Combination treatment with anti-CD20 and oral anti-CD3 prevents and reverses autoimmune diabetes

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ABSTRACT

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 the therapeutic efficacy remains to be explored. Herein we show that the combined treatment with intravenous anti-human CD20 (hCD20) and oral anti-CD3 significantly delayed diabetes development in pre-diabetic hCD20 transgenic NOD mice. More importantly, the combined treatment reversed diabetes in more than 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 enhanced the suppressive function of Treg. Interestingly, the oral anti-CD3 treatment induced a fraction of IL-10-producing CD4 T cells in the small intestine through IL-10 and IL-27-producing dendritic cells. Thus, our findings demonstrated 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 type 1 diabetes.
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 including fever, rash and anemia (5), as well as reactivation of EBV infection (6, 7), were reported. Recently, oral administration of CD3 specific antibody has proven to be an effective strategy to treat autoimmune diseases (8, 9). Interestingly, the side effects of intravenous anti-CD3 treatment were not observed when it was given orally (10). Importantly, the therapeutic effects of oral administration of anti-CD3 monoclonal antibody were demonstrated in both Streptozotocin-induced and spontaneous diabetes mouse models (8, 11).

In addition to the pivotal role of T cells in T1D development, the contribution of B cells to the pathogenesis of T1D is increasingly recognized. B cells are essential for the development of T1D in the NOD mouse model (12-14). B cell-deficient µMT-/- NOD mice fail to develop diabetes (12, 13). Furthermore, several preclinical studies have demonstrated that B cell-targeted therapies can prevent and reverse autoimmune diabetes (15-17). In line with these findings, a recent 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, the 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 have been terminated due to failure to reach their primary endpoints. Given that both T and B cells are essential in the development of T1D, it is very 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 combined treatment using intravenous anti-CD20 and oral anti-CD3 on the prevention and reversal of type 1 diabetes 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 type 1 diabetes in the hCD20/NOD mouse. Mechanistic studies demonstrated that the combined therapy enhanced immune tolerance by improving the Foxp3+ Treg compartment quantitatively and qualitatively, as well as by inducing IL-10 and IL-27-producing dendritic cells to promote the induction of IL-10+ CD4 T cells in the small intestine.

**Research Design and Methods**

*Mice.* Mice used in this study were kept in specific pathogen–free conditions in a 12-hour dark/light cycle and housed in individually ventilated filter cages with autoclaved food at the Yale University animal facility. HCD20/NOD mice were generated as described previously (15). The hCD20/FIR NOD mice were generated by intercrossing hCD20/NOD with Foxp3-IRES-mRFP (FIR) (FIR/NOD) mice (20). The original FIR mice were kindly
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 monoclonal antibody 2H7 was prepared by Bio X Cell Inc. (22). Hamster anti-mouse CD3 monoclonal antibody (clone 145-2C11) and control Hamster IgG were purchased from Bio X Cell Inc. Control mouse IgG used in the in vivo studies was purchased from Rockland Inc. The IL-27 ELISA kit was purchased from eBioscience Inc. IL-10 and TGF-β ELISA kits were obtained from BD Bioscience.

**Anti-hCD20/oral anti-CD3 combination treatment and its effect on spontaneous diabetes development.** Pre-diabetic female hCD20/NOD mice (9 weeks old) were treated with 2H7 (4 intravenous injections from D0 to D9 as described previously (15)) and simultaneously with 5 doses of 0.5 µg/kg anti-CD3 (treated from D0 to D4) daily 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 greater than 250 mg/dl (13.9 mmol/l).

**Treg cell suppression assay.** Bead-purified BDC2.5 TCR transgenic CD4 T cells were used as responder cells in the assay. The CD4 T cells (1 x 10^5/well) were co-cultured with
irradiated bone marrow-derived dendritic cells (BMDC) (1 x 10^4/well), as antigen presenting cells, in the presence of 1 µg of BDC2.5 mimotope (23, 24), at 37ºC, 5% CO₂, for 4 days. ^3H-thymidine was added during the last 16 hours of culture. To study the suppressive function in vitro, Treg cells were purified using a CD4^+CD25^+ Treg purification kit from Stem Cell Inc. Purified Treg were co-cultured 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 RNeasy mini kit (QIAGEN). Complementary DNA was generated using SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. The mRNA levels of Il-10, Il-27, STAT3, STAT4, GATA3, c-Maf, AhR (aryl hydrocarbon receptor) were quantified by quantitative PCR (qPCR) on an iCycler (Bio-Rad). Primer pairs used for the PCR are listed in supplementary table 2.

