treatment. Median values are indicated to the right of the legends. *C*: Percentage of skin graft survival of NOD and B6 skin placed 6 months after the start of αCD3 and BDC2.5 T_{reg} infusion. Mice were monitored for skin rejection and diabetes development for 70 days. Inset shows blood glucose values for the transplanted mice. Survival curves were subjected to Mantel-Cox log-rank test. The *P* value is indicated on the graph. d, day; UD, undefined.

4*A*). Although our combinatorial regimen was very effective at promoting engraftment in prediabetic NOD mice, long-term diabetes remission was not achieved in recently diagnosed NOD mice treated with immunomodulation alone or in combination with polyclonal T_{regs} (Fig. 4*B* and *C*). We found that in mice treated with the αCD3+CyP+polyclonal T_{reg}±IAC regimen, the time to disease relapse significantly correlated with the levels of donor T_{regs} in blood ($r = 0.72223$, $P = 0.028$). Therefore, we examined T_{reg} engraftment in blood and tissues; donor, congenic Thy1.1$^+$ NOD T_{regs} were readily detected in blood and SP, LN, pLN, and pancreas 4 weeks post-infusion (αCD3+CyP+T_{reg}+IAC) (Fig. 5*A* and *B* and Supplementary Table 2), and donor T_{regs} had a similar level of Foxp3/CD25 expression as host T_{regs} in blood and tissues (Supplementary Fig. 6*B*). However, there was a decrease (2.0–2.6-fold) in the level of T_{reg} engraftment observed in recent-onset mice compared with late prediabetic NOD mice at the end of follow-up (Supplementary Fig. 7*E* and *F*), but overall engraftment with αCD3/CyP treatment was

