Immunomodulation Followed By Antigen-Specific T\textsubscript{reg} Infusion Controls Islet Autoimmunity

Cecilia Cabello-Kindelan\textsuperscript{1}, Shane Mackey\textsuperscript{1}, Alexander Sands\textsuperscript{1}, Jennifer Rodriguez\textsuperscript{1}, Claudia Vazquez\textsuperscript{1}, Alberto Pugliese\textsuperscript{1,2,3}, and Allison L. Bayer\textsuperscript{1,2} \textsuperscript{*}

Diabetes Research Institute\textsuperscript{1}, Department of Microbiology and Immunology\textsuperscript{2}, Department of Medicine, Division of Diabetes, Endocrinology and Metabolism\textsuperscript{3}, University of Miami Miller School of Medicine, Miami, FL 33136

Corresponding Author:
Allison L. Bayer, Ph.D.
Department of Microbiology and Immunology and Diabetes Research Institute
Miller School of Medicine, University of Miami
1450 NW 10\textsuperscript{th} Avenue, Miami, Florida 33136
Phone: 305-243-6743
Fax: 305-243-4404
e-mail: abayer@med.miami.edu

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Abstract

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 non-autoimmune models required ablative host conditioning. Here, we evaluated T_{reg} engraftment and therapeutic efficacy in the non-obese diabetic (NOD) mouse model of autoimmune diabetes using non-ablative, combinatorial regimens involving 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 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 IL-2 (interleukin-2) support. A single-dose of CyP after αCD3 depleted rebounding host-T_{regs}, 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} following 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.
Introduction

The key role played by T\(_\text{regs}\) 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\(_\text{reg}\) therapy for recent onset type 1 diabetes (T1D) and in transplant settings (7-10). Several groups have established *in vitro* T\(_\text{reg}\) expansion protocols (11-15); clinical trials with autologous, expanded T\(_\text{regs}\) are ongoing in T1D (9, 10) using unselected, polyclonal-T\(_\text{regs}\), (14, 16). A phase 1 study revealed that *in vitro* expanded, autologous T\(_\text{regs}\) were safe and tolerable in children with recent-onset T1D, with evidence of improved fasting C-peptide and reduced insulin requirement 4-months post-treatment. Therapeutic effects correlated with increased T\(_\text{regs}\) post-infusion, but only persisted for a short time. A subsequent trial confirmed limited persistence of expanded T\(_\text{regs}\) even after a second infusion (9, 10, 17).

Data emerging from these trials highlight limitations of protocols that rely solely on T\(_\text{reg}\) infusion without recipient manipulation, including immunomodulation and homeostatic support. In fact, our previous work identified critical requirements for infused T\(_\text{reg}\) engraftment and function: 1) generation of peripheral space for engraftment, 2) overcoming competition from host-T\(_\text{regs}\), 3) sufficient IL-2 and 4) antigen availability to select disease-relevant T\(_\text{regs}\) (18-20). Our observations are supported by other studies (for review, (21)), and should be considered when designing T\(_\text{reg}\)-based clinical trials, whether these T\(_\text{regs}\) are expanded *in vitro* or infused after isolation for *in vivo* expansion. Our previous protocols involved ablative (radiation) conditioning of the host for T\(_\text{reg}\) engraftment and therapeutic efficacy. While effective, ablative conditioning bears 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\(_\text{regs}\). To this end, we explored novel, non-ablating, clinically applicable immunomodulatory regimens which included combinations of a short-course of intact $\alpha$CD3, a single injection of 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\textsubscript{regs} were autoantigen-specific rather than polyclonal, and this setting immunomodulation with \(\alpha\text{CD3}\) alone resulted in 100\% durable diabetes remission (>6 months), with robust T\textsubscript{reg} engraftment within the islets. Thus, our results demonstrate that robust engraftment and disease relevant antigen-specific T\textsubscript{regs} are key requirements for successful immunotherapy in a preclinical model of autoimmune diabetes.
Materials and Methods

Mice

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

Cell purifications

CD4\(^+\)CD25\(^+\)-T\(_\text{regs}\) were purified from Spleen (SP) and lymph nodes (LNs) as previously described (22) from NOD or Thy1.1\(^+\) NOD mice. T\(_\text{regs}\) on average were >92% pure. For remission studies, islet-specific CD4\(^+\)TCR\(\beta\)4\(^+\)CD25\(^+\) T\(_\text{regs}\) were purified from SP and LNs of NOD.Cg-Tg(TcraBDC2.5,TcrbBDC2.5)1Doi/DoiJ mice by cell sorting and on average >98.5% pure. Briefly, CD4 T-cells were enriched by anti-CD4 MACS microbeads (Miltenyi Biotec, San Diego, CA), stained with Alexa Flour 700-conjugated CD4, PE-conjugated V\(\beta\)4 antibody, and PE-CF594-conjugated CD25 (BD Bioscience, San Diego, CA) and cell sorted on Beckman Coulter Astrios. Purities were assessed by flow cytometry.

Adoptive T\(_\text{reg}\) transfer

Freshly-isolated T\(_\text{regs}\) (0.5x10\(^6\)), either polyclonal or islet-specific T\(_\text{regs}\), were adoptively transferred by i.v. injections through tail vein to NOD mice that received immunomodulation in various combinations using intact \(\alpha\)CD3 (50 \(\mu\)g, clone 2C11: Leinco Technologies St. Louis, MO) 1x/day for 5 consecutive days, a single injection of cyclophosphamide (CyP, 200mg/kg: Sigma Aldrich,
St. Louis, MO, and IL-2 complex (IAC) given every other day for 1 week starting at the time of
Tregs infusion, or mice left untreated (Figure 3A). IAC was prepared using recombinant murine
(rm) IL-2 with anti-IL-2 mAb (clone JES6-1A12) (e-Bioscience, San Diego, CA) and incubated
at a molar ratio of 2 to 1 (1μg IL-2 and 5 μg Ab) for 15 minutes at room temperature. The resulting
IAC was intraperitoneally injected in 200 μL phosphate-buffered saline (PBS) as indicated in each
illustration.