**Intracellular staining.** Foxp3 staining was performed using a Foxp3 staining kit (eBioscience) following the manufacturer’s instructions. For cytokine staining, 10^6 cells were cultured for 5 hours in the presence of 50 ng/ml PMA (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 minutes at room temperature. After 2 washes with permeabilization buffer (eBioscience), cells were stained with anti-cytokine 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 naive NOD or BDC2.5 NOD mice were cultured together with modified DCs (T : DC = 5 : 1) in the presence of anti-CD3 (1 µg/ml)/anti-CD28 (1 µg/ml) (for NOD naïve CD4⁺) or low affinity mimotope (3 µg/ml) (for BDC2.5 naïve CD4⁺) for 3 d before flow cytometry. Splenic dendritic cells were purified from spleen of control or treated mice using CD11c⁺ purification kit (STEMCELL Inc.). Bone marrow derived dendritic cells were generated by culture of bone marrow cells from control or treated mice in the presence of (20 ng/ml) GM-CSF and IL-4 (100 IU/ml) for 6 days.

**Histopathology and insulitis score.** Pancreata were fixed in 10% buffered formalin and then paraffin-embedded. Tissues were sectioned and stained with H&E. Insulitis was scored under light microscopy using the following grading: 0, no insulitis; 1, insulitis affecting less than 25% of the islet; 2, insulitis affecting 25~75% of the islet; 3, more than 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 1. The statistical analysis was performed with χ² analysis.

**Statistics.** Statistical analysis was performed using GraphPad Prism software. P values of less than 0.05 were 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 had suggested that anti-CD20 monotherapy can prevent type 1 diabetes (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 pre-diabetic female hCD20/NOD mice with 4 doses of mouse anti-hCD20 monoclonal antibody intravenously starting from day 0 to day 9, as described previously (15). Simultaneously, mice were treated with 5 doses of 0.5 µg/kg of hamster anti-mouse CD3 daily by oral gavage from day 0 to day 4. Groups of mice treated with mouse IgG/oral anti-CD3, or 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 treatment were significantly protected from diabetes, only 20% of the mice developed diabetes (Figure 1), although mouse IgG/oral anti-CD3, and anti-hCD20/oral hamster IgG treatment also delayed diabetes development in comparison to the mouse IgG/oral hamster IgG control treatment. Compared to 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 type 1 diabetes.** Previously we showed that anti-CD20 treatment reversed diabetes in about 1/3 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 ranging from 250 to 500 mg/dl), within one week of diagnosis, with anti-hCD20 and/or
oral anti-CD3 ($n = 18$) or control mouse IgG/hamster IgG ($n = 8$) using the same regimen as in the prevention study. Diabetic hCD20/NOD mice were given daily sub-therapeutic subcutaneous insulin while on treatment to maintain the animals in a hyperglycemic state but in relatively good general health. Their blood glucose was monitored every 24 (± 1 to 2) hours, and insulin was withdrawn if blood glucose was less than 250 mg/dl. Of the 18 anti-hCD20/oral anti-CD3 treated diabetic mice, 12 mice (66.67%) demonstrated declining blood glucose and required no further insulin treatment, remaining euglycemic for over one month after treatment (Table 1). Four of these 12 mice remained euglycemic for over 120 days when the experiment was terminated (data not shown). There were 6 mice that 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 was that the islets in these 6 mice had insufficient remaining beta cells, since 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, and all continued to require treatment with insulin (Table 1). The combination treatment greatly increased the disease remission rate compared to 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 control IgGs at different time points (15 days, 1 month and 3 months post treatment). Insulitis scores in these mice are shown in Figure 2. It is interesting that there was a significant reduction of insulitis one 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 the mouse IgG/hamster IgG-treated group (15-day, 
P=0.133; 3-month, P=0.264), although there was a trend toward fewer islets having over 75% 
infiltration in treated mice.

