Combination Treatment With Anti-CD20 and Oral Anti-CD3 Prevents and Reverses Autoimmune Diabetes

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Type 1 diabetes (T1D) is a T cell–mediated autoimmune disease, although B cells also play an important role in T1D development. Both T cell– and B cell–directed immunotherapies have shown efficacy in the prevention and reversal of T1D. However, whether the combined strategy of targeting both T and B cells could further improve therapeutic efficacy remains to be explored. We show that combined treatment with intravenous antihuman CD20 (hCD20) and oral anti-CD3 significantly delays diabetes development in prediabetic hCD20 transgenic NOD mice. More importantly, the combined treatment reverses diabetes in >60% of mice newly diagnosed with diabetes. Further mechanistic studies demonstrated that the addition of oral anti-CD3 to the B-cell depletion therapy synergistically enhances the suppressive function of regulatory T cells. Of note, the oral anti-CD3 treatment induced a fraction of interleukin (IL)-10–producing CD4 T cells in the small intestine through IL-10– and IL-27–producing dendritic cells. Thus, the findings demonstrate that combining anti-CD20 and oral anti-CD3 is superior to anti-CD20 monotherapy for restoring normoglycemia in diabetic NOD mice, providing important preclinical evidence for the optimization of B cell–directed therapy for T1D. ***Diabetes*** **62:2849–2858, 2013**

Type 1 diabetes (T1D) is an autoimmune disease characterized by selective destruction of insulin-secreting β-cells in genetically predisposed individuals (1,2). T1D has been demonstrated to be a T cell–mediated disease. Therapeutic targeting of T cells by CD3-specific antibody prevented and reversed new-onset T1D in NOD mice (3,4). Clinical trials also suggested efficacy of anti-CD3 for patients with recent-onset T1D (5,6). Although these T cell–targeted therapies have been efficacious in newly diagnosed patients, side effects such as fever, rash, and anemia (5) as well as Epstein-Barr virus reactivation (6,7) were reported. Recently, oral administration of CD3-specific antibody has proven to be an effective strategy to treat autoimmune diseases (8,9). Of note, the side effects of intravenous anti-CD3 treatment were not observed when anti-CD3 was given orally (10), and important therapeutic effects of oral administration of anti-CD3 monoclonal antibody were demonstrated in both streptozotocin-induced and spontaneous diabetes mouse models (8,11).

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In addition to the pivotal role of T cells in T1D development, the contribution of B cells to the pathogenesis of T1D has increasingly been recognized. B cells are essential for the development of T1D in the NOD mouse model (12–14). B cell–deficient $\mu MT^{-/-}$ NOD mice fail to develop diabetes (12,13). Furthermore, several preclinical studies demonstrated that B cell–targeted therapies can prevent and reverse autoimmune diabetes (15–17). In line with these findings, a clinical trial of the anti-B-cell antibody rituximab in patients with T1D has further confirmed the critical role of B cells in the development of T1D (18). Although the clinical trial report for patients with T1D was promising, rituximab therapy only partially preserved islet β-cell function. Thus, further improvement of therapeutic efficacy, together with reduction of potential side effects of treatment, is still needed. In addition, phase III clinical trials of anti-CD3 therapy suggested that targeting the T-cell arm of the immune response alone is not sufficient to block T1D progression (19), and both the phase III clinical trials of teplizumab and otelixizumab were terminated because of failure to reach their primary end points. Given that both T and B cells are essential in the development of T1D, it is likely that combined therapy targeting both T- and B-cell compartments may further improve the therapeutic efficacy for patients with T1D. Thus, we tested the effect of intravenous anti-CD20 and oral anti-CD3 combined treatment for the prevention and reversal of T1D in the human CD20 transgenic NOD (hCD20/NOD) animal model.

Herein, we show that oral administration of anti-CD3 together with intravenous injection of anti-CD20 has a synergistic effect on the prevention and reversal of T1D in the hCD20/NOD mouse. Mechanistic studies demonstrated that the combined therapy enhanced immune tolerance by improving the $Foxp3^+$ regulatory T cell (Treg) compartment quantitatively and qualitatively as well as by inducing interleukin (IL)-10– and IL-27–producing dendritic cells (DCs) to promote the induction of IL-10^+ CD4 T cells in the small intestine.

RESEARCH DESIGN AND METHODS

Mice. The mice used in this study were kept in specific pathogen-free conditions in a 12-h dark/light cycle, housed in individually ventilated filter cages, and fed autoclaved food at the Yale University animal facility. The hCD20/NOD mice were generated as described previously (15). The hCD20/Foxp3-IRES-mRFP (FIR) NOD mice were generated by intercrossing hCD20/NOD with FIR (FIR/NOD) mice (20). The original FIR mice were provided by Richard A. Flavell (Yale University) (21). The use of the animals in this study was approved by the Yale University Institutional Animal Care and Use Committee.

Antibodies and reagents. All fluorochrome-conjugated monoclonal antibodies (mAbs) were purchased from BioLegend, Inc. Affinity-purified mouse anti-hCD20 mAb 2H7 was prepared by Bio X Cell (22). The original hybridoma was kindly provided by Dr. Ed Clark (University of Washington). Hamster anti-mouse CD3 mAb (clone 145-2C11) and control hamster IgG were purchased from Bio X Cell. Control mouse IgG used in the in vivo studies was purchased from Rockland Immunochemicals Inc. The IL-27 ELISA kit was purchased from eBioscience, Inc. IL-10 and tumor growth factor-β (TGF-β) ELISA kits were obtained from BD Biosciences.

Anti-hCD20/oral anti-CD3 combination treatment and its effect on spontaneous diabetes development. Prediabetic female hCD20/NOD mice (9 weeks of age) were treated daily with 2H7 (four intravenous injections from days 0–9 as described previously [15]) and simultaneously with five doses of 0.5 μg/kg anti-CD3 (treated from days 0–4) by oral gavage. Groups of age- and sex-matched mice treated with the same dose of anti-hCD20/hamster IgG, mouse IgG/oral anti-CD3, or mouse IgG/hamster IgG were set up as controls. All the treated mice were observed for diabetes development up to 35 weeks of age. They were screened for glycosuria twice a week. Diabetes was confirmed by blood glucose levels >250 mg/dL (13.9 mmol/L).

Treg suppression assay. Bead-purified BDC2.5 TCR transgenic CD4 T cells were used as responder cells in the assay. The CD4 T cells (1×10^5/well) were cocultured with irradiated bone marrow-derived dendritic cells (BMDCs) (1×10^4/well) as antigen-presenting cells in the presence of BDC2.5 mimotope 1 μg (23,24) at 37°C in 5% CO_2 for 4 days. ^{3}H-thymidine was added during the last 16 h of culture. To study the suppressive function in vitro, Tregs were purified with a $CD4^+CD25^+$ Treg purification kit from STEMCELL Technologies. Purified Tregs were cocultured with responder CD4 T cells (Tresp) at a ratio of 1:5 and 1:2 (Treg:Tresp).