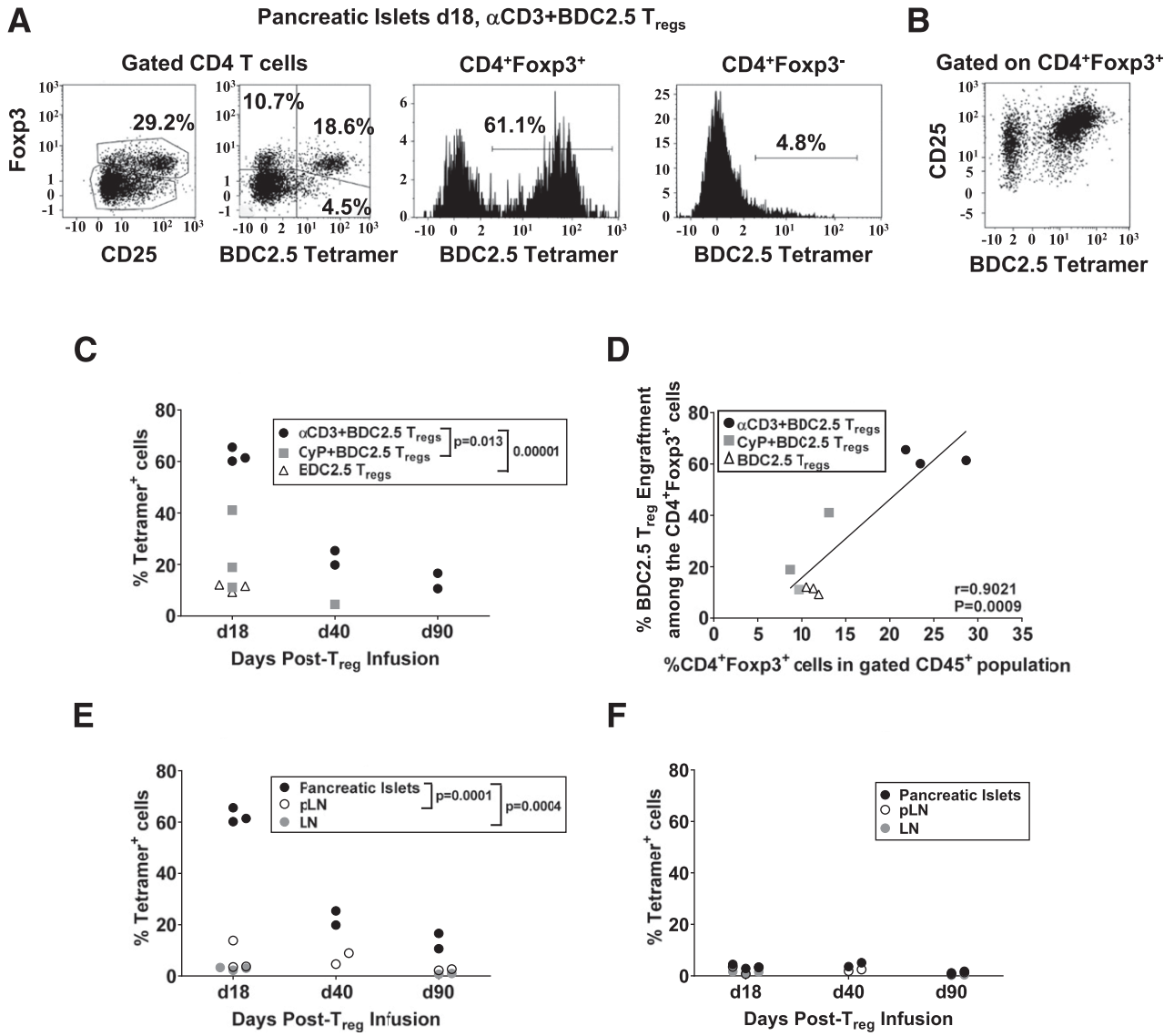

Figure 7—Antigen-specific donor T_{reg} engraftment after immunomodulation in recent diabetic NOD mice. *A*: BDC2.5 tetramer staining of $CD45^+$ cells from isolated pancreatic islets of recent-onset NOD mice subjected to immunomodulation and adoptive transfer of BDC2.5 T_{regs} of gated $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells 7 days after T_{reg} infusion (day 18). *B*: Representative staining of CD25 and BDC2.5 tetramer on gated $CD4^+Foxp3^+$ cells in the pancreatic islets 7 days after T_{reg} infusion after αCD3 treatment. *C*: Percentage of BDC2.5 $tetramer^+$ cells among the $CD4^+Foxp3^+$ cells from pancreatic islets 18, 40, and 90 days after αCD3 or CyP treatment or in mice left untreated. *D*: Correlation analysis between the level of BDC2.5 T_{reg} engraftment among the gated $CD4^+Foxp3^+$ cells and percentage of total $CD4^+Foxp3^+$ in the gated $CD45^+$ population within islets on day 18 after the start of αCD3 treatment. Percentage of BDC2.5 $tetramer^+$ cells among the $CD4^+Foxp3^+$ cells (*E*) or $CD4^+Foxp3^-$ cells (*F*) in the pancreatic islets, pLN, or other LN 18, 40, and 90 days post–T_{reg} infusion after αCD3 treatment. *P* values are indicated in *C* and *E* following unpaired Student *t* test. d, day.

still robust compared with prediabetic mice treated with αCD3 alone (Fig. 3*A*–*C* and Supplementary Fig. 7*A* and *B*). Several studies showed that inflammatory environments promote unstable T_{regs}, termed ex-T_{regs}, which become pathogenic (32–34). However, the observed lower engraftment was not explained by a loss of Foxp3 expression in donor T_{reg} populations ($CD4^+Foxp3^+Thy1.1^+$ cells), as there were very few $Thy1.1^+Foxp3^-$ cells in blood, SP, LN, pLN, or pancreas (Fig. 5*C*). Lower engraftment was not due to a failure of αCD3 to effectively deplete T cells, as CD4, CD8, and $CD4^+Foxp3^+$ cells were decreased post-αCD3 (Fig. 5*D* and *E*). Engraftment was not impacted by rebounding host T_{regs}, as CyP was very effective at depleting rebounding cells post-αCD3 (Fig. 5*E*, shaded area, and Fig. 5*F*, shaded area 1). However, unlike in prediabetic mice in which host T_{regs} recovered after CyP (Fig. 1*F*, shaded area 2), such a recovery was not observed in recent-onset mice (Fig. 5*F*, shaded area 2). Together, these results suggest that in recent-onset mice, despite initial insulin treatment, the host may not fully support the T_{reg} compartment, which could represent an obstacle to successful T_{reg} therapy with polyclonal T_{regs}.