Remission studies
Recent onset diabetic NOD mice (>250mg/dL for 2 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 Treg
infusion (autologous, polyclonal NOD or islet-specific NOD Tregs) or mice were left untreated as
indicated in each illustration. Diabetes reoccurrence was monitored for 6 months. Mice were tested
2-3x/week for weight and glycosuria. The absence of glycosuria and hyperglycemia indicates lack
of diabetes relapse.

Skin Transplantation
Skin grafting was performed as modification of Bellingham and Medawar technique (23).
Anesthetized mice received full thickness donor skin (prepared from dorsal tissue from ear) on
separate graft beds on back of NOD mice 6 months after αCD3+BDC2.5-Treg infusion. Each
recipient received 2-grafts, syngeneic-NOD and allogeneic-B6 grafts. Bandage was placed over
these 2-grafts for 7 days. Grafts were monitored every other day and scored rejected when >75%
of original graft was lost or become necrotic as assessed by visual examination.
**Antibodies and Flow Cytometry Analysis**

SP and LN were made into single cell suspension by mechanic disruption. Pancreatic LN (pLN) and pancreas were digested with collagenase D (2 mg/ml, Roche.com) at 37°C for 30min. Equal volumes of collected blood was used for PBMCs and purified from whole blood on Ficoll-Paque™Plus gradient (GElifesciences.com). Red blood cells from tissues were lysed with ACK lysing buffer. Total cell counts were performed on tissues and blood using hemocytometer.

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

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 main bile duct until distension was achieved. Digestion was performed at 37°C for 10-15min with gentle agitation and terminated by addition of cold RPMI–10% FCS and 2mmol/l L-glutamine. The tissue was filtered through 70-μm mesh and placed on Euro-Ficoll (Mediatech) gradients by centrifugation at 2000rpm 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 (NIH Tetramer Core, I-A(g7) BDC2.5 mimotope RTRPLWVRME) for 3-hours at 37°C and fluorescence-conjugated Abs against cell surface mouse CD45, Thy1.2, CD4, CD8, and CD25 added to last 30 minutes of incubation. Cells were then stained with live/dead fixable Near-IR followed by intracellular Foxp3 staining.

FACS analysis was performed using BD Bioscience LSRII and analyzed with Diva® or Kaluza® software. Total number of events collected was between 50,000 to 200,000 cells, except for pancreas samples in which entire samples were collected and CD45+ cells set as storage gate.

**Statistical Analysis**

One-way ANOVA followed by Dunnett’s Multiple Comparison Test. Two-way ANOVA followed by Sidak’s Multiple Comparison Test. Unpaired T Test, αCD3 compared to αCD3+CYP at each time point. Comparisons yielding p<0.05 were considered to be statistically significant. Survival curves subjected to Mantel-Cox Log-rank test. P value is indicated on graph.
Results

A combinatorial regimen of αCD3 and CyP creates space in the host-T\textsubscript{reg} compartment in female NOD mice

In previous studies, intact αCD3 was more effective at depleting T-cells than the F(ab’)\textsubscript{2} form (25-27); while regimens varied in timing and duration of administration, depletion was dose-dependent. Yang et al. 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 (50µg) than C57BL/6 mice; depletion was age-dependent in NOD mice. Moreover, in NOD mice, 50µg of intact-αCD3 more efficiently depleted CD4 T-cells than 100µg of F(ab’)\textsubscript{2}-αCD3 (27). Hence, we used intact αCD3 antibody (50µg dose) as a depleting agent in our study. We investigated the effects of αCD3 therapy on conventional T-cell and T\textsubscript{reg} compartments in the circulation of 5-6 weeks-old prediabetic NOD female mice. We used a 5-day course of αCD3 and followed effects for 39-days (Fig. 1A). αCD3 readily decreased conventional T-cells, including CD4\textsuperscript{+}Foxp3\textsuperscript{−} and CD8 T-cells (Figs. 1B-D, solid-lines); CD4\textsuperscript{+}Foxp3\textsuperscript{−} T-cells return to normal levels around 39-days after the 1\textsuperscript{st} αCD3-dose; CD8 T-cells did not fully recovered. Our assessments at earlier time points (as early as 2-days) revealed that T\textsubscript{reg} depletion occurs very early post-αCD3 (Fig. 1E); in fact, T\textsubscript{regs} return to normal levels within 7-days of the 1\textsuperscript{st}-dose (Fig. 1E, solid-lines, grey-arrow). Thus, we demonstrated a previously unknown early T\textsubscript{reg} depletion post-αCD3. We also show that T\textsubscript{regs} rebound faster than conventional T-cells, which recover around day 32-39, leading to increased proportion of CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells in the CD4\textsuperscript{+} T-cell compartment (Fig. 1F, solid line-shaded area 1). Paneranda et al. noted that treatment with F(ab’)\textsubscript{2}-αCD3 induced a transient increase in the proportion of Foxp3\textsuperscript{+} cells in the CD4 T-cell compartment that was accompanied by slight reduction in the total count of T\textsubscript{regs}, but that the increase Foxp3\textsuperscript{+} proportion was due to greater
depletion of the CD4⁺Foxp3⁻ conventional T-cells (28). In our study, most T-cells are initially proliferating after depletion with intact αCD3 (Fig. 1G); however, only Tₕ helps maintain robust proliferation, including a much higher Ki67 MFI, over the 39-day follow-up [Supp. Fig. 1 (S1)], which may contribute to the increased proportion of Foxp3⁺ cells in the CD4⁺ T-cell compartment following αCD3-treatment together with the slow recovery of the CD4⁺Foxp3⁻ T-cells.