Real-time PCR. Total RNA was extracted from sorted cells from Peyer's patches with the RNeasy Mini Kit (QIAGEN). Complementary DNA was generated with SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The mRNA levels of *IL10*, *IL27*, *STAT3*, *STAT4*, *GATA3*, *c-Maf*, and aryl hydrocarbon receptor *AhR* were determined by quantitative PCR (qPCR) on an iCycler (Bio-Rad). Primer pairs used for the PCR are listed in Supplementary Table 1.

Intracellular staining. Foxp3 staining was performed using a Foxp3 staining kit (eBioscience) according to the manufacturer's instructions. For cytokine staining, 10^6 cells were cultured for 5 h in the presence of 50 ng/mL of phorbol myristic acid (Sigma), 500 ng/mL of ionomycin (Sigma), and 1 μL/mL of Golgiplug (BD Bioscience). After staining of surface markers, cells were fixed in IC Fixation Buffer (eBioscience) for 20 min at room temperature. After two washes with permeabilization buffer (eBioscience), cells were stained with anticytokine antibodies.

Generation of IL-10–producing T cells. For the generation of IL-10–producing T cells in vitro, $CD4^+CD25^-CD62L^+$ cells purified from naïve NOD or BDC2.5 NOD mice were cultured together with modified DCs (T:DC = 5:1) in the presence of 1 μg/mL anti-CD3/1 μg/mL anti-CD28 (for NOD naïve $CD4^+$) or 3 μg/mL low-affinity mimotope (for BDC2.5 naïve $CD4^+$) for 3 days before flow cytometry. Splenic DCs were purified from spleen of control or treated mice using a $CD11c^+$ purification kit (STEMCELL Technologies). BMDCs were generated by culture of bone marrow cells from control or treated mice in the presence of 20 ng/mL granulocyte macrophage colony-stimulating factor and 100 IU/mL IL-4 for 6 days.

Histopathology and insulitis score. Pancreata were fixed in 10% buffered formalin and then paraffin embedded. Tissues were sectioned and stained with hematoxylin-eosin. Insulitis was scored under light microscopy with the following grading scale: 0, no insulitis; 1, insulitis affecting <25% of the islet; 2, insulitis affecting 25–75% of the islet; and 3, >75% islet infiltration. Fifty-three to 205 islets were scored for insulitis in each group (n = 5 mice) by an individual blinded to the experimental design. The insulitis scores with number of islets are shown in Supplementary Table 2.

Statistical analysis. The statistical analysis was performed with χ^2 test with the use of GraphPad Prism software. $P < 0.05$ was considered significant.

RESULTS

Combination treatment with anti-hCD20 and oral anti-CD3 delayed and prevented autoimmune diabetes development in hCD20/NOD mice. Our previous study suggested that anti-CD20 monotherapy can prevent T1D (15). To further improve the efficacy of treatment, we tested the combination treatment of anti-hCD20/oral anti-CD3 in hCD20/NOD mice. We treated a group of 9-week-old prediabetic female hCD20/NOD mice with four doses of mouse anti-hCD20 monoclonal antibody intravenously from days 0–9, as described previously (15). Simultaneously, mice were treated with five doses of 0.5 μg/kg of hamster anti-mouse CD3 daily by oral gavage from days 0–4. Groups of mice treated with mouse IgG/oral anti-CD3, anti-hCD20/oral hamster IgG, or mouse IgG/oral hamster IgG were set up as controls. By 35 weeks of age, mice treated with anti-hCD20/oral anti-CD3 were significantly protected from diabetes. Only 20% of the mice developed diabetes (Fig. 1), although mouse IgG/oral anti-CD3 and anti-hCD20/oral hamster IgG treatment also delayed diabetes development compared with the mouse IgG/oral hamster IgG control treatment. Compared with anti-CD20 monotherapy, the anti-hCD20/oral anti-CD3 treatment improved protection from disease development.

Anti-hCD20/oral anti-CD3 combination therapy reversed diabetes in the majority of hCD20/NOD mice with newly diagnosed T1D. Previously, we showed that anti-CD20 treatment reversed diabetes in about one-third of newly diagnosed diabetic mice. To determine whether the addition of oral anti-CD3 can improve the therapeutic efficacy of anti-CD20 treatment, we treated new-onset diabetic hCD20/NOD mice (blood glucose level 250–500 mg/dL) within 1 week of diagnosis with anti-hCD20, oral anti-CD3, or both (n = 18) or control mouse IgG/hamster IgG (n = 8), using the same regimen as in the prevention study. Diabetic hCD20/NOD mice were given daily subtherapeutic subcutaneous insulin while being treated to maintain the animals in a hyperglycemic state but in relatively good general health. Their blood glucose level was monitored every 24 ± 1–2 h, and insulin was withdrawn if blood glucose was <250 mg/dL. Of the 18 diabetic mice treated with anti-hCD20/oral anti-CD3, 12 (66.67%) demonstrated declining blood glucose levels and required no further insulin treatment, remaining euglycemic for >1 month after treatment (Table 1). Four of these 12 mice remained euglycemic for >120 days when the experiment was terminated (data not shown). Six mice did not become euglycemic after treatment with combined therapy, although they had the same immunological response to the antibody treatment as the responders. A possible reason for this finding was that the islets in these six mice had insufficient remaining β-cells because their blood glucose levels were already very high when they received treatment. In contrast, none of the IgG-treated mice had a sustained decline of blood glucose level, and all continued to require treatment with insulin (Table 1). The combination treatment greatly increased the disease remission rate compared with anti-hCD20 or oral anti-CD3 monotherapy (Table 1).

To investigate whether the combined treatment suppressed cellular infiltration in the pancreatic islets, we collected pancreata randomly from mice treated with anti-hCD20/oral anti-CD3 or mouse IgG/hamster IgG control at

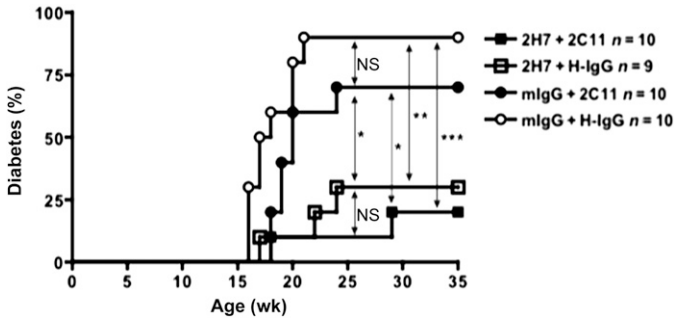

FIG. 1. Anti-hCD20/oral anti-CD3 combined treatment prevents T1D. Nine-week-old prediabetic hCD20/NOD mice were treated with anti-hCD20/oral anti-CD3 or control combinations as described in RESEARCH DESIGN AND METHODS. Glycosuria was monitored twice a week, and diabetes was confirmed by a blood glucose level of ≥250 mg/dL. Statistical analysis was performed with log-rank test. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. 2C11, oral anti-CD3; 2H7, anti-hCD20; H-IgG, hamster IgG; mIgG, mouse IgG; NS, not significant; wk, weeks.