Anti-hCD20/oral anti-CD3 treatment expanded the CD4⁺Foxp3⁺ regulatory T cells (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 suggested 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 to mice treated with mouse 
IgG/oral hamster IgG and mouse IgG/oral anti-CD3 groups, we found significantly more 
CD4⁺Foxp3⁺ Treg in both spleen and pancreatic draining lymph nodes in anti-hCD20 
treated groups, combined either with oral anti-CD3 or hamster IgG (Figure 3A). 
Significantly more CD4⁺Foxp3⁺ Treg were also observed in islet infiltrating cells from 
anti-CD20 treated mice (data not shown). The result indicates that anti-hCD20, rather than 
oral anti-CD3, induced the expansion of CD4⁺Foxp3⁺ Treg, which is consistent with our 
previous studies showing that B cell depletion induced CD4⁺Foxp3⁺ Treg (15) but oral anti-
CD3 treatment did not (25).

Anti-hCD20/oral anti-CD3 treatment improved the function of CD4⁺Foxp3⁺ Treg cells
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 T cell as responder cells in a ratio of 1:5 and 1:2 in the presence of irradiated BMDC and antigenic peptide. As shown in Figure 3B, CD4^+Foxp3^+ Treg 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^+ Treg cells. The improved suppressor function of CD4^+Foxp3^+ Treg by anti-hCD20/oral anti-CD3 treatment was also observed in vivo when CD4^+Foxp3^+ Treg cells were co-transferred into NOD/SCID mice together with splenocytes from diabetic NOD mice (Figure 3C). CD4^+Foxp3^+ Treg purified from treated mice showed significant improvement in its suppressor function, although both control and treated Treg significantly delayed the transferred disease. Moreover, upregulation of CTLA-4 was detected in Treg from mice treated with anti-hCD20/oral anti-CD3, supporting the enhanced function (supplementary figure 1). These results indicated that anti-CD20 treatment quantitatively and qualitatively improved CD4^+Foxp3^+ Treg, while oral anti-CD3 treatment improved the function of CD4^+Foxp3^+ Treg rather than inducing CD4^+Foxp3^+ Treg expansion.

*Anti-hCD20/oral anti-CD3 treatment increased conversion of CD4^+Foxp3^- cells to CD4^+Foxp3^+ Treg cells*
To further understand the mechanism of CD4$^{+}$Foxp3$^{+}$ Treg induction in treated mice, we performed an experiment to test whether the combination treatment induced conversion of CD4$^{+}$Foxp3$^{-}$ into CD4$^{+}$Foxp3$^{+}$ Treg. Since oral anti-CD3 treatment did not significantly induce CD4$^{+}$Foxp3$^{+}$ Treg, we focused on two groups of mice, namely mice treated with anti-hCD20/oral anti-CD3 and mice treated with mouse IgG/oral hamster IgG as a control. We treated a cohort of hCD20/NOD mice with either anti-hCD20/oral anti-CD3 or control IgGs. 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 post treatment. One week after cell transfer, the presence of CD4$^{+}$FIR$^{+}$ Treg in recipients was analyzed by flow cytometry. Interestingly, CD4$^{+}$Foxp3$^{+}$ Treg cells were observed in both treated and control mice. However, more CD4$^{+}$Foxp3$^{+}$ Treg cells were detected in pancreatic draining lymph nodes, but not in spleens, of anti-hCD20/oral anti-CD3 treated recipients, compared to control recipients (Figure 3D). These data indicated that the combined treatment promoted a local niche for the conversion of CD4$^{+}$Foxp3$^{-}$ CD4 T cells into CD4$^{+}$Foxp3$^{+}$ Treg cells.