We next examined T-cell compartments following αCD3-treatment up to 15-days in pancreas, SP, LN, pLN and Peyers’ patches (PP), lamina proprialymphocytes (LPLs), intraepithelial lymphocytes (IELs) in the small intestine (Fig 2A). Similar to blood, αCD3 therapy rapidly depleted CD4⁺Foxp3⁻ and CD8⁺ T-cells in LN and pLN with less than 30% recovery from baseline at the end of 15-day follow-up (Figs. 2B-C, S2). 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 following αCD3-treatment (Figs. S2-S3). There was a slow recovery in small intestine with less than 35% of CD4⁺Foxp3⁻ T-cells recovering from baseline, excluding T-cells in SP with ~36-58% recovery (Figs. S2-S3). 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 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 following αCD3-treatment (Fig. 2D).

Resembling effects in blood (Fig. 2E, left panel, grey-arrow), αCD3-treatment is immediately followed of by a decrease of CD4⁺Foxp3⁺ Tₕ in LN and pLN with 44-60% recovery by day 15 (Figs. 2E-F, S2 grey-arrows); the slower recovery of CD4⁺Foxp3⁻ T-cells in these tissues compared to Tₕ helps leads to increased proportions of CD4⁺Foxp3⁺ in the CD4⁺ compartment (Figs. 2E-F, S2). There was delayed Tₕ depletion in SP (Fig. S2, white-arrow) compared to blood and LNs, but recovery was still around 50% at the end of follow-up (Figs. 2 and S2). In contrast,
αCD3-treatment results in an initial T_{reg} increase in pancreas (Fig. 2G, 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} do begin to recover (Fig. S3). 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 (Fig. S4A), pancreas, LPL and peripheral blood (not shown). However, there was no correlation observed in LN (Fig. S4B), 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 spleen or pancreas of treated, prediabetic mice exhibit 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 spleen (Fig. S5). Circulating cells of αCD3-treated mice show little production of these cytokines. Lymphocytes from untreated mice show very little production of these cytokines from any of tissues examined. Overall, this extended longitudinal evaluation in tissues reveals 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 pancreas or lymphoid tissues, suggesting that this treatment alone may not promote robust polyclonal-T_{reg} engraftment.

A single-dose of cyclophosphamide (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\textsubscript{reg} depletion that is followed by rebound, achieving optimal engraftment of autologous donor-T\textsubscript{regs} may require further manipulation to control rebounding populations. CyP is known to eliminate proliferating cells in response to alloantigen, but also depletes T\textsubscript{regs} because of their higher proliferation rates compared to naïve T-cells (30, 31). To determine whether CyP could effectively deplete rapidly rebounding host-T\textsubscript{regs} post-αCD3, a single CyP injection was given at day 10 (Fig. 1A). CyP following αCD3 significantly decreased host-T\textsubscript{regs} by 62% (day 11, 2-way ANOVA, p=0.0215) for ~5 days post-CyP in blood (Fig. 1E, dashed-lines); thus, creating a more supportive T\textsubscript{reg} environment (Fig. 1E, shaded-area). CyP also depleted CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells, which remained lower compared to αCD3 alone 39-days after the 1\textsuperscript{st} αCD3-dose (Figs. 1B-D, dashed-lines). After CyP, a substantial increase in the proportion of CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells in the CD4\textsuperscript{+} T-cell compartment was observed during days 18-32 (Fig. 1F, dashed line-shaded area 2). Moreover, CyP following αCD3 decreases rebounding host-T\textsubscript{regs} by 21-82% in SP, LN, PP, and IEL (Figs. S2, S3). In contrast, host-T\textsubscript{regs} showed a modest increase in pancreas and pLN following CyP (Figs. 2F-G). Overall, these findings highlight that a single-dose of CyP following αCD3 can effectively deplete rebounding host-T\textsubscript{regs}, which are expected to compete with infused donor-T\textsubscript{regs}, and can be used to synergize with αCD3 therapy to open the T\textsubscript{reg} compartment to promote robust T\textsubscript{reg} engraftment.

A combinatorial regimen of αCD3 and CyP promotes robust engraftment of polyclonal, autologous donor-T\textsubscript{regs} in NOD mice

We tested whether these combinatorial therapies (Fig. 3A) supported engraftment of polyclonal, autologous donor-T\textsubscript{regs} in 5-6 weeks-old, female NOD mice. Despite robust T-cell depletion with
αCD3 alone, donor congenic Thy1.1+ NOD-T_{regs} are minimally detected 1-4 weeks post-infusion in blood (Figs. 3B-C, Table S1 αCD3+T_{reg}), SP, LN, pLN, and pancreas (Fig. 3D). The addition of IL-2 support with a short-course IAC treatment after αCD3 enhanced engraftment during 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. 1E, dashed-line, shaded-area), resulted in a 43-fold increase in donor-T_{reg} engraftment compared to αCD3; the addition of IAC further enhanced engraftment by several folds (αCD3+CyP+T_{reg}+IAC) (Figs. 3B-C, Table S1). The significant increase in proportion of host CD4+Foxp3+cells in the CD4+ T-cell compartment during the rebound phase following αCD3+CyP (Fig.1F, shaded-area 2) did not inhibit donor T_{reg} engraftment, which was robust (Fig. 3D); 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 to all other regimens tested (Fig. 3F), and was accompanied by increased proportion of CD4~Foxp3~ cells in the CD4+ T-cell compartment (Fig. 3G). While the total T_{reg} compartment returned to pre-treatment levels at the end of the 39-day follow-up (Figs. 3F-G), donor-T_{regs} comprised 8-25% of the T_{reg} compartment in mice given αCD3 and CyP with or without IAC (Fig. 3B 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 (Supp. Fig. 6A).

