Long-Term Remission of Diabetes in NOD Mice Is Induced by Nondepleting Anti-CD4 and Anti-CD8 Antibodies

Zuoan Yi,[1] Ramiro Diz,[1] Aaron J. Martin,[1] Yves Maurice Morillon,[1] Douglas E. Kline,[1] Li Li,[1] Bo Wang,[1] and Roland Tisch[1,2]

Residual β-cells found at the time of clinical onset of type 1 diabetes are sufficient to control hyperglycemia if rescued from ongoing autoimmune destruction. The challenge, however, is to develop an immunotherapy that not only selectively suppresses the diabetogenic response and efficiently reverses diabetes, but also establishes long-term β-cell–specific tolerance to maintain remission. In the current study, we show that a short course of nondepleting antibodies (Abs) specific for the CD4 and CD8 coreceptors rapidly reversed clinical disease in recent-onset diabetic NOD mice. Once established, remission was maintained indefinitely and immunity to foreign antigens unimpaired. Induction of remission involved selective T-cell purging of the pancreas and draining pancreatic lymph nodes and upregulation of transforming growth factor (TGF)-β1 by pancreas-resident antigen-presenting cells. Neutralization of TGF-β blocked the induction of remission. In contrast, maintenance of remission was associated with tissue-specific immunoregulatory T cells. These findings demonstrate that the use of nondepleting Ab specific for CD4 and CD8 is a robust approach to establish long-term β-cell–specific T-cell tolerance at the onset of clinical diabetes. *Diabetes* **61:2871–2880, 2012**

T ype 1 diabetes is marked by the progressive infiltration of the islets (i.e., insulitis) by immune effectors and subsequent destruction of the β-cells (1,2). Clinical diabetes is diagnosed when ~80–90% of β-cell mass has been destroyed or rendered nonfunctional. Notably, a sufficient number of residual β-cells typically exist at the time of diagnosis so that diabetes can be reversed if the autoimmune response is rapidly suppressed (3). Studies in NOD mice, and indirect evidence from diabetic patients, indicate that CD4^{+} and CD8^{+} T cells are the primary mediators of β-cell destruction (4–6). Pathogenic β-cell–specific CD4^{+} and CD8^{+} T cells often exhibit a type 1 phenotype marked by interferon-γ (IFN-γ) secretion. The differentiation and expansion of pathogenic autoreactive T cells in type 1 diabetes are partly due to dysregulation of immunoregulatory T cells (Treg). Foxp3-expressing CD25^{+}CD4^{+} Treg (Foxp3^{+} Treg), for instance, have impaired survival and/or suppressor activity in NOD mice and type 1 diabetic patients (7–10).

Efforts to prevent and treat type 1 diabetes have focused on immunotherapies that directly tolerize or deplete pathogenic T effectors and/or enhance Treg populations. Anti-CD3 antibodies (Abs) and antithymocyte globulin induce remission to varying degrees in recent-onset diabetic NOD mice by depleting the autoreactive T effectors and increasing the frequency of CD25^{+}CD4^{+} Treg (11–13). Treatment of recent-onset diabetic patients with non-Fc receptor binding (NFB) anti-CD3 Abs also rescues residual β-cell mass; however, the protective effect is transient, and euglycemia and insulin independence are not achieved (14–16). Furthermore, T cell-depleting Abs may compromise normal protective immunity. Systemic albeit transient depletion of T cells following NFB anti-CD3 Ab treatment has been linked to recurrent viral infections in some patients (15).

Nondepleting Abs specific for the CD4 and CD8 T-cell coreceptor molecules have been used to establish persistent T-cell tolerance (17). Waldmann and colleagues (17–19) demonstrated that nondepleting anti-CD4 and anti-CD8 coupled with donor-derived splenocytes induce long-lasting tolerance in allograft models. Systemic T-cell numbers are unaffected by the nondepleting Abs, and transplantation tolerance is mediated by alloantigen-specific Foxp3^{+} Treg (18,20). Nondepleting anti-CD4 or anti-CD8 has also been used to prevent type 1 diabetes. YTS105, a rat IgG2a anti-CD8α, blocks insulitis and diabetes in young NOD mice (21). Furthermore, YTS177, a rat IgG2a anti-CD4 prevents diabetes in NOD mouse adoptive transfer models (22,23). The nondepleting nature of YTS177 and YTS105 is attributed to these two rat IgG2a Abs exhibiting an inability to bind murine Fc receptors and fix complement efficiently. In this study, we tested whether tolerance induced by nondepleting Abs specific for CD4 and CD8 was sufficiently robust to elicit remission and long-term β-cell tolerance in recent-onset diabetic NOD mice.

RESEARCH DESIGN AND METHODS

Mice. NOD/LtJ, NOD.CB17-Prkdc[scid]/J (NOD.*scid*), NOD.129P2(C)-Tcra[tm1Mjo]/Doi/J (NOD.Cα[null]), NOD.Cg-Tg(TcraTcrbBDC2.5)1Doi/DoiJ (NOD.BDC2.5) (24), NOD.Cg-Tg(TcraTcrbNY8.3)1Pesa/DvsJ (NOD.8.3) (25), NOD.129P2(B6)-Il4[tm1Cgn]/DvsJ (NOD.IL4[null]) (26), NOD.Foxp3.GFP mice (27), and C57BL/6 mice were bred and maintained in specific pathogen-free facilities at the University of North Carolina at Chapel Hill (UNC-CH). All animal experiments were approved by the UNC-CH Institutional Animal Care and Use Committee.

Ab preparation and treatment. YTS105.8 and YTS177.9 hybridomas were a gift from Dr. Waldmann (Oxford, U.K.). YTS105 and YTS177 Abs were purified from ascites with the Melon Gel Monoclonal IgG Purification Kit (Thermo Scientific) or from culture supernatants with saturated ammonium sulfate. YTS105 and YTS177 were quantitated using a rat IgG2a-specific enzyme-linked immunosorbent assay (ELISA). Purified YTS177.9 was also purchased (BioXCell).

For remission experiments, female NOD mice with blood glucose levels ≥250 mg/dL (Abbott Diabetes Care) for 2 consecutive days were injected intraperitoneally twice (1 day apart) with 600–800 µg of YTS177 and YTS105 or rat IgG2a isotype control monoclonal Ab (mAb) 2A3 (BioXCell). NOD female

mice were injected three times per week for 3 weeks with 200 µg of anti-transforming growth factor-β (TGF-β; 1D11.16.8), 300 µg of anti-interleukin (IL)-10 receptor (1B1.3A; BioXCell), or anti-rat IgG1 mAb (HRPN; BioXCell) an isotype control for both mAbs.

Intraperitoneal glucose tolerance test was performed as described (28). Briefly, mice fasted for 12 h, during which baseline blood glucose levels measured, they were injected intraperitoneally with 10% glucose in PBS (3 g/kg body weight), and blood glucose was determined.