TABLE 1
Therapeutic effect of anti-CD20/anti-CD3 on T1D

Treatment group	Treated mice	Euglycemic mice (1 mo posttreatment)	Remission rate (%)
mIgG/H-IgG	8	0	0
Anti-CD3/mIgG	29	7	24.1
Anti-CD20/H-IgG	10	3	30
Anti-CD20/anti-CD3	18	12	66.6

Anti-hCD20/oral anti-CD3 combined treatment reverses diabetes in new-onset diabetic mice. Newly diagnosed diabetic hCD20/NOD mice were treated with anti-hCD20/oral anti-CD3 or control IgGs within 1 week of disease diagnosis while using the same regimen as in the prevention study. H-IgG, hamster IgG; mIgG, mouse IgG; mo, month.

different time points (15 days, 1 month, and 3 months posttreatment). Insulitis scores in these mice are shown in Fig. 2. It is interesting that there was a significant reduction of insulitis 1 month after treatment ($P = 0.017$), but otherwise, there were no significant differences in cellular infiltration in mice treated with anti-hCD20/oral anti-CD3 compared with those treated with control IgGs (15 days $P = 0.133$; 3 months $P = 0.264$), although there was a trend toward fewer islets having >75% infiltration in the mice treated with anti-hCD20/oral anti-CD3.

Anti-hCD20/oral anti-CD3 treatment expanded the CD4$^+$Foxp3$^+$ Treg compartment. As shown earlier, we observed a synergistic protective effect of anti-hCD20/oral anti-CD3 treatment on diabetes prevention and reversal. However, the insulitis data suggest that the combined treatment had only a transient effect on the clearance of infiltrates. One possible explanation is that the combined treatment enhanced immune tolerance to control the inflammation in pancreatic islets. To understand the mechanisms by which the antibodies improved diabetes prevention and reversal, we studied the effect of combined treatment on the CD4$^+$Foxp3$^+$ Treg compartment. Compared with the mouse IgG/oral hamster IgG and mouse IgG/oral anti-CD3 groups, we found significantly more CD4$^+$Foxp3$^+$ Tregs in both spleen and pancreatic draining lymph nodes in anti-hCD20–treated groups combined either with oral anti-CD3 or hamster IgG (Fig. 3*A*). Significantly more CD4$^+$Foxp3$^+$ Tregs were also observed in islet-infiltrating cells from mice treated with anti-hCD20 (data not shown). The result indicates that anti-hCD20 rather than oral anti-CD3 induces the expansion of CD4$^+$Foxp3$^+$ Tregs, which is consistent with our previous studies showing that B-cell depletion induced CD4$^+$Foxp3$^+$ Tregs (15) but that oral anti-CD3 treatment did not (25).

Anti-hCD20/oral anti-CD3 treatment improved the function of CD4+Foxp3$^+$ Tregs. To investigate whether the treatment also improves CD4$^+$Foxp3$^+$ Treg function, we treated a group of hCD20/NOD mice with anti-hCD20/oral anti-CD3. Groups of mice treated with mouse IgG/oral hamster IgG, anti-hCD20/oral hamster IgG, or mouse IgG/oral anti-CD3 were set up as controls. Three months after the treatment, CD4$^+$CD25$^+$ Tregs were purified and tested with purified diabetogenic BDC2.5 CD4 Tresp cells in a ratio of 1:5 and 1:2 in the presence of irradiated BMDC and antigenic peptide. As shown in Fig. 3*B*, CD4$^+$Foxp3$^+$ Tregs from mice that received anti-hCD20/oral anti-CD3 treatment most effectively suppressed BDC2.5 CD4 T-cell proliferation in a dose-dependent manner, although anti-hCD20/oral hamster IgG and mouse IgG/oral anti-CD3 treatment also improved the suppressor function of CD4$^+$Foxp3$^+$ Tregs. The improved suppressor function of CD4$^+$Foxp3$^+$ Tregs by anti-hCD20/oral anti-CD3 treatment was also observed in vivo when CD4$^+$Foxp3$^+$ Tregs were cotransferred into NOD/SCID mice together with splenocytes from diabetic NOD mice (Fig. 3*C*). CD4$^+$Foxp3$^+$ Treg purified from treated mice showed significant improvement in its suppressor function, although both control and treated Tregs significantly delayed the transferred disease. Moreover, upregulation of cytotoxic T lymphocyte antigen 4 (CTLA-4) was detected in Tregs from mice treated with anti-hCD20/oral anti-CD3, supporting the enhanced function (Supplementary Fig. 1). These results indicate that anti-hCD20 treatment quantitatively and qualitatively improved

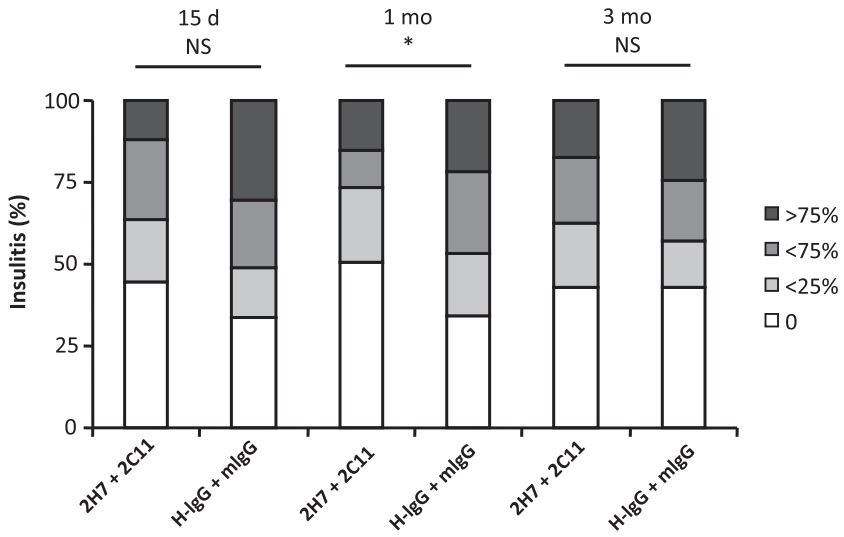

FIG. 2. Anti-hCD20/oral anti-CD3 combined treatment affects insulitis in hCD20/NOD mice. Mice were treated with anti-hCD20/oral anti-CD3 or control IgGs. Pancreata were collected at 15 days, 1 month, and 3 months after treatment, and insulitis was scored as follows: 0 = no insulitis, 1 = <25% infiltration, 2 = 25–75% infiltration, and 3 = >75% infiltration. Islets were examined from five euglycemic mice in each group, and insulitis was scored in 53–205 islets. The statistical analysis was performed with χ^2 test. *$P < 0.05$. 2C11, oral anti-CD3; 2H7, anti-hCD20; H-IgG, d, days; hamster IgG; mIgG, mouse IgG; mo, months; NS, not significant.