We then treated late, prediabetic NOD mice aged 16-22 weeks old 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 the SP, LN, pLN, and pancreas (Supp. Figs 7A, B, E). Again, optimal T_{reg} engraftment was observed in the αCD3+CyP+T_{reg}+IAC group with a 53-fold increase in engraftment compared to αCD3 alone (day 18 after the start of αCD3) and led to the greatest increase in the total CD4+Foxp3+ population (Supp. Figs. 7A-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 (Supp. Fig. 7A). 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. 4A). 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} (Figs. 4B-C). We found that mice treated with the αCD3+CyP+polyclonal-T\textsubscript{reg}+/-IAC regimen, the time to disease relapse significantly correlated with the levels of donor-T\textsubscript{regs} in blood (r=0.72223, p=0.028). Therefore, we examined T_{reg} engraftment in blood and tissues: donor, congeneric Thy1.1+ NOD-T_{regs} were readily detected in blood and SP, LN, pLN, and pancreas 4wks post-infusion (αCD3+CyP+T_{reg}+IAC) (Figs. 5A, B and Table S2) and donor-T_{regs} have similar level of Foxp3/CD25 expression as host-T_{regs} in blood and tissues (Supp. Fig. 6B). However, there was a
decrease (2.0-2.6-fold) in the level of \( T_{\text{reg}} \) engraftment observed in recent onset mice compared to late prediabetic NOD mice at end of follow-up (Supp. Fig. 7E,F), but overall engraftment with \( \alpha\text{CD3/CyP} \) treatment was still robust compared to prediabetic mice treated with \( \alpha\text{CD3} \) alone (Fig. 3A-C and Supp. Fig. 7A,B). Several studies showed that inflammatory environments promote unstable \( T_{\text{reg}} \), termed ex-\( T_{\text{reg}} \), which become pathogenic (32-34). However, the observed lower engraftment was not explained by a loss of Foxp3-expression in donor-\( T_{\text{reg}} \) populations (CD4\(^+\)Foxp3\(^+\) Thy1.1\(^+\) cells), as there were very few Thy1.1\(^+\) cells Foxp3-negative cells in blood, SP, LN, pLN, or pancreas (Fig. 5C). Lower engraftment was not due to a failure of \( \alpha\text{CD3} \) to effectively deplete T-cells, as CD4, CD8 and CD4\(^+\)Foxp3\(^+\) cells were decreased post-\( \alpha\text{CD3} \) (Fig. 5D, E): Engraftment was not impacted by rebounding host-\( T_{\text{reg}} \), as CyP was very effective at depleting rebounding cells post-\( \alpha\text{CD3} \) (Fig. 5E, shaded area and Fig. 5F, shaded area 1). However, unlike in prediabetic mice in which host-\( T_{\text{reg}} \) recovered after CyP (Fig. 1F, shaded area 2), such a recovery was not observed in recent onset mice (Fig. 5F, shaded area 2). Together, these results suggest that in recent onset mice, despite initial insulin treatment, the host may not fully support the \( T_{\text{reg}} \) compartment, which could represent an obstacle to successful \( T_{\text{reg}} \) therapy with polyclonal-\( T_{\text{reg}} \).

**Combinational therapies that include antigen-specific \( T_{\text{reg}} \) leads to durable remission in newly diagnosed diabetic NOD mice.**

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

**Combinational regimen of αCD3 and antigen-specific T\textsubscript{regs} results in robust, long-term engraftment within islets**
We determined the level of islet-specific T\textsubscript{reg} engraftment following immunomodulation. BDC2.5-T\textsubscript{reg} engraftment was assessed from isolated islets 7-days post-T\textsubscript{reg} infusion (d18 after start of \(\alpha\)CD3) and up to 90 days post-\(\alpha\)CD3 or CyP treatment or in unmanipulated recent onset NOD mice. We detected highest engraftment in pancreatic islets with \(\alpha\)CD3-treatment and persisted up to 90-days compared to CyP treatment; mice that did not receive immunomodulation had lowest engraftment (Figs. 7A,C). These BDC2.5 tetramer-positive cells express high levels of CD25 (Fig. 7B). Importantly, this level of BDC2.5-T\textsubscript{reg} engraftment in the pancreas 40-days post-\(\alpha\)CD3 treated mice was similar to the level of polyclonal-T\textsubscript{reg} engraftment in the pancreas when \(\alpha\)CD3, CYP and IAC were given (Fig. 5B). There was a significant correlation between the level of BDC2.5-T\textsubscript{reg} engraftment and percentage of total CD4\textsuperscript{+}Foxp3\textsuperscript{+} T-cells within islets (Fig. 7D). BDC2.5-T\textsubscript{regs} were detected in pLN or non-draining LNs, but much less compared to islets (Fig. 7E) and was similar to the level of engraftment observed with polyclonal, autologous-T\textsubscript{regs} after \(\alpha\)CD3-treatment alone (Fig. 3D). Moreover, tetramer-positive CD4\textsuperscript{+}Foxp3\textsuperscript{+} T-cells were minimally detected suggesting that BDC2.5-T\textsubscript{regs} are stable within these tissues (Figs. 7E,F). Collectively, our data suggest that antigen-specific T\textsubscript{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 has focused on understanding critical factors required for the generation/homeostasis of thymic-T$_{reg}$s 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 successful outcome of a T$_{reg}$-based immunomodulatory regimen in a preclinical T1D model. While therapeutic success in NOD mice does not guarantee 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 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$_{reg}$s to treat islet autoimmunity.