Flow cytometry. Spleen, pancreatic lymph node (PLN), and pancreas suspensions were prepared by grinding tissue between frosted slides (29). Cells were blocked with anti-CD16/32 (2.4G2) and stained with anti-CD90.2 (53–2.1), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), and anti-CD25 (PC61.5) (BD Biosciences). Binding of YTS105 and YTS177 was determined using mouse anti-rat IgG2a (2A8F4). YTS177- and YTS105-bound $CD4^+$ and $CD8^+$ T cells were detected with anti-CD4 RM4–5 and anti-CD8β 53–5.8, respectively; binding of the respective Abs was reduced relative to unmanipulated T cells. Supplementary Fig. 1 provides representative gating schemes and fluorescence-activated cell sorter (FACS) profiles for detection of YTS-bound $CD4^+$ and $CD8^+$ T cells. Anti-CD11c (B418), anti-CD11b (M1/70), and anti-F4/80 (BM8) (BD Biosciences) were used to stain dendritic cells (DC) and macrophages. Dead cells were excluded by LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen) and absolute cell counts determined by AccuCheck Counting Beads (Invitrogen). T-cell apoptosis was measured with the Apo-Direct Kit TUNEL assay (BD Biosciences). Data were acquired at the UNC-CH Flow Cytometry Facility using LSRII (BD Biosciences) or CyAn (DakoCytomation) cytometers and analyzed with FlowJo (Tree Star) or Summit (DakoCytomation) software.

Analyses of the pancreas. Pancreases were snap frozen in Tissue-Tek OCT (Sakura), sectioned, fixed with acetone/methanol, and treated with Superblock Blocking buffer (Thermo Scientific) in PBS (1:1 ratio) containing 2.4G2 anti-FcγR Ab. Slides were stained with anti-CD90.2–FITC, anti-B220–PE (BD Biosciences), and rabbit anti-insulin (Cell Signaling Technology). Insulin staining was detected by goat F(ab')₂ anti-rabbit Alexa 647 (Invitrogen). Transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using the In Situ Cell Death Detection kit (Roche); sections were stained with anti-CD90.2 biotin/streptavidin-phycoerythrin (PE) and anti-insulin as above. NOD mice were also injected with 50 µL 10 mmol/L dexamethasone i.p. and thymi removed 12 h later. Analyses were performed using a digital deconvolusion microscope (Intelligent Imaging Innovations), and images collected and analyzed using Slidebook software (Intelligent Imaging Innovations).

To measure insulitis, pancreases were fixed with formalin, serially sectioned 90 µm apart, and stained with hematoxylin and eosin. A minimum of 10 islets was scored per pancreas.

To measure cytokines, pancreases were prepared as described (30). IFN-γ and IL-2 were measured using OptEIA Ab pair ELISA (BD Biosciences). Measurements of TGF-β1 were performed using the DuoSet Ab pair ELISA (R&D Systems).

Measurement of TGF-β1 mRNA. NOD.BDC2.5 mice were treated with YTS177 and YTS105 or 2A3 and, 6 days later, $CD4^+$ T cells, DC ($CD11c^+F4/80^-$), and macrophages ($CD11c^-CD11b^+F4/80^+$) sorted with a MoFlo high-speed sorter (DakoCytomation) from the PLN and islets of a given mouse. RNA was extracted with RNeasy Protocol (Qiagen) and cDNA synthesized using SuperScript II (Invitrogen). RT-PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using SYBR Green (Applied Biosystems). cDNA levels were determined with a standard curve and normalized to β-actin. TGF-β1 primers were: forward 5′-CACTGATACGCCTGAGTG-3′ and reverse 5′-GTGAG CGCTGAATCGAAA-3′; and β-actin primers were: forward 5′-TGGCACCCAGCA-CAATGAA-3′ and reverse 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′.

Coadoptive transfers. Cotransfer experiments were performed as described using NOD.scid mice as recipients (31). YTS-treated remission NOD mice (>200 days) and diabetic control NOD mice were used as donors. Splenocytes (10×10^6) or PLN cells (2×10^6) were coinjected intraperitoneally with splenocytes from diabetic NOD mice (10×10^6). $CD4^+CD25^+$ T cells (3.5×10^5), purified from the spleen or PLN using a $CD4^+CD25^+$ Treg purification kit (Miltenyi Biotec), were cotransferred with diabetogenic spleen cells (3.5×10^6).

Measuring β-cell–specific T-cell responses. ELISPOT plates (Millipore) were coated with anti-cytokine Ab and blocked as described (31). A total of 5×10^5 splenocytes/well was cultured for 48 h at 37°C in HL-1 medium and stimulated with peptide (20 µg/mL). Alternatively, 5×10^5 PLN cells were stimulated with irradiated antigen-presenting cells (APC) pulsed with 20 µg/mL peptide. Plates were incubated with biotinylated anti-mouse cytokine Abs plus streptavidin-horseradish peroxidase and spot-forming units detected by an ImmunoSpot Analyzer (Cellular Technology). Supernatants were harvested from individual wells and TGF-β measured via ELISA.

BDC2.5 $CD4^+$ T cells (5×10^6), labeled with 5 µmol/L 5- (and 6-)carboxy-fluorescein diacetate succinimidyl ester (CFSE; eBioscience), were injected intravenously into 16-week-old nondiabetic NOD female mice or YTS-treated remission (>150 days) NOD female mice. Proliferation and activation of CFSE-labeled BDC2.5 $CD4^+$ T cells in individual mice was assessed 4 days later via FACS and staining with mAbs specific for Vβ4, CD3, CD44, and CD62L.

Measurement of in vivo hemagglutinin-specific cytotoxic T lymphocyte activity and keyhole limpet hemocyanin-specific Ab. Remission or 16-week-old nondiabetic NOD female mice were immunized subcutaneously with 50 µg of hemagglutinin peptide $(HA)_{512–520}$ (IYSTVASSL) peptide in complete Freund's adjuvant (CFA). Splenocytes were pulsed with 10 µg of $HA_{512–520}$ or influenza nucleoprotein (NP; TYQRTRALY) peptide, labeled with 5 µmol/L and 0.5 µmol/L CFSE, respectively, mixed at 1:1 ratio (5×10^6 cells each), and injected intravenously. CFSE-labeled target cells were analyzed 16 h later by FACS and percent specific lysis calculated as: $100 - (100 \times$ [percent HA-pulsed target/percent NP-pulsed target in immunized animals]/[percent HA-pulsed target/percent NP-pulsed target in unimmunized animals]).

Mice were injected subcutaneously with 50 µg of keyhole limpet hemocyanin (KLH) (Calbiochem) in CFA (Sigma Chemical) and boosted 2 weeks later with 50 µg of KLH in incomplete Freund's adjuvant (21). KLH-specific IgM and IgG in sera was measured 7 days later by ELISA.

Statistics. Data represent mean ± SEM. Statistical comparisons of differences between sample means used the two-tailed Student t test and one-way ANOVA with Bonferroni posttesting. Kaplan-Meier disease incidence and log-rank test comparisons of diabetes onset used Prism software (version 4.0; GraphPad).

RESULTS

YTS treatment induces rapid and long-term remission in recent-onset diabetic NOD mice. Recent-onset diabetic NOD female mice received two injections of 600–800 µg of YTS177 and YTS105 over 48 h. No insulin was provided prior to or at the time of YTS treatment. Strikingly, diabetes was reversed in 19 out of 24 (79%) YTS-treated NOD mice, whereas no remission was observed in animals receiving 2A3, an isotype control mAb (Fig. 1A). Remission was rapid, detected in 13 out of 24 (54%) NOD mice within 72 h of YTS treatment (Fig. 1B); the average blood glucose level for the 24 NOD mice was reduced from 365 mg/dL, seen prior to treatment, to 231 mg/dL (Fig. 1B). Furthermore, the majority of NOD mice receiving YTS105 or YTS177 alone failed to undergo remission, or, if induced, remission was relatively short-term (Fig. 1C).