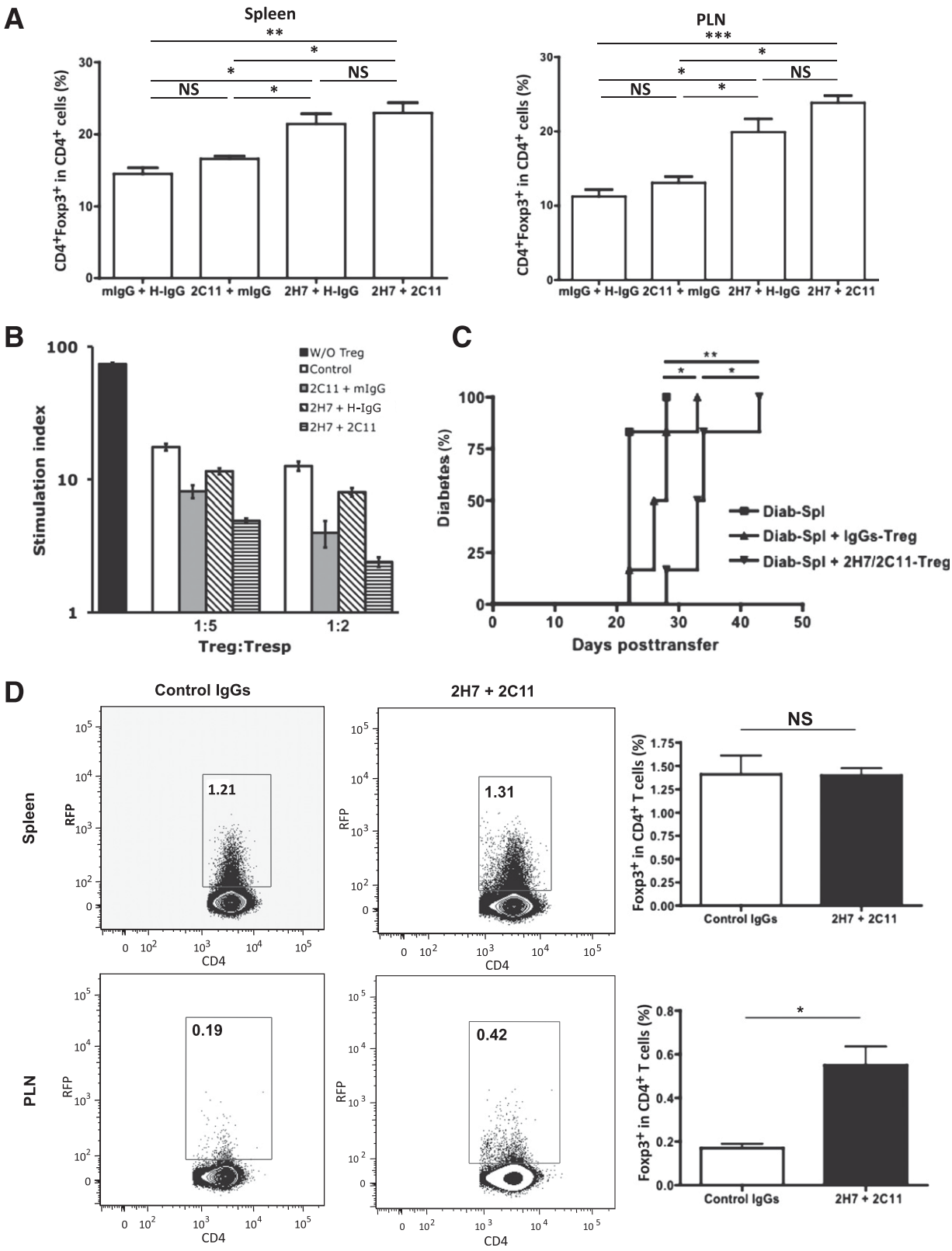

FIG. 3. Anti-hCD20/oral anti-CD3 combined treatment promotes CD4$^+$Foxp3$^+$ Tregs. *A*: Anti-hCD20/oral anti-CD3 combined treatment induces CD4$^+$Foxp3$^+$ Tregs in spleens and pancreatic draining lymph nodes (n = 3–4 mice each group). *B*: Anti-hCD20/oral anti-CD3 combined treatment enhanced suppressive function of Tregs. Tregs from mice treated with anti-hCD20/oral anti-CD3 or control were cocultured with BDC2.5 CD4$^+$ Tresp at 1:5 and 1:2 ratios for 4 days. One representative experiment of two is shown. The results are shown as the stimulation index, which was calculated as counts per minute (cpm) in the presence of antigen/cpm in the absence of antigen. The background counts were 200–500 cpm. *C*: Anti-hCD20/oral anti-CD3 treatment enhances Treg function in vivo. hCD20 FIR/NOD mice were treated with anti-hCD20/oral anti-CD3 or control IgGs. Three months posttreatment, CD4$^+$Foxp3$^+$ Tregs were purified from splenocytes of mice treated with anti-hCD20/oral anti-CD3 or control IgG and adoptively transferred into NOD/SCID mice together with splenocytes from diabetic NOD mice at a 1:7 ratio. Diabetes development was monitored as described in RESEARCH DESIGN AND METHODS (n = 6 each group). The statistical analysis was performed with log-rank test. *D*: Sorted CD4$^+$Foxp3$^-$

$CD4^+Foxp3^+$ Tregs, whereas oral anti-hCD3 treatment improved the function of $CD4^+Foxp3^+$ Tregs rather than induced $CD4^+Foxp3^+$ Treg expansion.

Anti-hCD20/oral anti-CD3 treatment increased conversion of $CD4^+Foxp3^-$ cells to $CD4^+Foxp3^+$ Tregs. To further understand the mechanism of $CD4^+Foxp3^+$ Treg induction in treated mice, we tested whether the combination treatment induced conversion of $CD4^+Foxp3^-$ cells into $CD4^+Foxp3^+$ Tregs. Because oral anti-CD3 treatment did not significantly induce $CD4^+Foxp3^+$ Tregs, we focused on mice treated with anti-hCD20/oral anti-CD3 and mice treated with mouse IgG/oral hamster IgG as a control. Treg-depleted $CD4^+Foxp3^-$ naïve T cells were sorted from FIR/NOD mice and then adoptively transferred into treated or control hCD20/NOD mice 1 month posttreatment. One week after cell transfer, the presence of $CD4^+FIR^+$ Tregs in recipients was analyzed by flow cytometry. Of note, $CD4^+Foxp3^+$ Tregs were observed in both treated and control mice. However, more $CD4^+Foxp3^+$ Tregs were detected in pancreatic draining lymph nodes but not in spleens of mice treated with anti-hCD20/oral anti-CD3 compared with controls (Fig. 3*D*). These data indicate that the combined treatment promoted a local niche for the conversion of $CD4^+Foxp3^-$ CD4 T cells into $CD4^+Foxp3^+$ Tregs.