Our experimental studies utilized the intact αCD3ɛ antibody for T-cell de-bulking 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, 5µg) led to higher percentage of CD4 and CD8 cell apoptosis accompanied by greater depletion of CD4 T cells and higher rebound of CD8 T cells compared to non-mitogenic F(ab’)$_2$ fragments (50µg). Similar results on T cell apoptosis, depletion, and rebound were observed when single dose of mitogenic (YTH12.5, 2µg) and non-mitogenic (Otelixizumab, 100µg) human CD3ɛ 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 (50µg) led to significant depletion of CD4 and CD8 T cell which was accompanied by slow recovery, while the T$_{reg}$s were depleted early after αCD3 treatment with a rapid
recovery. However, intact αCD3 treatment allowed only limited T_{reg} engraftment following T-cell debulking and was likely due to competition with rapidly rebound host-T_{regs}. In fact, when 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 non-autoimmune 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 following immunomodulation and clinical outcome in recent onset NOD mice, suggesting 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 clinical setting given that it is more challenging to utilize 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 from utilizing 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 presence of target antigen in pancreas led to islet-specific T_{regs} to be largely found 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 to a previous study that utilized 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 availability of sufficient number of disease-relevant T_{regs}, 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 (HIPs) and other modified peptides highlights the importance of further investigation on antigen specificities needed for therapeutic gains. (43, 44). Although current methodologies are limited in terms of 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 efficacy and durability of T\textsubscript{reg}-based cell therapies.
Author Contribution

ALB designed and conceived the studies, performed experiments, analyzed data and wrote paper. CC performed experiments and provided intellectual feedback. JR, CV, AS, and SM performed experiments. AP provided intellectual feedback and reviewed manuscript. ALB 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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Data and Resource Availability

The datasets 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.
References


Abbreviations

BMT, bone marrow transplantation  
CyP, cyclophosphamide  
HIP, hybrid insulin peptides  
IEL, Intraepithelial lymphocytes  
IL-2R, IL-2 receptor  
LPL, Lamina Propria lymphocytes  
NOD, nonobese diabetic  
PP, Peyer’s patches  
T_{regs}, T regulatory cells  
T1D, type 1 diabetes.
Figure Legends

Figure 1. T-cell depletion and rebound following immunomodulation with \( \alpha CD3 \), CyP and IAC in the circulation. (A) Experimental Scheme. Percentage of (B) Thy1.2, (C) CD4, (D) CD8, (E) CD4\(^+\)Foxp3\(^+\) in the gated CD45\(^+\) population and (F) \%CD4\(^+\)Foxp3\(^+\) in the gated CD4 T-cells (shaded area 1 and 2 indicate increase in the \%CD4+Foxp3\(^+\) in the CD4 T-cells) in prediabetic female NOD mice receiving \( \alpha CD3 \) (solid-lines) or \( \alpha CD3 + CyP \) (dashed-lines). (G) Percentage of Ki67\(^+\) in the gated CD4\(^+\)Foxp3\(^+\), CD4\(^+\)Foxp3\(^-\), 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/group. In (E) grey arrow indicates recovery in \( T_{reg} \) compartment and shaded area indicates the decrease in CD4\(^+\)Foxp3\(^+\) host-\( T_{reg} \)s following single CyP injection after \( \alpha CD3 \)-treatment. In (F) shaded area 1 indicates increase in CD4\(^+\)Foxp3\(^+\) cells in the gated CD4 T-cells after CD3 alone and shaded area 2 indicates increase in CD4\(^+\)Foxp3\(^+\) cells in total CD4 T-cells after CD3+CyP. *P<0.0001, +P<0.001, @P<0.01, and ^P<0.05 One-way ANOVA followed by Dunnett’s Multiple Comparison Test compared to day 0. Two-was ANOVA followed by Sidak’s Multiple Comparison Test, \( \alpha CD3 \) compared to \( \alpha CD3+CYP \) at each time point.

Figure 2. \( T_{reg} \) depletion and rebound in the tissues after \( \alpha CD3 \) and CyP immunomodulation. (A) Experimental Scheme. Number of CD8\(^+\) and CD4\(^+\)Foxp3\(^-\) T-cells (per 1x10\(^6\) lymphocytes) in (B) peripheral blood, (C) pancreatic lymph nodes, and (D) pancreas Number of CD4\(^+\)Foxp3\(^+\) cells (per 1x10\(^6\) CD45\(^+\) lymphocytes) and percentage of CD4\(^+\)Foxp3\(^+\) in the gated CD4 T-cells in (E) peripheral blood, (F) Lymph Nodes, and (G) pancreas in the presence or absence of CyP of young, prediabetic NOD mice (aged 5-6 weeks). n=3-6 mice/group. In (E and F) grey-arrows indicates
and early decrease in the number of CD4^+Foxp3^+ cells after the start of αCD3 in the blood and pancreatic LNs. 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, and ^P<0.05 One-way ANOVA followed by Dunnett’s Multiple Comparison Test compared to day 0. Unpaired T-Test, αCD3 compared to αCD3+CYP at each time point. n=3-6 mice/group.