YTS177 and YTS105 were no longer detected on the surface of T cells 4 to 5 weeks after treatment (data not shown). Nevertheless, 18 out of 19 of remission NOD mice remained diabetes-free >200 days post-YTS injection; remission was maintained in some animals monitored up to 400 days (Fig. 1A). Remission NOD mice exhibited stable albeit increased blood glucose levels relative to unmanipulated, nondiabetic animals (Fig. 1D). Histological analysis demonstrated that islets were relatively small in size and that >60% of islets were insulitis-free compared with the <5% seen in recent-onset diabetic NOD mice. Remission NOD mice also controlled a glucose challenge with similar kinetics as nondiabetic mice despite the generally small-sized islets (Fig. 1D). These results demonstrate that YTS177 and YTS105 induce rapid remission that is maintained long-term and that both YTS177 and YTS105 are required for efficient protection.

YTS treatment induces T-cell purging of the pancreas. Pancreatic IFN-γ and IL-2 were reduced at the time of remission in NOD mice (Fig. 2A), suggesting that the response of pancreatic T cells was readily blocked by YTS treatment. Next, the number of pancreatic $CD4^+$ and $CD8^+$ T cells was examined cytometrically in individual diabetic NOD mice following YTS treatment. $CD4^+$ and $CD8^+$ T-cell numbers were significantly reduced (approximately three- to fivefold) in the pancreas of NOD mice 6 days after the first YTS injection (Fig. 2B). Clearance of pancreatic T cells was confirmed via immunofluorescence (Supplementary Fig. 2). YTS-mediated purging was independent of T-cell subset
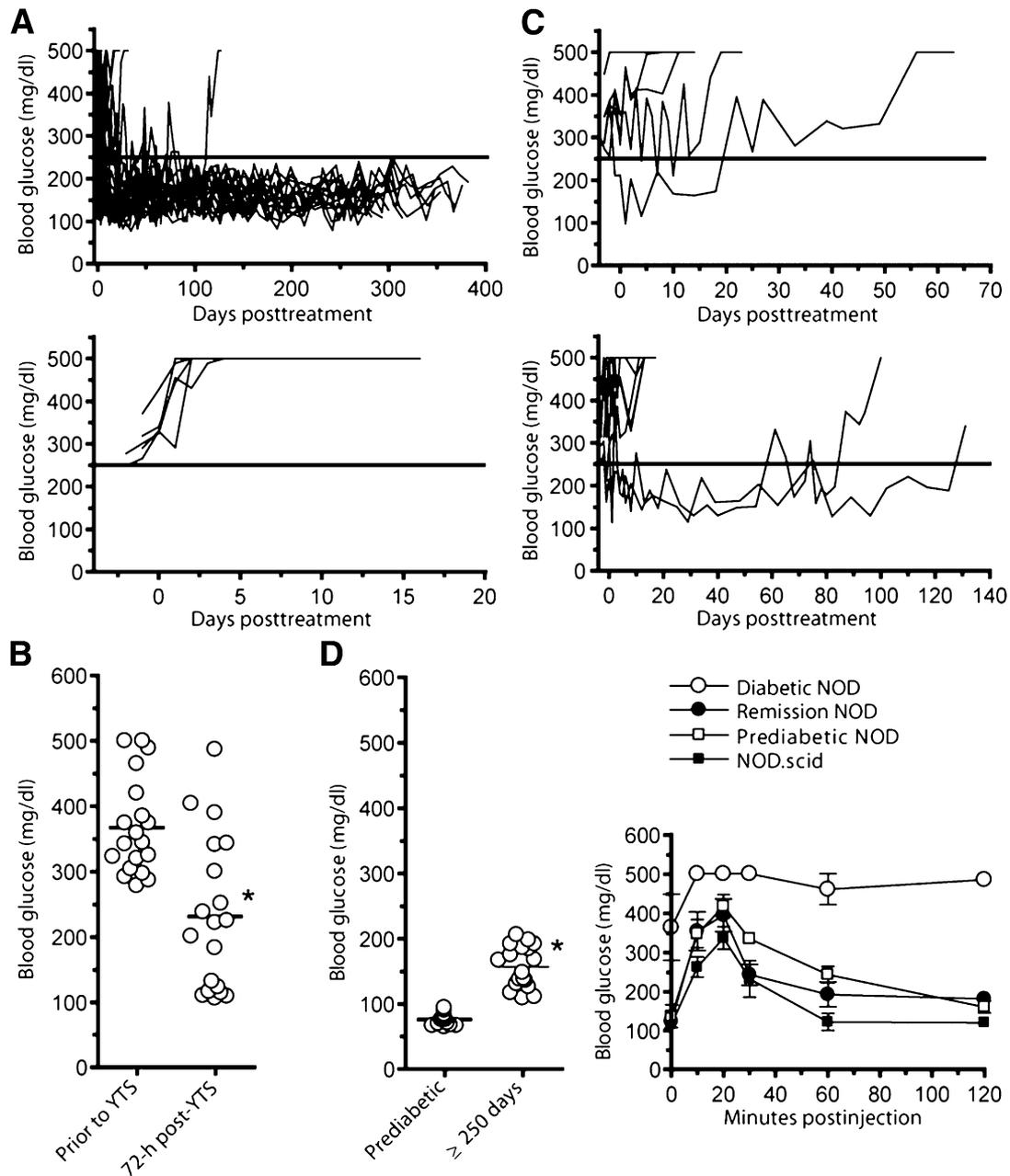
FIG. 1. Short-course treatment with YTS177 and YTS105 rapidly induces remission in recent-onset diabetic NOD mice that is maintained long-term. *A*: Recent-onset diabetic NOD female mice were treated with YTS177 and YTS105 ($n = 24$; *top panel*) or control 2A3 ($n = 5$; *bottom panel*) and blood glucose was monitored. *B*: Blood glucose levels of individual recent-onset diabetic NOD mice prior to and 72 h after YTS treatment. *$P < 10^{-3}$. *C*: Recent-onset diabetic NOD female mice were treated with only YTS105 ($n = 5$; *top panel*) or YTS177 ($n = 8$; *bottom panel*) and blood glucose was monitored. *D*: Blood glucose levels were measured in individual 12–14-week-old prediabetic ($n = 17$) or YTS-treated, long-term remission (≥ 250 days; $n = 18$) NOD female mice (*left panel*) *$P < 10^{-3}$. Tolerance to injected glucose was assessed in groups of three to six animals (*right panel*); YTS-treated NOD female mice remaining free of recurrent diabetes ≥ 150 days were tested.

because naive (CD62L$^+$CD44$^-$CD69$^-$), recently activated (CD44$^+$CD69$^+$), and effector/memory (CD44$^+$CD69$^-$) CD4$^+$ and CD8$^+$ T cells were reduced in the pancreas (Supplementary Figs. 3 and 4). CD4$^+$ T-cell numbers were also decreased approximately twofold in the PLN of YTS-treated NOD mice; CD8$^+$ T cells, in contrast, were only marginally decreased (Fig. 2*B* and Supplementary Fig. 4). In contrast, an approximately twofold increase in the number of CD4$^+$ and CD8$^+$ T cells was observed in the spleen of YTS-treated NOD mice (Fig. 2*B*). The increase in splenic T cells was due to transiently (~2 to 3 weeks) elevated numbers of naive CD4$^+$ and CD8$^+$ T cells (Supplementary Fig. 4).