TGF-β is required for the increased conversion of $CD4^+Foxp3^-$ cells to $CD4^+Foxp3^+$ Tregs. The regulatory cytokine TGF-β is essential for the differentiation of $CD4^+Foxp3^+$ Tregs. To understand whether anti-hCD20/oral anti-CD3 treatment promoted TGF-β expression, we performed an ELISA to determine the active TGF-β level in sera. In sera from mice treated with anti-hCD20/oral anti-CD3, there was a significantly higher level of TGF-β compared with sera from control mice (Fig. 4*A*). Of note, the increase in TGF-β was only observed at the early time points of 15 and 30 days posttreatment (data not shown). We also observed significantly higher IgG2b levels in sera from treated mice than in that from control mice (Fig. 4*B*), which further supports the induction of TGF-β by the combined treatment because TGF-β can promote IgG2b isotype switch (26,27).

Anti-hCD20/oral anti-CD3 combined treatment induced IL-10^+ CD4 T cells in the small intestine. To further understand other mechanisms behind the synergistic effect of the combination therapy in addition to the observed modulation in the $CD4^+Foxp3^+$ Treg compartment, we tested the effect of anti-hCD20/oral anti-CD3 treatment on mucosal immunity. Weiner and colleagues (9,25) reported that oral or nasal anti-CD3 can induce $CD4^+CD25^-LAP^+$ helper T 3 (Th3) regulatory cells. Of note, we did not observe LAP^+ Th3 cells in hCD20/NOD mice that had received the combined therapy (data not shown). However, compared with the groups of mice without oral anti-CD3 treatment, more IL-10^+ CD4 T cells were detected in Peyer's patches from the small intestine of the groups that received oral anti-CD3 treatment combined with either mouse IgG or anti-hCD20 (Fig. 5*A*). Also interesting, IL-10–secreting CD4 T cells were induced in luminal cells of the small intestine (Supplementary Fig. 2*A*) and to a lesser extent in the spleen (Supplementary Fig. 2*B*) by anti-hCD20/oral anti-CD3 treatment. Furthermore, significantly more $CD4^+$IL-10^+ T cells were observed in islet-infiltrating cells from mice treated with anti-CD3 (data not shown). The serum level of IL-10 detected by ELISA further supported the induction of IL-10 by the combined treatment (Fig. 5*B*). A higher level of IgA in sera from mice treated with anti-hCD20/oral anti-CD3 also indicated upregulation of IL-10 in CD4 T cells (Fig. 5*C*) because IL-10 promotes IgA isotype switch in combination with TGF-β (28).

Because IL-10^+ CD4 T cells from Peyer's patches were only observed in mice treated with oral anti-CD3, we focused on the anti-hCD20/oral anti-CD3 group to further delineate the molecular mechanisms responsible for the induction of IL-10 expression in CD4 T cells from Peyer's patches. qPCR results showed that upregulation of *IL10* was detected in $CD4^+$ T cells from Peyer's patches of mice treated with anti-hCD20/oral anti-CD3 compared with control mice (Fig. 5*D*). The transcription factor c-Maf is essential for the induction of IL-10 in CD4 T cells (29). Of note, $CD4^+$ T cells from treated Peyer's patches showed a significantly higher level of transcription factor c-Maf

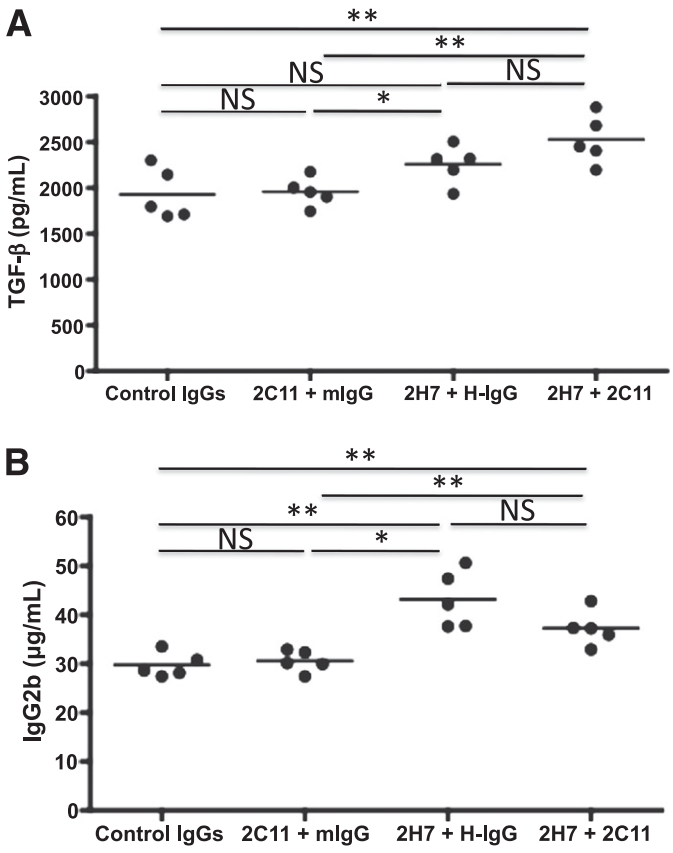

FIG. 4. Anti-hCD20/oral anti-CD3 combined treatment induces TGF-β production. *A*: Sera were collected from hCD20/NOD mice treated with anti-hCD20/oral anti-CD3 or control mouse IgG/oral hamster IgG, and active TGF-β level in sera was detected by ELISA. *B*: IgG2a and IgG2b levels in sera were detected by ELISA. The statistical analysis was performed with Student *t* test. *$P < 0.05$; **$P < 0.001$. 2C11, oral anti-CD3; 2H7, anti-hCD20; H-IgG, hamster IgG; mIgG, mouse IgG; NS, not significant.

T cells from FIR/NOD mice were adoptively transferred into hCD20/NOD mice treated with anti-hCD20/oral anti-CD3 or control mouse IgG/oral hamster IgG. One week later, $CD4^+Foxp3^+$ Tregs were detected in spleens and PLNs from recipients by flow cytometry, with representative plots of $CD4^+RFP^+$ cells shown. One representative experiment of three is shown. The statistical analysis was performed with Student *t* test. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. 2C11, oral anti-CD3; 2H7, anti-hCD20; Diab-Spl, splenocytes from diabetic mice; H-IgG, hamster IgG; mIgG, mouse IgG; NS, not significant; RFP, red fluorescence protein; W/O, without.