Figure 3. Adoptive transfer of T regs leads to engraftment following 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/1x10^6 CD45^+ lymphocytes in peripheral blood, and (D) Percentage of Thy1.1^+ donor T reg engraftment in the total gated CD4^+Foxp3^+ T-cells 4wk post-T reg infusion in the SP, LN, pancreatic (p)LN, 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)+rmIL-2 complex (E) Percentage of Thy1.1^+ Donor Tregs in the gated CD4^+Foxp3^+ T-cells in mice receiving αCD3+CyP+T_reg+IAC regimen and CD4^+Foxp3^+ in the gated total CD4 T-cells in mice receiving αCD3 regimen in the peripheral blood. (F) Percentage of CD4^+Foxp3^+ in gated CD45^+ population, and (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, αCD3+CyP+T_reg+IAC. n=5-6 mice/group. *P<0.0001, +P<0.001, @P<0.01, and ^P<0.05 in (A) and (B) Multiple T-test t compared to αCD3+T_reg and in (F) and (G) Two-way ANOVA followed by Dunnett’s Multiple Comparison Test compared to αCD3.
Figure 4. Diabetes Remission following immunomodulation and infusion of polyclonal-T\textsubscript{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\textsubscript{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.

Figure 5. Donor T\textsubscript{reg} Engraftment following Immunomodulation in Recent Diabetic NOD Mice. (A) %Thy1.1\textsuperscript{+} donor NOD T\textsubscript{reg} in CD4\textsuperscript{+}Foxp3\textsuperscript{+} in PB or (B) 4-8wk in the SP, LN, pancreatic (p)LN, and pancreas (Pan), (C) Foxp3 & Thy1.1 staining in gated CD4 T-cells d22-39 after T\textsubscript{reg} infusion in recent onset diabetic NOD mice receiving αCD3+CyP+T\textsubscript{reg}+IAC.#Donor T\textsubscript{reg}/1x10\textsuperscript{6} lymphocytes, (D) Percent positive Thy1.2\textsuperscript{+} total T-cells, CD4\textsuperscript{+}Foxp3\textsuperscript{−} T-cells, or CD8 T-cells in the peripheral blood of recent onset NOD mice receiving αCD3-treatment. *P<0.0001 and @P<0.01, One-way ANOVA followed by Dunnett’s Multiple Comparison Test compared to day 0. (E) %CD4\textsuperscript{+}Foxp3\textsuperscript{+} in gated CD45\textsuperscript{+} population and (F) %CD4\textsuperscript{+}Foxp3\textsuperscript{+} in the gated CD4 T-cells with αCD3 (solid) or αCD3+CyP (dashed). Diabetic NOD mice were maintained with an insulin pellets to control blood sugar. n=2-6 mice/group. In (E) shaded area indicates the decrease in CD4\textsuperscript{+}Foxp3\textsuperscript{+} host-T\textsubscript{regs} following single CyP injection after αCD3-treatment. In (F) shaded area 1 indicates increase in CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells among total in the gated CD4 T-cells after CD3 alone and shaded area 2 indicates increase in CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells in the gated CD4 T-cells after CD3+CyP.
Figure 6. Diabetes Remission following immunomodulation and infusion of antigen-specific T\(_{\text{regs}}\) in recently diabetic NOD mice. (A) Experimental Scheme. (B) Percentage of diabetic mice after \(\alpha\)CD3, CyP, and/or IAC immunomodulation and adoptive transfer of antigen-specific T\(_{\text{regs}}\) 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 \(\alpha\)CD3-treatment to control diabetes. Urine and blood glucose levels was monitored for 6 months after the start of \(\alpha\)CD3-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 \(\alpha\)CD3 and BDC2.5 T\(_{\text{reg}}\) infusion. Mice were monitored for skin rejection and diabetes development for 70 days. Inset shows blood glucose values for the transplanted mice. Survival curves subjected to Mantel-Cox Log-rank test. P value is indicated on graph.

Figure 7. Antigen-specific donor T\(_{\text{reg}}\) engraftment following immunomodulation in recent diabetic NOD Mice. (A) BDC2.5 Tetramer staining of CD45\(^+\) cells from isolate pancreatic islets of recent onset NOD mice subjected to immunomodulation and adoptive transfer of BDC2.5 T\(_{\text{regs}}\) of gated CD4\(^+\)Foxp3\(^+\), CD4\(^+\)Foxp3\(^-\) cells 7-days after T\(_{\text{reg}}\) infusion (d18). (B) Representative staining of CD25 and BDC2.5 Tetramer on gated CD4\(^+\)Foxp3\(^+\) cells in the pancreatic islets 7-days after T\(_{\text{reg}}\) infusion following \(\alpha\)CD3-treatment. (C) Percentage of BDC2.5 tetramer\(^+\) cells among the CD4\(^+\)Foxp3\(^+\) cells from pancreatic islets 18, 40, 90 days post-T\(_{\text{reg}}\) infusion following \(\alpha\)CD3 or CyP treatment or in mice left untreated. (D) Correlation analysis between the level of BDC2.5-T\(_{\text{reg}}\) engraftment among the gated CD4\(^+\)Foxp3\(^+\) cells and percentage of total CD4\(^+\)Foxp3\(^+\) in gated CD45\(^+\) population within islets on d18 after the start of \(\alpha\)CD3-treatment. Percentage of BDC2.5
tetramer$^+$ cells among the (E) CD4$^+$Foxp3$^+$ cells or (F) CD4$^+$Foxp3$^-$ cells in the pancreatic islets, pLN or other LN 18, 40, 90 days post- T$_{\text{reg}}$ infusion following $\alpha$CD3-treatment. P values in (B) and (D) following unpaired T-test.
Figure 1