To establish the generality of the above observations, TCR transgenic NOD.BDC2.5 (24) and NOD.8.3 mice (25) were treated with YTS. Progression of insulitis is synchronized, and large numbers of islet-infiltrating CD4$^+$ and CD8$^+$ T cells are found in the pancreas of NOD.BDC2.5 and NOD.8.3 mice, respectively. Analogous to NOD mice, CD4$^+$ T cells in YTS177-treated NOD.BDC2.5 mice (Fig. 2*C*) and CD8$^+$ T cells in YTS105-treated NOD.8.3 mice (Fig. 2*D*) were reduced in the pancreas (~6–10-fold) and PLN (approximately two- to threefold). The decrease in T-cell numbers in the pancreas and PLN of NOD.BDC2.5 and NOD.8.3 mice was also independent of T-cell phenotype
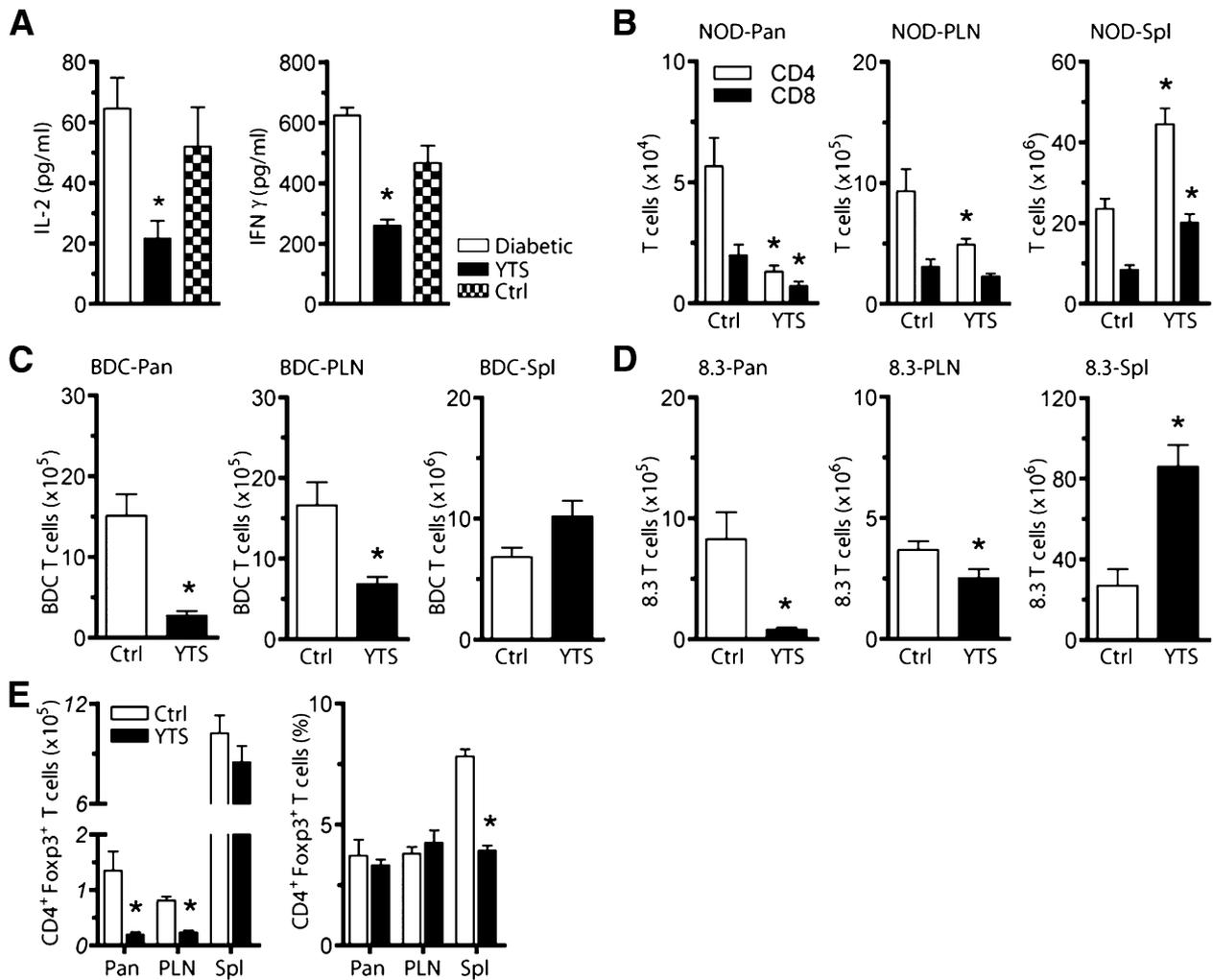
FIG. 2. YTS177 and YTS105 treatment induces T-cell loss in the pancreas and PLN. *A*: IL-2 and IFN-γ were measured via ELISA in pancreatic homogenates prepared from groups of recent-onset diabetic NOD mice treated with YTS177 and YTS105 (YTS), control 2A3 (Ctrl), or left untreated (Diabetic). Data are the average of eight individual mice. *P ≤ 0.008. The number of CD4+ and CD8+ T cells in the pancreas (Pan), PLN, and spleen (Spl) of NOD (*P ≤ 0.02) (*B*), NOD.BDC.2.5 (*P < 10⁻³) (*C*), and NOD.8.3 (*P ≤ 0.037) (*D*) was determined via FACS 6 days post-YTS or control 2A3 treatment. Data are the average of 8–15 individual mice. *E*: The number (*left panel*) and frequency (*right panel*) of Foxp3+ Treg in the respective tissues was determined in groups of six NOD.BDC.Foxp3.GFP female mice. *P ≤ 0.01.

(Supplementary Figs. 3 and 4); the increase in splenic T cells was similarly attributed to elevated naive T-cell numbers (Fig. 2*D*).

A decrease in the number (three- to fivefold) but not the frequency of Foxp3+CD4+ T cells was also observed in the pancreas and PLN of YTS177- and YTS105-treated NOD.BDC2.5 female reporter mice expressing a Foxp3 promoter-driven green fluorescent protein transgene (27) (NOD.BDC.Foxp3.GFP) (Fig. 2*E*). In contrast, Foxp3+CD4+ T-cell numbers in the spleen of NOD.BDC. Foxp3.GFP were unaffected by YTS treatment, although the frequency was reduced, reflecting an increase in naive CD4+ T cells (Fig. 2*E*). Similar results were obtained in wild-type NOD mice (Supplementary Fig. 5). These findings demonstrate that conventional CD4+ and CD8+ T cells independent of activation status or phenotype are purged from the pancreas and PLN, but not the spleen, following YTS binding.

T-cell clearance of the pancreas by YTS treatment is independent of apoptosis. Although YTS177 and YTS105 are nondepleting (32), Ab binding may induce T-cell apoptosis under the proinflammatory conditions found in the

pancreas and PLN. To rule out this possibility, the frequency of apoptotic T cells in the pancreas and PLN of NOD.BDC2.5 and NOD.8.3 mice was measured following YTS treatment. Apoptotic thymocytes were readily detected using FACS- and histological-based TUNEL assays in NOD mice treated with dexamethasone (Fig. 3*A* and *C*). No marked increase in the frequency of apoptotic cells over time, however, was detected in the PLN and pancreas of YTS177-treated NOD.BDC2.5 mice and YTS105-treated NOD.8.3 mice (Fig. 3*B–D*). The reduction in T cells was first detected at 36 and 24 h post-YTS treatment in NOD. BDC2.5 and NOD.8.3 mice, respectively. These results indicate that YTS-mediated T-cell purging of the pancreas and PLN is independent of T-cell apoptosis.