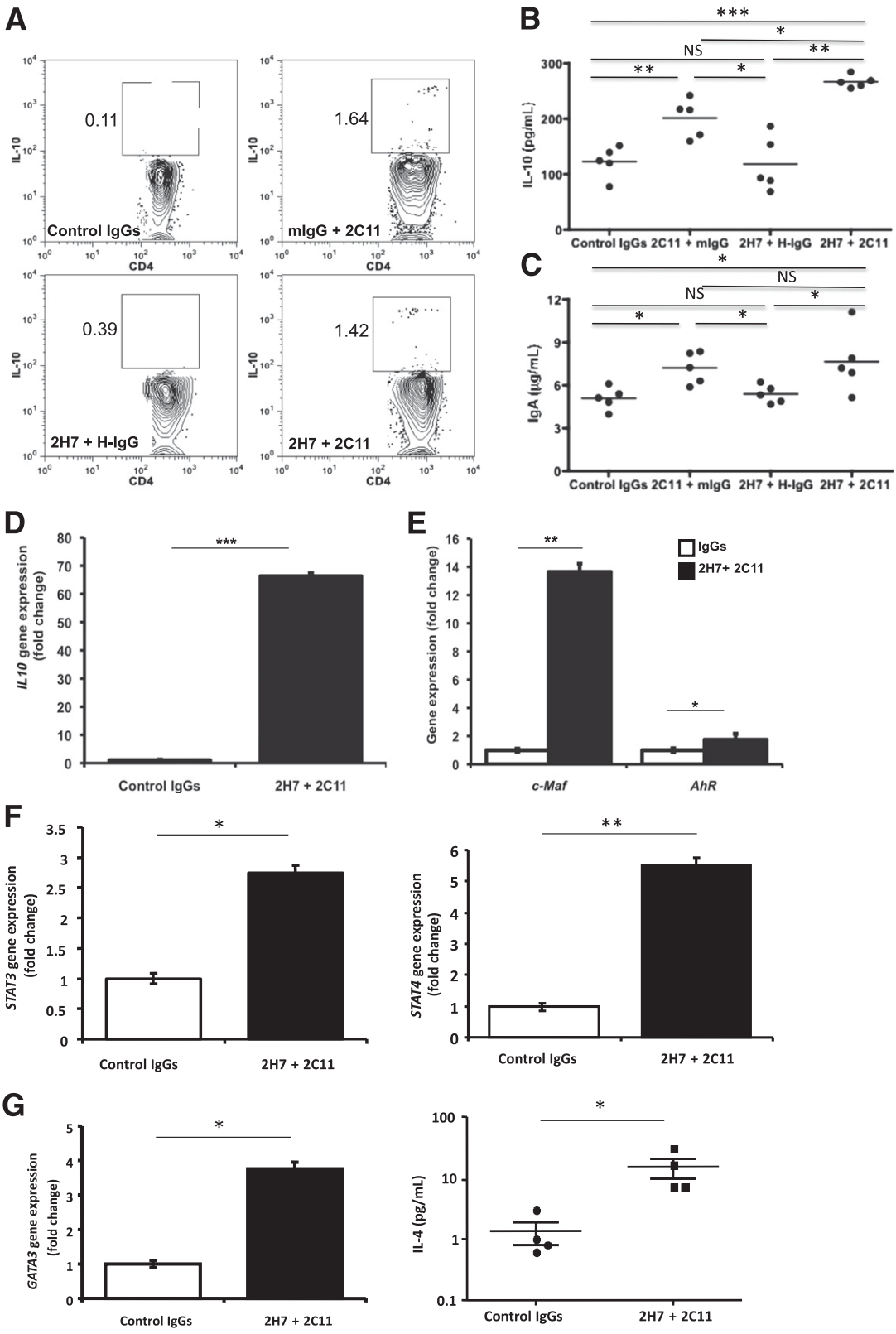

FIG. 5. Anti-hCD20/oral anti-CD3 combined treatment induces IL-10–producing CD4$^+$ T cells. *A*: hCD20/NOD mice were treated with anti-hCD20/oral anti-CD3 or control mouse IgG/hamster IgG. Peyer's patches were collected. IL-10 production was detected by intracellular cytokine staining. *B*: Sera were collected from hCD20/NOD mice 15 days after anti-hCD20/oral anti-CD3 or control IgG treatment, and IL-10 levels in sera were detected by ELISA. *C*: IgA levels in sera were detected by ELISA. *D*: CD4$^+$ T cells were sorted from Peyer's patches of mice treated with anti-hCD20/oral anti-CD3 or control IgGs and *IL10* transcripts were detected by qPCR in purified CD4 T cells. *E*: *c-Maf* and *AhR* mRNA were detected in purified CD4 T cells from Peyer's patches by qPCR. *F*: Transcription factors *STAT3* and *STAT4* mRNA were detected in purified CD4 T cells from Peyer's patches by qPCR. *G*: *GATA3* mRNA was detected in purified CD4 T cells in Peyer's patches by qPCR, and IL-4 protein levels in sera were detected by ELISA. The statistical analysis was performed with Student *t* test. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. 2C11, oral anti-CD3; 2H7, anti-hCD20; H-IgG, hamster IgG; mIgG, mouse IgG; NS, not significant.

compared with control, and there was only a marginal increase in *AhR* transcripts (Fig. 5*E*). Transcription factors STAT3, STAT4, and GATA3 have also been shown to be involved in the induction of IL-10 in CD4 T cells (30–32). In the mice treated with anti-hCD20/oral anti-CD3, we observed upregulation of these three transcription factors in CD4 T cells from Peyer's patches compared with control mice (Fig. 5*F* and *G*). We also detected a higher level of IL-4 in sera from the anti-hCD20/oral anti-CD3 group compared with controls, which further supports the upregulation of GATA3 (Fig. 5*G*).

Combined antibody treatment induced IL-27– and IL-10–producing DCs that can promote IL-10–producing CD4 T cells. Next, we investigated which cell subset is responsible for the induction of IL-10$^+$ CD4 T cells. It has been shown that IL-27 together with TGF-β can induce *AhR* and act in synergy with c-Maf to promote the differentiation of type 1 regulatory (Tr1) cells (29,33). IL-27– and IL-10–producing DCs also enhanced IL-10 expression in T cells in an oral tolerance model (34). To understand the molecular mechanisms that lead to the induction of IL-10$^+$ CD4 T cells by anti-hCD20/oral anti-CD3 treatment, we performed qPCR to analyze the gene expression in DCs from spleen and Peyer's patches as well as in BMDCs. A higher level of *IL10* and *IL27* transcripts was observed in anti-hCD20/oral anti-CD3–treated DCs (Fig. 6*A*). Upregulation of IL-27 was further confirmed at the protein level in sera and supernatants from DC culture by ELISA (Fig. 6*B*).