(A) CD8+ cells

Female NOD d0 d1 d2 d3 d4 d10 d11 d15 d18 d22 d25 d32 d39

FACS: d0 d4 d7 d11 d15 d21 d22 d25 d32 d39

(B) T-cell Depletion Post-αCD3

Peripheral Blood

% Thy1.2+ cells

αCD3
αCD3+CyP

0 2 4 7 9 11 15 18 22 25 32 39

(C) CD4+Foxp3+ T-Cell Depletion Post-αCD3

Peripheral Blood

% CD4+Foxp3+ cells

αCD3
αCD3+CyP

0 2 4 7 9 11 15 18 22 25 32 39

(D) CD8+ T-cells Depletion Post-αCD3

Peripheral Blood

% CD8+ cells

αCD3
αCD3+CyP

0 2 4 7 9 11 15 18 22 25 32 39

(E) CD4+Foxp3+ cells in CD45 cells Post-αCD3

Peripheral Blood

% CD4+Foxp3+ in gated CD45 cells

CD4+Foxp3+ + gaged CD45 cells

0 2 4 7 9 11 15 18 22 25 32 39

(F) CD4+Foxp3+ cells in CD4 T cells Post-αCD3

Peripheral blood

% CD4+Foxp3+ in gated CD4 T-cells

αCD3
αCD3+CyP

0 2 4 7 9 11 15 18 22 25 32 39

(G) T cell Proliferation Post-αCD3

Ki67+

CD4+Foxp3+ T cells
CD8+ T cells
CD4+Foxp3+ Treg cells

0 2 4 7 9 11 15 18 22 25 32 39

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Diabetes
Figure 2

Legend:  
- $\#_{\text{T_{reg}/10^6}}$  
- $\#_{\text{T_{reg}/10^6}, +\text{CyP}}$  
- $\%_{\text{CD4^{+}Foxp3^{+}}}$ in gated CD4 T cells
Figure 3

(A) Donor Treg Engraftment

*CD4+Foxp3+ cells in CD4 T-cells

Prediabetic NOD (5-6 wks)

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FACS: d0 d2 d4 d7 d11 d15 d18, 22, 25, 32 d39

(B) Donor Treg Engraftment
Peripheral Blood

% Donor Treg among CD4+Foxp3+ cells

(C) Number of Donor Treg cells
Peripheral Blood

#Thy1.1+ CD4+Foxp3+ cells/10^6 lymphocytes (x10^4)

(D) Donor Treg Engraftment Tissues
4 weeks Post-Treg Infusion

% Donor Treg cells among CD4+Foxp3+ cells

(E) Peripheral Blood

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(F) CD4+Foxp3+ in CD45 Cells
Peripheral Blood

% CD4+Foxp3+ in gated CD45+ cells

(G) CD4+Foxp3+ cells in CD4 T-cells
Peripheral Blood

% CD4+Foxp3+ cells in gated CD4 T-cells

Figure 3
Figure 4
**Figure 5**

(A) Recent Onset Diabetics
\( \alpha_{CD3}+CyP+T_{reg}+IAC \)
Peripheral Blood

(B) Recent Onset Diabetics
\( \alpha_{CD3}+CyP+T_{reg}+IAC \)
d39 Tissues

(C) Recent Onset NOD
(Gated on CD4 T cells)

(D) T cell Depletion following \( \alpha_{CD3} \) in Recent Onset NOD
Peripheral Blood

(E) \( CD^4^+Foxp^3^+ \) cells in \( CD45^+ \) cells Post-\( \alpha_{CD3} \)
in Recent Onset NOD
Peripheral Blood

(F) \( CD^4^+Foxp^3^+ \) in CD4 T cells Post-\( \alpha_{CD3} \)
in Recent Onset MOD mice
Peripheral Blood
Female NOD Recent Onset

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Diabetes Remission After Immunomodulation and Infusion of Islet-Specific Tregs

Skin graft

αCD3+BDC2.5 T_{reg}
Figure 7

(A) Pancreatic Islets d18, αCD3+BDC2.5 T_{reg}

Gated CD4 T cells

- CD25
- BDC2.5 Tetramer

Donor T_{reg} Engraftment: Pancreatic Islets

- CD4^+Foxp3^+

(B) Gated on CD4^+Foxp3^+

- CD25
- BDC2.5 Tetramer

(C) Donor T_{reg} Engraftment: Pancreatic Islets

- CD4^+Foxp3^+

(D) Pancreatic Islets d18

- % BDC2.5 T_{reg} Engraftment among the CD4^+Foxp3^+ cells

(E) Donor T_{reg} Engraftment: αCD3+BDC2.5 T_{reg}

- CD4^+Foxp3^+

(F) Donor T_{reg} Engraftment: αCD3+BDC2.5 T_{reg}

- CD4^+Foxp3^+

Diabetes
Supplemental Figure 1. $T_{reg}$ proliferation is much higher compared to CD4+Foxp3- and CD8 T cells following aCD3 treatment. (A) Representative histograms showing Ki67 staining in the peripheral blood at d22 following the start of aCD3 treatment. Mean Fluorescent Intensity (MFI) is indicated in each histogram for gated CD4+Foxp3+ $T_{reg}$ cells, and CD4+Foxp3- and CD8+ T cells.
Supplemental Figure 2. T<sub>reg</sub> depletion and rebound in the pancreatic lymph nodes and spleen after αCD3 and CyP immunomodulation. (A) Experimental Scheme. Number of CD8<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> T-cells (per 1x10<sup>6</sup> lymphocytes) in (B) lymph nodes and (C) spleen. Number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells (per 1x10<sup>6</sup> lymphocytes) and percentage of Foxp3<sup>+</sup> CD4 T-cells in (D) lymph nodes and (E) spleen in the presence or absence of CyP of young, prediabetic NOD mice (aged 5-6 weeks). n=3-6 mice/group. *P<0.0001, +P<0.001, @P<0.01, and ^P<0.05 One-way ANOVA followed by Dunnett's Multiple Comparison Test compared to day 0. Unpaired T-Test, αCD3 compared to αCD3+CYP at each time point. n=3-6 mice/group. In (D) grey-arrow indicates an early decrease in the number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells after the start of αCD3 LNs. In (E) white-arrow indicates a late decrease in the number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells after the start of αCD3.
Supplemental Figure 3. 