TGF-β1 is required for the induction of YTS177- and YTS105-mediated remission. Waldmann and colleagues (18,33) showed that YTS-mediated allograft protection was dependent on TGF-β1. Accordingly, levels of TGF-β1 were measured in pancreas homogenates from YTS-treated NOD mice within 3 days of diabetes reversal. A more than twofold increase in TGF-β1 was detected in remission versus diabetic NOD mice and nonautoimmune control
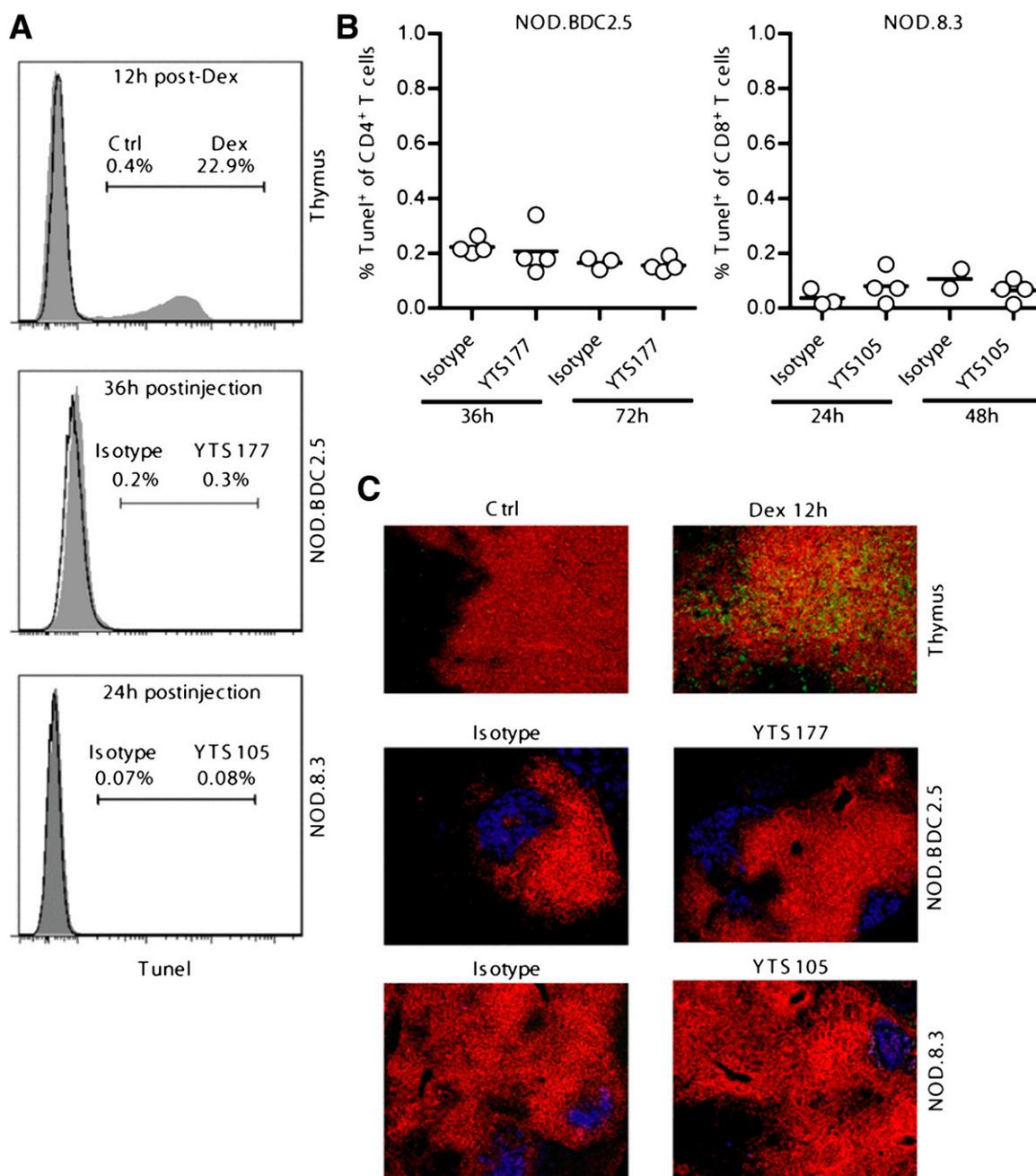
FIG. 3. YTS177- and YTS105-induced T-cell loss in the pancreas and PLN is not due to apoptosis. *A*: Representative FACS histograms for TUNEL staining of CD8^{+} thymocytes in NOD mice 12 h postdexamethasone (Dex) treatment and CD4^{+} and CD8^{+} T cells in the PLN of NOD.BDC2.5 and NOD.8.3 mice, respectively, treated with YTS or control 2A3. *B*: The frequency of TUNEL staining T cells in PLN of individual YTS and isotype control mAb-treated NOD.BDC2.5 and NOD.8.3 mice over time. *C*: Representative immunofluorescence of thymic sections from control and Dex-treated NOD mice or pancreatic sections from YTS versus control 2A3-treated NOD.BDC2.5 (36 h) and NOD.8.3 (24 h) mice stained with TUNEL (FITC), anti-CD90.2 (PE), and anti-insulin (Alexa 647). (A high-quality color representation of this figure is available in the online issue.)

mice (Fig. 4*A*). Serum TGF-β, however, was not increased in remission NOD mice (Fig. 4*A*).

To test a functional role for TGF-β1 in remission induction, recent-onset diabetic NOD female mice were treated with YTS177 and YTS105 and anti–TGF-β or HPRN, an isotype control mAb for anti–TGF-β. Transient remission was seen in three out of nine NOD mice receiving YTS177 and YTS105 plus anti–TGF-β (Fig. 4*B*). In contrast, remission was detected in all animals receiving YTS and HPRN (Fig. 4*B*). YTS177 and YTS105 also induced remission in diabetic NOD mice receiving anti–IL-10 receptor or lacking IL-4 expression (NOD.IL4[null]) (Fig. 4*C*). Interestingly, the

neutralizing TGF-β1 Ab had no effect on YTS177-mediated purging of CD4^{+} T cells in the pancreas of NOD.BDC2.5 mice (Fig. 4*D*).