Because upregulation of IL-10 and IL-27 in DCs of mice treated with combination antibody was observed, we speculated that DCs from treated mice could more potently induce IL-10–producing CD4 T cells. To test this hypothesis, we performed fluorescence activated cell sorting of polyclonal naïve CD4 T cells (CD44^{lo}CD62L^{hi}CD25$^-$) from NOD mice or oligo/monoclonal naïve CD4 T cells from BDC2.5 mice and then cocultured them with purified splenic DCs or BMDCs of treated or control mice. The IL-10 expression in cultured CD4 T cells was detected by intracellular cytokine staining after 5 days of coculture. Of note, both splenic DCs (Fig. 6*C* and *E*) and BMDCs (Fig. 6*D* and *F*) from mice treated with anti-hCD20/oral anti-CD3 showed an enhanced ability to induce IL-10–producing polyclonal (Fig. 6*C* and *D*) or monoclonal CD4 T cells (Fig. 6*E* and *F*) compared with DCs from control antibody-treated mice.

DISCUSSION

To further improve the therapeutic efficacy of B cell–directed therapy for T1D, we combined B-cell depletion with oral anti-CD3 to treat hCD20/NOD mice. The results demonstrated that oral anti-CD3 had a synergistic effect when combined with anti-hCD20 antibody on the prevention and reversal of T1D in the hCD20/NOD mouse model. Only 20% of the mice treated with anti-CD20/oral anti-CD3 developed diabetes, and about two-thirds of newly diagnosed mice that received combined treatment became euglycemic. The combined therapy showed improved efficacy for the prevention and reversal of T1D compared with anti-hCD20 monotherapy. The mechanistic studies showed that the combined therapy promoted immune tolerance by increasing CD4$^+$Foxp3$^+$ Treg numbers and function and inducing IL-10– and IL-27–producing DCs to promote an additional subset of IL-10$^+$ Tr1 CD4 T cells that were Foxp3$^-$ in the small intestine. These cells not only were detectable in the pancreatic lymph nodes (PLNs) but also were found in the islet infiltration such that they could exert their effects locally at the site of damage.

Our previous studies have shown that temporary B-cell depletion by anti-hCD20 antibody improved immune regulation by induction of Foxp3$^+$ Treg after B-cell repopulation (15,35). Consistent with the effects seen with anti-hCD20 monotherapy, the combined treatment of anti-hCD20/oral anti-CD3 also expanded CD4$^+$Foxp3$^+$ Tregs. Moreover, the combined treatment improved the suppressor function of CD4$^+$Foxp3$^+$ Tregs. Of note, the expansion of CD4$^+$Foxp3$^+$ Treg numbers was mainly induced by anti-hCD20 treatment rather than by oral administration of anti-CD3. Nevertheless, both the intravenous anti-hCD20 and the oral anti-hCD3 treatment contributed to the enhanced function of CD4$^+$Foxp3$^+$ Tregs. The improved function of CD4$^+$Foxp3$^+$ Tregs was demonstrated by improved suppression of diabetogenic CD4 T cells in vitro and further delay of disease development in vivo when adoptively transferred together with diabetogenic splenocytes into NOD/SCID mice. It has been suggested that levels of Foxp3 in Tregs reflect their suppressive function (36). In the present study, however, we did not observe a difference in Foxp3 expression between control and treated Tregs. Instead, we observed upregulation of CTLA-4 in CD4$^+$Foxp3$^+$ Tregs. CTLA-4 has been implicated in the control of CD4$^+$Foxp3$^+$ Treg function (37). Impaired CD4$^+$Foxp3$^+$ Treg function has been attributed to decreased expression of CTLA-4 in locations that have been linked to pathogenesis of diabetes (PLNs and small intestinal lamina propria) rather than to downregulation of Foxp3 expression (38). In the present study, not only was upregulation of CTLA-4 detected in a proportion of CD4$^+$Foxp3$^+$ Tregs, but also a population of CD4$^+$CTLA-4$^+$Foxp3$^-$ cells were induced in mice treated with anti-hCD20/oral anti-CD3. The findings imply that CTLA-4 plays a role in enhancing the suppressive function of Foxp3$^+$ Tregs from treated mice and that CD4$^+$CTLA-4$^+$Foxp3$^-$ cells may also contribute to reestablishing immune tolerance, although the latter needs further investigation. This treatment is clearly immunoregulatory, and the enhancement of effects is likely to be best seen when treatment is started as early as possible after diabetes is diagnosed. The mice that showed a response, with a return to euglycemia, were those in which treatment was started at a lower blood glucose level within the diabetic range. Reducing the effects of diabetogenic T cells would allow any remaining β-cells to recover function. There is no evidence thus far that this treatment stimulates β-cell replication and regeneration. The effects of the treatment are likely to be enhanced by further combination with therapy that could improve β-cell mass.

Oral anti-CD3 has been shown to be an effective agent in the treatment of lupus in (NZB × SWR) F1 mice as well as in streptozotocin-induced autoimmune diabetes in AKR/J male mice through the induction of CD4$^+$CD25$^-$LAP$^+$ Tregs (8,9). In the current study, however, we could not detect induction of CD4$^+$CD25$^-$LAP$^+$ Tregs in hCD20/NOD mice treated with oral anti-CD3. The discrepancy observed among these animal models might be due to the genetic background differences because NOD mice have several defects in the regulation of immune tolerance (2). However, a subset of IL-10–producing CD4 T cells in the small intestine was effectively induced by the anti-hCD20/oral anti-CD3 treatment. IL-10–producing CD4 T cells were also induced in the spleens of treated mice, albeit to a lesser extent, in contrast to the control spleens. These IL-10–producing CD4 T cells