Combinational Therapies That Include Antigen-Specific T_{regs} Lead to Durable Remission in Newly Diagnosed Diabetic NOD Mice

Selection of therapeutic T_{regs} by antigen could potentially overcome the less supportive environment observed in recent-onset mice. Moreover, antigen-specific T_{regs} are known to be more effective than polyclonal T_{regs} at regulating diabetes and responses to alloantigen (35–37). Therefore, we tested whether the infusion of islet-specific T_{regs} together with αCD3 and/or CyP immunomodulation could lead to durable diabetes remission in recently diabetic NOD mice (Fig. 6*A*). In sharp contrast to polyclonal T_{regs}, infusion of islet-specific (chromogranin A) T_{regs} isolated from BDC2.5 TCR transgenic NOD mice after immunomodulation induced durable diabetes remission (Fig. 6*B*). This remission was observed after immunomodulation with αCD3 alone or in combination with CyP with or without a short course of IAC. All the NOD mice receiving an islet-specific T_{reg} infusion after αCD3 alone went into durable remission (6 months). αCD3 prior to BDC2.5 T_{reg} infusion is critical for achieving durable remission as infusion of BDC2.5 T_{regs} after CyP injection resulted in only 38% of mice experiencing long-term remission, and infusion of equal numbers of BDC2.5 T_{regs} without any prior immunomodulation failed to reverse diabetes (Fig. 6*B*). These data support that manipulation of the host immune system is necessary to achieve a therapeutic benefit with adoptive T_{reg} therapy. Antigen specificity of infused T_{regs} is critical, since equal numbers of polyclonal T_{regs} failed to achieve durable remission in most mice (Fig. 4*B*). Importantly, the lack of diabetes relapse with αCD3+BDC2.5 T_{reg} therapy was not due to loss of effective immune responses. Fully allogeneic B6 skin grafts placed 6 months after the start of therapy on mice given αCD3+BDC2.5 T_{reg} therapy were all rejected, whereas syngeneic NOD grafts were maintained (Fig. 6*C*), and mice continued to be diabetes free at end of the 70-day follow-up (Fig. 6*C*, inset). Therefore, the destructive autoimmune response had been reset without long-term immunosuppression and these mice had a fully competent immune system.

Combinational Regimen of αCD3 and Antigen-Specific T_{regs} Results in Robust, Long-Term Engraftment Within Islets