To identify the source(s) of TGF-β1, DC, macrophages, and T cells were FACS sorted from individual pancreas and PLN of YTS177 and YTS105 or 2A3-treated NOD.BDC2.5 mice and mRNA levels measured. TGF-β1 mRNA in pancreatic T cells was unaffected by YTS (Fig. 4*E*). In contrast, increased TGF-β1 mRNA was detected in pancreas-but not PLN-derived DC and macrophages of NOD.BDC2.5 mice treated with YTS (Fig. 4*E*). These results demonstrate that TGF-β1 but not IL-4 or IL-10 is necessary for the
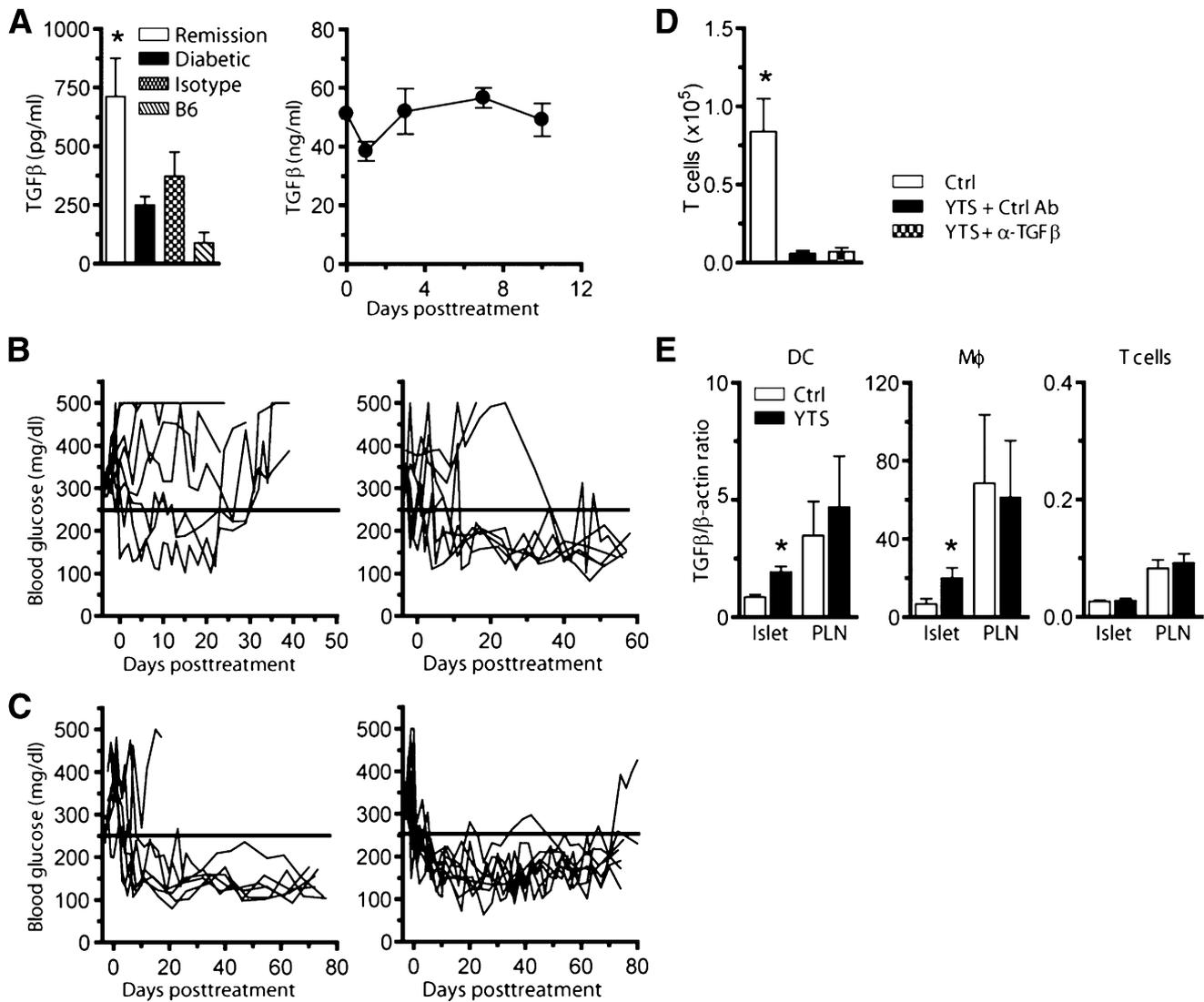
FIG. 4. TGF-β is necessary for YTS177- and YTS105-induced diabetes reversal. *A*: TGF-β was measured in individual pancreases from groups of three recent-onset diabetic NOD mice treated with YTS, control 2A3, or left untreated and from unmanipulated B6 mice (*left panel*; *P ≤ 0.0017) and serum at the time of onset and post-YTS treatment in five remission NOD female mice (*right panel*). The data are representative of at least three experiments. *B*: Recent-onset diabetic NOD female mice were treated with YTS177 and YTS105 plus anti–TGF-β (*n* = 9; *left panel*) or isotype control mAb (*n* = 10; *right panel*) and blood glucose was monitored. *C*: Recent-onset diabetic NOD (*n* = 7; *left panel*) and NOD. IL4^{null} (*n* = 8; *right panel*) female mice were treated with YTS plus anti–IL-10 receptor mAb or YTS alone, respectively, and blood glucose was monitored. *D*: NOD.BDC2.5/Cα$^{-/-}$ mice were treated with YTS177 plus anti–TGF-β or isotype control mAb, and 6 days later, the number of CD4$^+$ T cells in the pancreas determined via FACS. *P < 10^{-3}. *E*: Fold increase in TGF-β1 mRNA in DC, macrophages (MΦ), and T cells sorted from handpicked islets and PLN of individual NOD.BDC2.5 mice (*n* = 4 to 5/group) 6 days after treatment with YTS or isotype control. DC: *P = 0.004; MΦ: *P = 0.048.

induction of remission by YTS treatment and that pancreatic APC and not T cells are a source of elevated TGF-β1 levels.

β-Cell–specific immunoregulatory T cells selectively reside in the PLN of long-term remission NOD mice. To characterize β-cell autoimmunity in remission NOD mice, T-cell reactivity to insulin B chain (InsB$_{9–23}$) and the mimetic sBDC peptides was examined. No significant difference in the frequency of IFN-γ–secreting T cells was detected in the spleen of remission and control NOD mice (Fig. 5*A*), nor were IL-4–, IL-10–, and TGF-β1–secreting T cells increased (data not shown). However, in the PLN of remission NOD mice, InsB$_{9–23}$ and sBDC-specific IFN-γ–secreting T cells were reduced concomitant with an increase in T cells secreting IL-4 (Fig. 5*B*) but not IL-10 (data not shown). Furthermore, TGF-β1 was elevated in InsB$_{9–23}$ and

sBDC-stimulated PLN from remission NOD mice (Fig. 5*B*). The frequency of Foxp3$^+$CD25$^+$CD4$^+$ T cells was also increased in the PLN but not the spleen of remission NOD mice (Fig. 5*C*), although the number of Foxp3$^+$CD25$^+$CD4$^+$ T cells in the PLN of remission NOD mice was similar to control animals (Fig. 5*C*). These findings indicate that the frequency of immunoregulatory β-cell–specific T cells and Foxp3$^+$CD25$^+$CD4$^+$ T cells is increased in the PLN but not the spleen of long-term remission NOD mice.