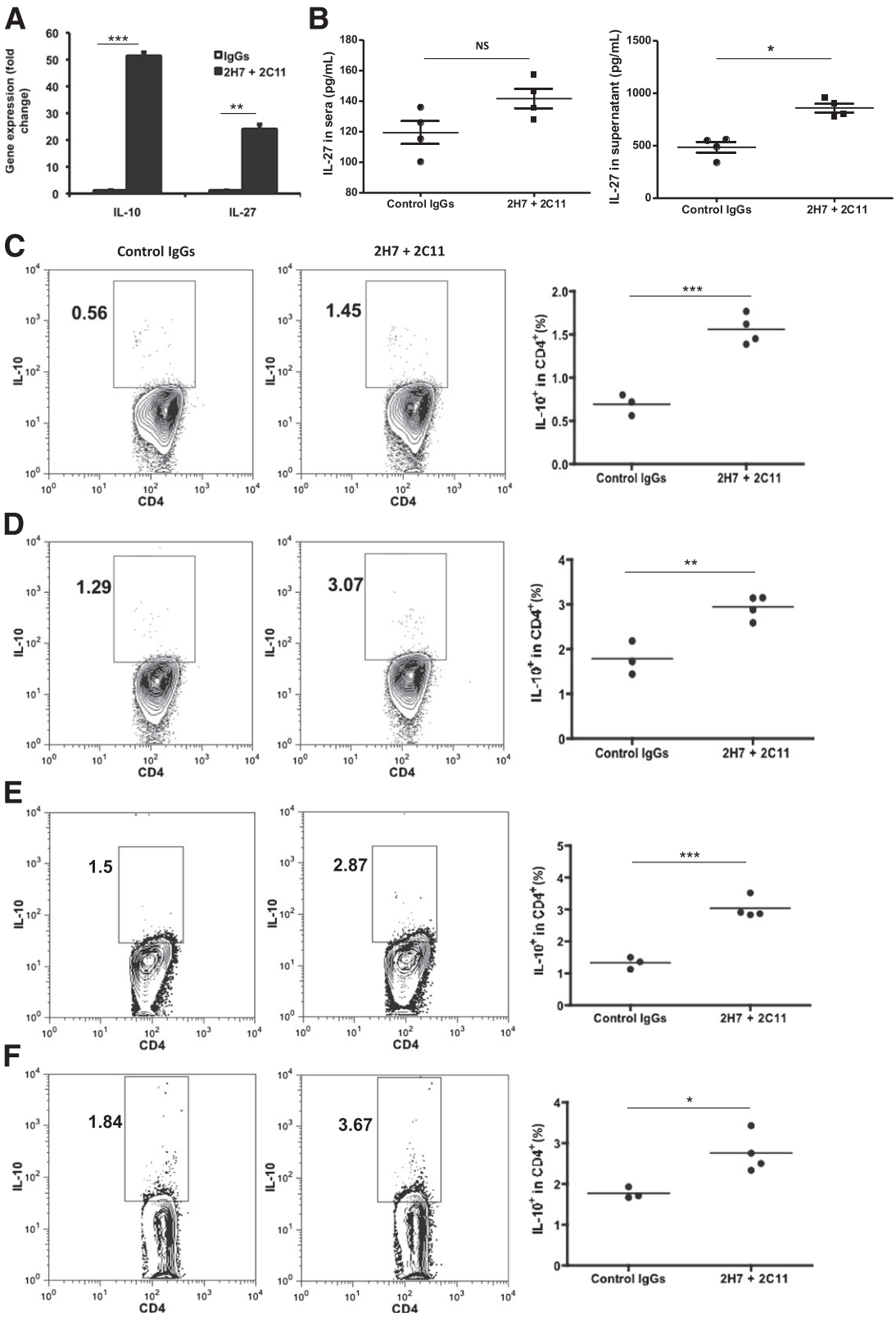

FIG. 6. Anti-hCD20/oral anti-CD3 combined treatment promotes IL-10– and IL-27–producing DCs to induce IL-10–secreting CD4 T cells. *A*: *IL10* and *IL27* mRNA were determined by qPCR in DCs from mice treated with anti-hCD20/oral anti-CD3 or control mouse IgG/hamster IgG. *B*: IL-27 protein was detected by ELISA from sera of mice treated with anti-hCD20/oral anti-CD3 or control IgG and supernatant of BMDC culture. Splenic DCs from mice treated with anti-hCD20/oral anti-CD3 or control IgG (*C* and *E*) and BMDC (*D* and *F*) were cocultured with sorted polyclonal CD4$^+$ T cells (*C* and *D*) and BDC2.5 monoclonal CD4$^+$ T cells (*E* and *F*) in the presence of anti-CD3 and anti-CD28 for 3 days. IL-10–producing CD4 T cells were detected by intracellular cytokine staining. The statistical analysis was performed with Student *t* test. *$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. 2C11, oral anti-CD3; 2H7, anti-hCD20; NS, not significant.

induced by anti-CD3 treatment were Foxp3$^-$ and IL-4$^-$ (data not shown), and thus more likely to be induced Tr1 cells, rather than skewing the immune response toward Th2. Of note, IL-10–producing CD4 T cells in Peyer's patches could only be detected in groups that were treated with oral anti-CD3 combined with either anti-hCD20 or mouse IgG but not in the groups without oral anti-CD3 treatment. The IL-10–producing CD4 T cells were also identified in small intestinal luminal cells. These data indicate that the induction of IL-10–producing CD4 T cells was due to oral anti-CD3 treatment rather than to B-cell depletion. The oral route of anti-CD3 administration appears not to be essential for the induction of IL-10–producing CD4$^+$ T cells because anti-CD3 injected intraperitoneally induced IL-10–producing CD4$^+$ T cells and attenuated colitis (39). Taken together, the present findings suggest that oral anti-CD3 acts synergistically with anti-hCD20 in the prevention and reversal of T1D by both enhancement of CD4$^+$Foxp3$^+$ Treg function and induction of IL-10–producing CD4 Tr1 cells. How the induced IL-10–producing CD4 T cells participate in the control of pancreatic islet autoimmunity is under further investigation.

DCs play a role in the induction of IL-10$^+$ CD4 T cells. We detected upregulation of IL-27 and IL-10 in DCs on anti-hCD20/oral anti-CD3 treatment. When cocultured with sorted naïve CD4$^+$ T cells, these treated DCs showed an enhanced ability to induce IL-10 in CD4$^+$ cells compared with control DCs. Shiokawa et al. (34) showed that IL-27– and IL-10–producing DCs enhanced IL-10 expression in T cells in an oral tolerance model. The induction of IL-10 was further supported by the upregulation of transcription factors STAT3, STAT4, GATA3, AhR, and c-Maf in CD4$^+$ cells. It has been shown that IL-27 induces physical association of AhR with c-Maf and subsequently transactivates the *IL10* during Tr1 cell differentiation (40). The present data further support the notion that IL-27 from DCs is a growth and differentiation factor for Tr1 cells through the induction of the transcription factor c-Maf (29,33). The mechanisms by which anti-hCD20 and oral anti-CD3 treatment induces the expression of IL-27 and IL-10 in DCs are under further investigation.

In summary, we have reported that oral anti-CD3 acts synergistically with anti-hCD20 to improve the therapeutic efficacy of B cell–directed therapy through promoting immune tolerance by increasing the number and function of Foxp3$^+$ Tregs and inducing IL-10$^+$ CD4 Tr1 cells through the induction of IL-10– and IL-27–producing DCs. These findings provide important preclinical evidence for the enhanced therapeutic efficacy of the combined treatment compared with anti-hCD20 monotherapy. We also identified mechanisms responsible for the improved prevention and reversal of T1D in NOD mice. These findings will guide the design and evaluation of novel combinatorial immunotherapy that removes a critical antigen-presenting cell while altering the T-cell repertoire by targeting both B and T cells for patients with T1D.

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C.H. designed and performed the experiments and wrote the manuscript. H.D. and X.Z. performed the experiments. F.S.W. wrote the manuscript. L.W. designed the experiments and wrote the manuscript. L.W. 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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