We determined the level of islet-specific T_{reg} engraftment after immunomodulation. BDC2.5 T_{reg} engraftment was assessed from isolated islets 7 days post–T_{reg} infusion (day 18 after start of αCD3) and up to 90 days post-αCD3 or CyP treatment or in unmanipulated recent-onset NOD mice. We detected the highest engraftment in pancreatic islets with αCD3 treatment and persisted up to 90 days compared with CyP treatment; mice that did not receive immunomodulation had the lowest engraftment (Fig. 7*A* and *C*). These BDC2.5 tetramer-positive cells expressed high levels of CD25 (Fig. 7*B*). Importantly, this level of BDC2.5 T_{reg} engraftment in the pancreas of mice 40 days post–αCD3 treatment was similar to the level of polyclonal T_{reg} engraftment in the pancreas when αCD3, CyP, and IAC were given (Fig. 5*B*). There was a significant correlation between the level of BDC2.5 T_{reg} engraftment and percentage of total $CD4^+Foxp3^+$ T cells within islets (Fig. 7*D*). BDC2.5 T_{regs} were detected in pLN or nondraining LNs, but much less compared with islets (Fig. 7*E*), and this was similar to the level of engraftment observed with polyclonal, autologous T_{regs} after αCD3 treatment alone (Fig. 3*D*). Moreover, tetramer-positive $CD4^+Foxp3^-$ T cells were minimally detected, suggesting that BDC2.5 T_{regs} are stable within these tissues (Fig. 7*E* and *F*). Collectively, our data suggest that antigen-specific T_{regs} may be capable of better engraftment specifically in the pancreatic islets where their target antigen is present, and this may explain the therapeutic efficacy observed.

DISCUSSION

Our previous work focused on understanding critical factors required for the generation/homeostasis of thymic T_{regs} and demonstrated the importance of host environment as a key determinant of long-term T_{reg} engraftment and therapeutic efficacy in therapies involving T_{reg} infusion (18–20,22,38,39). Our data from this study demonstrate how these requirements also apply for a successful outcome of a T_{reg}-based immunomodulatory regimen in a preclinical T1D model. Although therapeutic success in NOD mice does not guarantee a translation to patient benefit, it is nonetheless important to conduct preclinical testing, when feasible, before starting clinical experimentation. The NOD mouse presents critical similarities at genetic and immunological levels with the human diseases and, despite its limitations, is the most widely used and accepted experimental model. Our findings provide the rationale for the design of future clinical trials to test the efficacy of T_{reg}-based therapies and highlight the importance of incorporating immunomodulation prior to T_{reg} infusion and the key role of antigen-specific T_{regs} to treat islet autoimmunity.

Our experimental studies used the intact αCD3ε antibody for T-cell debulking because it is more efficient in this setting than the $F(ab')_2$ form (25–27). Studies with a humanized CD3 NOD mouse model (NOD-huαCD3) (29) demonstrated that a single injection of intact αCD3 antibody (clone 2C11, 5 μg) led to a higher percentage of CD4 and CD8 cell apoptosis accompanied by greater depletion of CD4 T cells and a higher rebound of CD8 T cells compared with nonmitogenic $F(ab')_2$ fragments (50 μg). Similar results on T-cell apoptosis, depletion, and rebound were observed when a single dose of mitogenic (YTH12.5, 2 μg) and nonmitogenic (otelixizumab, 100 μg) human CD3ε antibodies were used. In the context of our studies examining the in vivo environment to support persistent T_{reg} engraftment, a 5-day course of a higher dose of intact αCD3 (50 μg) led to significant depletion of CD4 and CD8 T cells, which was accompanied by slow recovery, while the T_{regs} were depleted early after αCD3 treatment with a rapid recovery. However, intact αCD3 treatment allowed only

limited T_{reg} engraftment after T-cell debulking and was likely due to competition with rapidly rebounding host T_{regs}. In fact, when the recovering host T_{reg} population was decreased again with CyP just before T_{reg} infusion, robust engraftment of polyclonal T_{regs} occurred. This level of donor T_{reg} engraftment 4 weeks post–T_{reg} infusion in prediabetic NOD is similar to what was observed with harsher ablative conditioning in nonautoimmune C57BL/6 mice (20), yet relying on short courses of clinically relevant agents. Improving T_{reg} engraftment could have increased frequencies of disease-relevant T_{regs} and improve therapeutic benefit, but durable remission was not achieved despite robust polyclonal T_{reg} engraftment. However, we observed a strong correlation with the level of polyclonal T_{reg} engraftment after immunomodulation and clinical outcome in recent-onset NOD mice, suggesting that increased numbers of adoptively transferred polyclonal, autologous T_{regs}, perhaps through ex vivo expansion, could lead to improved benefit. This will require further investigation but could be more easily implemented in a clinical setting given that it is more challenging to use antigen-specific T_{regs}. Additionally, the positive effects of αCD3 in a recent prevention clinical trial (40), and our new data on engraftment in late prediabetic mice, support the hypothesis that this regimen could be tested in this preclinical model with polyclonal and antigen-specific T_{regs}.