Maintenance of remission involves tissue-specific immunoregulation. The above results suggested that active immunoregulation in a tissue-specific manner was ongoing in remission NOD mice. To test immunoregulatory activity in the PLN versus spleen, coadoptive transfers were carried out. NOD.*scid* mice receiving diabetogenic T effectors and splenocytes from control NOD
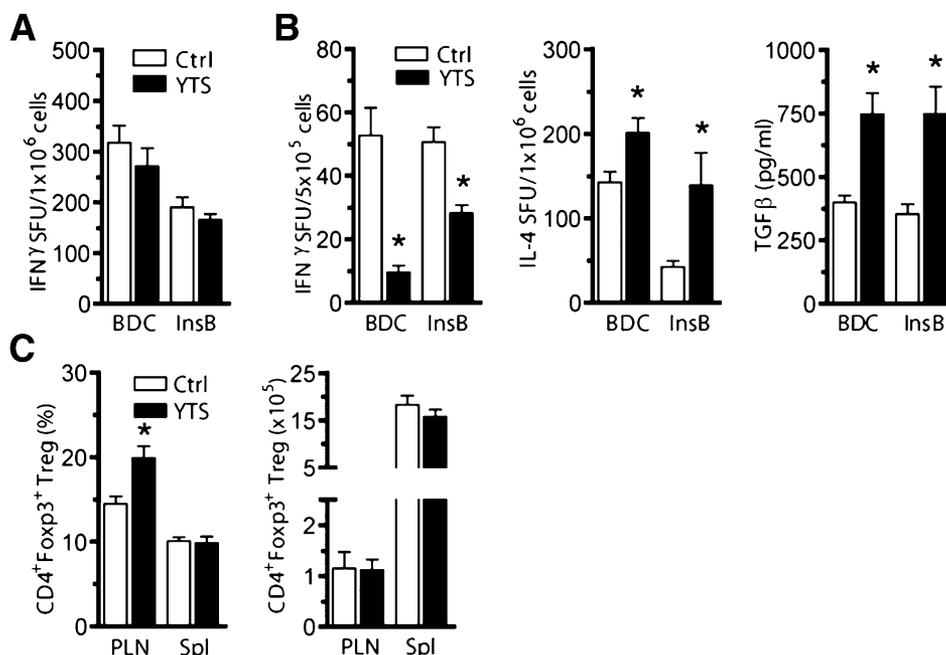
FIG. 5. Long-term remission NOD mice have altered β-cell–specific T-cell reactivity in the PLN. Cytokine production by β-cell–specific T cells was measured in the spleen (*A*) and PLN (*B*) (*$P \leq 0.04$) of YTS-treated animals in remission for >200 days and control recent-onset diabetic NOD mice. Shown is the average of two to four experiments. In medium-only controls, ≤10 IFN-γ or IL-4 spot-forming units (SFU) (*left and middle panels*) and ≤50 pg/mL of TGF-β (*right panel*) were detected in enzyme-linked immunospot and ELISA, respectively. *C*: The frequency (*left panel*) and number (*right panel*) of Foxp3+CD25+CD4+ T cells in the spleen (Spl) and PLN of diabetic (Ctrl; $n = 6–10$) and YTS-treated long-term remission (YTS; >200 days; $n = 7–10$) NOD female mice as determined by FACS. *$P = 0.01$.

mice developed diabetes analogously to NOD.*scid* recipients of diabetogenic T effectors alone (Fig. 6A). Similarly, splenic T cells from remission NOD mice failed to block diabetes transfer (Fig. 6A). Furthermore, no protection was detected with cells from the mesenteric lymph nodes of long-term remission NOD mice (data not shown). Moreover, CD25+CD4+ T cells sorted from the spleen of either remission or control NOD mice also failed to suppress diabetes transfer (Fig. 6A). These findings demonstrate that the immunoregulatory activity of splenic T cells from remission NOD mice is not increased under the conditions tested.

In contrast, PLN cells from YTS-treated but not control NOD donors significantly delayed diabetes onset in NOD.*scid* recipients (Fig. 6B). Furthermore, CD25+CD4+ T cells sorted from the PLN of remission versus control NOD mice significantly delayed the onset and reduced the incidence of diabetes (Fig. 6B). A similar frequency of Foxp3+CD4+ T cells (~90%) was found in the pool of CD25+CD4+ T cells isolated from the PLN of the respective groups (Supplementary Fig. 6).

Consistent with the coadaptive transfer experiments, proliferation of CFSE-labeled BDC2.5 CD4+ T cells was inhibited in the PLN of remission but not control NOD mice (Fig. 6C). These results demonstrate that CD25+CD4+ T cells are induced in a tissue-specific manner and confer protection against recurrent diabetes in long-term remission NOD mice.

To determine whether YTS-induced immunoregulation did not impair normal immune function, immunity to foreign antigens was assessed in remission NOD mice. Remission NOD mice were immunized with an H2K^d-restricted influenza HA peptide (HA$_{512–520}$) prepared in CFA and in vivo cytotoxic T lymphocyte (CTL) activity measured. No difference in HA$_{512–520}$-specific CD8+ CTL

activity was detected between HA$_{512–520}$-immunized nondiabetic control and long-term remission NOD female mice (Fig. 7A). Also, remission or untreated nondiabetic NOD female mice were immunized with KLH in CFA and then boosted 2 weeks later. Serum levels of KLH-specific IgM and IgG were similar between the two groups of immunized animals (Fig. 7B). These results demonstrate that long-term remission NOD mice respond normally to foreign antigens and that YTS177- and YTS105-induced immunoregulation is β-cell–specific.

DISCUSSION

We show that a short course of nondepleting YTS105 and YTS177 results in robust reversal of diabetes in NOD mice and that once induced, remission is β-cell–specific and long-term. Importantly, both YTS177 and YTS105 were required to efficiently reverse diabetes in NOD mice (Fig. 1C). A striking aspect of our findings was the prompt reversal of hyperglycemia following YTS treatment (Fig. 1B). Because the time to intervene after diabetes onset and rescue β-cells is limited, the rapid effect of YTS is advantageous. Indeed, delayed YTS administration or treating NOD mice with blood glucose levels >400 mg/dL failed to induce remission. Nevertheless, pancreatic T cells were efficiently purged in YTS-treated NOD mice remaining diabetic, further underscoring the importance of a sufficient β-cell mass at the time of intervention (Y.M.M and R.T., unpublished observations).

Results indicate that induction of remission by YTS treatment is driven by two key events. The first entails tissue-specific purging of conventional CD4+ and CD8+ T cells regardless of phenotype (Fig. 2 and Supplementary Fig. 4). This effect was independent of T-cell apoptosis, which differs from NFB anti-CD3 and antithymocyte
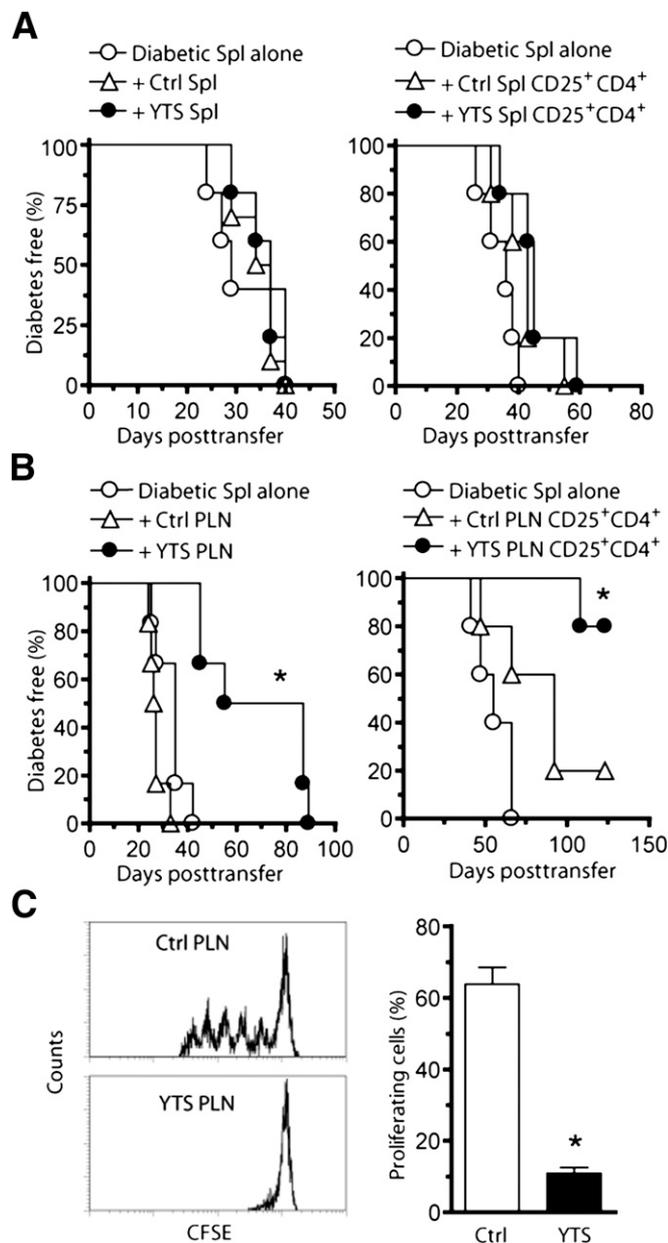
FIG. 6. PLN but not the spleen (Spl) of YTS-treated long-term remission NOD mice contains Treg. Groups of 5–10 NOD.*scid* mice received diabetogenic splenocytes plus splenocytes (*A, left panel*) or splenic CD25$^+$CD4$^+$ T cells (*A, right panel*) and PLN cells (*B, left panel*) or PLN-derived CD25$^+$CD4$^+$ T cells (*B, right panel*) isolated from YTS-treated long-term remission (>200 days) or control 2A3-treated diabetic NOD female mice. *$P \leq 0.002$. *C*: Proliferation of CFSE-labeled BDC2.5 CD4$^+$ T cells was measured via FACS (representative histograms provided) in the PLN of YTS Ab-treated long-term remission (>150 days) and nondiabetic 16-week-old NOD female mice. Data are the average of six mice/group. *$P = 10^{-4}$.