T<sub>reg</sub> depletion and rebound in the Peyer’s Patches, Lamina Propria and Intraepithelial of the small intestine after αCD3 and CyP immunomodulation. (A) Experimental Scheme. Number of CD8<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> T-cells (per 1x10<sup>6</sup> lymphocytes) in (B) Peyer’s Patches, (C) LPL, and (D) IEL. Number of CD4<sup>+</sup>Foxp3<sup>-</sup> cells (per 1x10<sup>6</sup> lymphocytes) and percentage of Foxp3<sup>+</sup> CD4 T-cells in (E) Peyer’s Patches, (F) LPL, and (G) IEL in the presence or absence of CyP of young, prediabetic NOD mice (aged 5-6 weeks). n=3-6 mice/group. *P<0.0001, +P<0.001, @P<0.01, and ^P<0.05 One-way ANOVA followed by Dunnett’s Multiple Comparison Test compared to day 0. Unpaired T Test, αCD3 compared to αCD3+CYP at each time point. n=3-6 mice/group.
Supplemental Figure 4. Link between the T_{reg} rebound and proliferation in some tissues but not all. Number of versus the percentage of Ki67+ in gated CD4+Foxp3+ T cells in the (A) pLN or (B) LNs of young, prediabetic NOD mice (aged 5-6 weeks) receiving 5-day course of αCD3.
Supplemental Figure 5. Depletion of T-cells with short-course αCD3 treatment in late-prediabetic NOD mice leads IFNγ- and TNFα-producing lymphocytes. Prediabetic NOD mice treated with 5-day course of intact αCD3 (50µg) were sacrificed 15 days after the start of treatment and compared to untreated, age- and sex-matched NOD mice. Histograms show the percentage of (A) IFNγ or (B) TNFα producing CD45+ cells isolated from pancreatic islets (Pan), spleen (SP), or peripheral blood after culturing cells directly ex-vivo for 4 hours in the presence of brefeldin A. n=3 mice/group.
Supplemental Figure 6. Representative staining of Foxp3 and CD25 expression on donor (Thy1.1+) or host (Thy1.2+) T\(_{\text{reg}}\) from the gated CD4^+Foxp3^+ population from the blood and SP, LN, pLN, and pancreas 4-weeks post-T\(_{\text{reg}}\) infusion in (A) young, prediabetic NOD mice or (B) recent onset NOD mice treated with various immunomodulation followed by adoptive transfer of congenic, polyclonal Thy1.1+ T\(_{\text{reg}}^+\).
Supplemental Figure 7. (A) Percentage of Thy1.1+ donor Treg engraftment among the total CD4+Foxp3+ T-cells in peripheral blood, (B) Number of donor Treg/1x10^6 lymphocytes in peripheral blood, and (C) Percentage of CD4+Foxp3+ cells in the gated CD4 T cells in peripheral blood of late, prediabetic NOD mice. Percentage of Thy1.1+ donor Treg engraftment among the total CD4+Foxp3+ T-cells 4wk post-Treg infusion in the SP, LN, pancreatic (p)LN, and pancreas (Pan) of (D) young, prediabetic NOD (E) late, female prediabetic NOD mice aged 16-22 weeks of age in mice treated with αCD3+Treg, αCD3+Treg+IAC, αCD3+CyP+Treg, or αCD3+CyP+Treg+IAC. IAC=anti-IL-2 (JES6)+rmIL-2 complex. (F) Percentage of Thy1.1+ donor Treg engraftment among the total CD4+Foxp3+ T-cells 4wk post-Treg infusion in the SP, LN, pancreatic (p)LN, and pancreas (Pan) of recent onset NOD mice treated with αCD3+CyP+Treg+IAC. n=3-4 mouse/treatment group.
Supplemental Table 1. Engraftment of Donor T\textsubscript{reg} following \(\alpha\)CD3+CyP Immunomodulation. Young, prediabetic female NOD mice were treated with combinational regimen of \(\alpha\)CD3+\(T_{\text{reg}}\), \(\alpha\)CD3+\(T_{\text{reg}}\)+IAC, \(\alpha\)CD3+CyP+\(T_{\text{reg}}\), \(\alpha\)CD3+CyP+\(T_{\text{reg}}\)+IAC, as described in Figure 3A. Shown is number of donor T\textsubscript{reg} \(\times 10^6\) lymphocytes in the peripheral blood d15, 18, 22, 25, 32, and 39 after the start of \(\alpha\)CD3 treatment.

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<tr>
<td>d39</td>
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<td>6,582 ± 1,940</td>
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Supplemental Table 2. Engraftment of Donor T\textsubscript{reg} following \(\alpha\)CD3+CyP Immunomodulation. Recent onset diabetic female NOD mice were treated with combinational regimen of \(\alpha\)CD3+CyP+\(T_{\text{reg}}\)+IAC as described in Figure 3A. Shown is number of donor T\textsubscript{reg} \(\times 10^6\) lymphocytes in the peripheral blood, spleen, LN, pLN, and pancreas d22 and 39 after the start of \(\alpha\)CD3 treatment.

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