Antigen-based therapies have been used in experimental models and clinical trials with mixed results. However, antigens could be helpful to drive the selection/expansion of infused antigen-specific T_{regs}. Indeed, diabetes was prevented in adoptive transfer studies with islet-specific NOD T_{regs} into NOD mice (NOD.Rag$^{-/-}$, NOD TCRα$^{-/-}$, or NOD.CD28$^{-/-}$) that lack either T cells or T_{regs}, respectively (35,37,41,42). Critically, these models resemble IL-2Rβ$^{-/-}$ mice, in that there is natural space with limited host T_{reg} competition. A major benefit of using antigen-specific T_{regs} is that these T_{regs} will likely act only where antigen is present, thereby providing local immunotherapy without impacting the remainder of the immune system. Here, we found that our combinational regimens allow engraftment of polyclonal T_{regs} throughout the immune compartments and in tissues, but the presence of the target antigen in the pancreas led to islet-specific T_{regs} being found largely within islets after infusion. Both αCD3 or αCD3+CyP treatments prior to islet-specific T_{reg} infusion led to durable remission; however, αCD3 alone with islet-specific T_{regs} was sufficient. The level of engraftment in these mice was similar to what was observed in mice treated with αCD3+CyP+polyclonal T_{regs}+IAC, but now islets also include disease-relevant T_{regs} in the pancreatic microenvironment. These NOD mice were still fully capable of rejecting allogeneic skin transplants without breaking the islet autoimmune tolerance established by our T_{reg} immunotherapy with islet-specific T_{regs}. Importantly, by creating this supportive environment for donor T_{regs}, durable diabetes remission was achieved using 20-fold less numbers of antigen-specific T_{regs} in our studies compared with a previous study that used in vitro expanded BDC2.5 T_{regs} in unmanipulated NOD mice (35). Like others (25,29), we found that intact αCD3 antibody led to IFNγ and TNFα production, but this did not hinder T_{reg} engraftment or the induction of durable diabetes remission with antigen-specific T_{reg} infusion.

Challenges with antigen-specific T_{reg}-based therapies still center on the availability of a sufficient number of disease-relevant T_{regs} and whether pools of antigen specificities, and what specificities, will be needed to achieve therapeutic benefit, given that autoimmunity is likely to be more heterogeneous in patients. The discovery of hybrid insulin peptides and other modified peptides highlights the importance of further investigation on the antigen specificities needed for therapeutic gains (43,44). Although current methodologies are limited in terms of the ability to isolate and expand a sufficient number of autologous antigen-specific T_{regs}, new strategies aimed at generating large numbers of antigen-specific T_{regs} are being explored. These include lentiviral TCR gene transfer into expanded polyclonal T_{regs}, Foxp3 gene editing into antigen-specific $CD4^+$ T cells, and conversion of effector T cells into T regulatory–like cells by CRISPR/Cas9-mediated integration of a Foxp3 transgene, and open the possibility of overcoming this limitation (45–47). Our work sets the conceptual framework and a key innovative approach that was aimed at addressing a critical and unmet need in the clinics, the need for new therapeutic protocols for T1D treatment that will enhance the efficacy and durability of T_{reg}-based cell therapies.

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Author Contributions. C.C.-K. performed experiments and provided intellectual feedback. S.M., A.S., J.R., and C.V. performed experiments. A.P. provided intellectual feedback and reviewed the manuscript. A.L.B. designed and conceived the studies, performed experiments, analyzed data, and wrote the manuscript. A.L.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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