globulin, which readily promote T-cell apoptosis (13–15). These data suggest that decreased tissue retention of T cells is the underlying mechanism of YTS-mediated purging. YTS binding may influence the response of T cells to retention cues present during inflammation in the pancreas and PLN and in this way promote protection in a tissue-specific manner. Consistent with this hypothesis, T-cell trafficking is affected by binding of the coreceptor molecules with Ab or natural ligands such as HIV gp120 in the absence of TCR ligation (34–36). Furthermore, the influx of YTS-bound naive T cells into the spleen may be

explained by cleavage of CD62L following CD4 cross linking (37) (Supplementary Fig. 3). However, it is also possible that additional nonapoptotic inducing events such as T-cell redistribution and/or cytotoxic cell death also contribute to tissue purging.

The second key event associated with remission induction by YTS treatment was upregulation of pancreatic TGF-β1. Importantly, the need for TGF-β1 was demonstrated by administration of anti–TGF-β (Fig. 4B). The role (s) TGF-β1 plays in inducing remission following YTS treatment needs to be further defined. TGF-β1 may inhibit the effector function of pancreatic APC (38) and/or directly enhance the survival and function of β-cells (39). However, T cells do not appear to be a target of TGF-β1, at least early in remission, because anti–TGF-β had no effect on YTS-mediated T-cell purging (Fig. 4D). Our findings also indicate that the source of TGF-β1 is Treg-independent. TGF-β1 was increased in the pancreas despite reduced numbers of CD4$^+$ T cells and Foxp3$^+$ Treg (Fig. 2E), and elevated TGF-β1 mRNA was detected in pancreatic DC and macrophages but not in the remaining T cells found post-YTS treatment (Fig. 4E).

Whereas induction events were largely Treg-independent, maintenance of YTS-mediated remission involved tissue-specific Treg. Enhanced immunoregulatory activity was associated with Foxp3$^+$ Treg derived from the PLN but not the spleen of remission NOD mice (Fig. 6). PLN contain β-cell antigen-laden APC trafficking from the islets that would be expected to promote maintenance of β-cell–specific Treg. Indeed, persistence of YTS-induced allograft-specific Treg is dependent on continued alloantigen presentation (40,41). Induction of immunoregulatory activity in PLN is likely due to purging of resident T cells by YTS treatment (Fig. 2), thereby resetting the PLN (and pancreas) to establish a microenvironment that is more amenable to Treg induction/expansion. YTS177 binding to conventional CD4$^+$ T cells in the presence of TGF-β1 induces Foxp3$^+$ Treg in vitro, which may also contribute to the pool of β-cell–specific Treg (18). Interestingly, CD25$^+$CD4$^+$ T cells from the PLN of remission NOD mice exhibited enhanced suppressor activity relative to control CD25$^+$CD4$^+$ T cells (Fig. 6B). Foxp3$^+$ Treg with increased suppressor activity would be expected to further enhance the efficacy of immunoregulation in long-term remission NOD mice.

In summary, we show that administration of nondepleting anti-CD4 and anti-CD8 effectively induces and maintains diabetes remission in NOD mice. Recently, the Cooke group (42) also showed that remission is induced following multiple injections of both YTS177 and YTS105 in a limited number of diabetic NOD mice; animals were monitored for a short period of time and the mechanism of protection was not investigated. We propose that induction of remission involves rapid T-cell purging of the pancreas due to the inability of YTS-bound T cells to respond to retention signals in the pancreas (and PLN). TGF-β1 is selectively upregulated in pancreatic APC to further suppress the proinflammatory milieu of the pancreas, in addition to enhancing β-cell viability and function. Maintenance of remission is mediated by β-cell–specific Treg and enhanced by functionally superior Foxp3$^+$ Treg. Importantly, YTS binding of the coreceptor molecules has no long-term effects on normal immune function. These findings provide rationale for testing nondepleting anti-CD4 and anti-CD8 in the clinic for the treatment of type 1 diabetes.
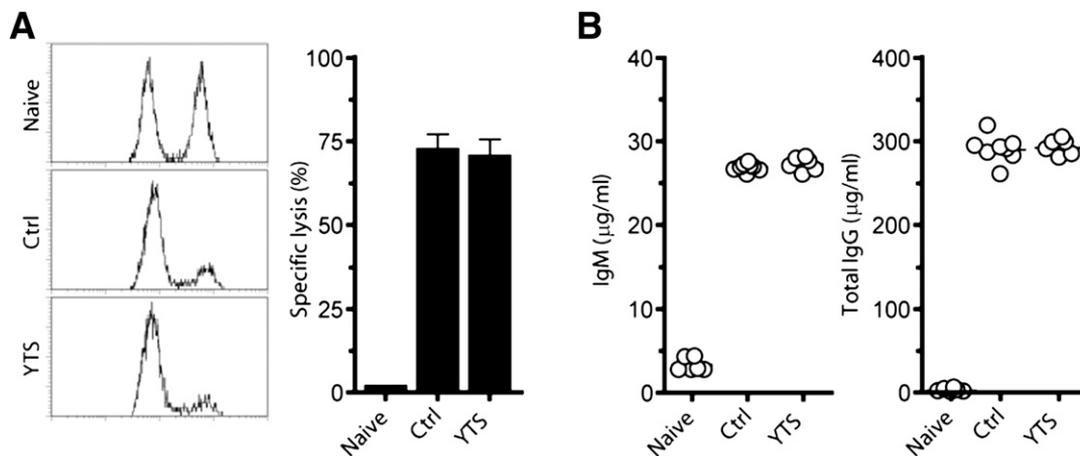
FIG. 7. Immunity to foreign antigens is unaffected by YTS Ab treatment. *A*: Representative FACS plots (*left panel*) of in vivo CTL activity specific for $HA_{512-520}$-pulsed targets in nondiabetic untreated (Naive) or peptide-immunized (Ctrl) NOD female mice and YTS Ab-treated long-term remission NOD mice (>150 days) immunized with $HA_{512-520}$ average specific lysis for groups of eight mice (*right panel*). *B*: KLH-specific serum IgM (*left panel*) and IgG (*right panel*) were measured in untreated or KLH-immunized NOD female mice or YTS Ab-treated long-term remission NOD mice (>150 days) immunized with KLH.

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