**TGF-β is required for the increased conversion of CD4$^{+}$Foxp3$^{-}$ cells to CD4$^{+}$Foxp3$^{+}$ Treg cells**

The regulatory cytokine TGF-β is essential for the differentiation of CD4$^{+}$Foxp3$^{+}$ Treg. To understand whether anti-hCD20/oral anti-CD3 treatment promoted TGF-β expression, we performed an ELISA (BD Bioscience) to determine active TGF-β level in sera. In sera from anti-hCD20/oral anti-CD3 treated mice, there was a significantly higher level of TGF-β compared with sera from control mice (Figure 4A). Interestingly, the increase in TGF-β
was only observed at the early time points of 15 and 30 days post treatment (data not shown). We also observed significantly higher IgG2b levels in sera from treated mice, compared to sera from control mice (Figure 4B), which further supported the induction of TGF-β by the combined treatment, since 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 CD4$^+$Foxp3$^+$ Treg compartment, we also tested the effect of anti-CD20/oral anti-CD3 treatment on mucosal immunity. Weiner and colleagues have reported that oral or nasal anti-CD3 can induce CD4$^+$CD25$^-$LAP$^+$ Th3 regulatory cells (9, 25). Interestingly, we did not observe LAP$^+$ Th3 cells in hCD20/NOD mice that had received the combined therapy (data not shown). However, compared to 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 of mice that had received oral anti-CD3 treatment, combined either with mouse IgG or with anti-hCD20 (Figure 5A). Intriguingly, IL-10-secreting CD4 T cells were also induced in luminal cells of small intestines (supplementary figure 2A), and to a lesser extent in spleens (supplementary figure 2B), by anti-hCD20/oral anti-CD3 treatment. Furthermore, significantly more CD4$^+$IL-10$^+$ T cells were also observed in islet infiltrating cells from anti-CD3 treated mice (data not shown). The serum level of IL-10 detected by ELISA further supported the induction of IL-10 by the combined treatment (Figure 5B). A higher level of IgA in sera
from anti-hCD20/oral anti-CD3 treated mice also indicated upregulation of IL-10 in CD4 T cells (Figure 5C) since IL-10 promotes IgA isotype switch in combination with TGF-β (28).

As IL-10+ CD4 T cells from Peyer’s patches were only observed in oral anti-CD3 treated mice, 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. We investigated the gene expression in CD4 T cells from Peyer’s patches. Quantitative PCR result showed that upregulation of *Il-10* was detected in CD4+ T cells from Peyer’s patches of mice treated with anti-hCD20/oral anti-CD3, compared to control mice (Figure 5D). The transcription factor c-Maf is essential for the induction of IL-10 in CD4 T cells (29). Interestingly, CD4+ T cells from treated Peyer’s patches showed a significantly higher level of transcription factor *c-Maf*, compared to control mice, and there was only a marginal increase in *AhR* transcripts (Figure 5E). 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 anti-hCD20/oral anti-CD3 treated mice, we observed upregulation of these three transcription factors in CD4 T cells from Peyer’s patches, in comparison to control mice (Figure 5F and 5G). We also detected a higher level of IL-4 in sera from anti-hCD20/oral anti-CD3 treated mice in comparison with controls, which further supported the upregulation of GATA3 (Figure 5G).

*Combined antibody treatment induced IL-27 and IL-10 producing dendritic cells 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 Tr1 cells (29, 33). IL-27 and IL-10 producing dendritic cells 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 quantitative PCR to analyze the gene expression in dendritic cells from spleen and Peyer’s patches, as well as bone marrow derived dendritic cells. A higher level of Il-10 and Il-27 transcripts was observed in anti-hCD20/oral anti-CD3 treated dendritic cells (Figure 6A). Upregulation of IL-27 was further confirmed at protein level in sera and supernatants from dendritic cell culture by ELISA (Figure 6B).

Since upregulation of IL-10 and IL-27 in dendritic cells of combination antibody treated mice was observed, we speculated that dendritic cells from treated mice could more potently induce IL-10 producing CD4 T cells. To test this hypothesis, we FACS sorted polyclonal naïve CD4 T cells (CD44loCD62LhiCD25-) from NOD mice or oligo/monoclonal naïve CD4 T cells from BDC2.5 mice, and then co-cultured 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 co-culture. Interestingly, both splenic dendritic cells (Figure 6C and 6E) and bone marrow derived dendritic cells (Figure 6D and 6F) from anti-hCD20/oral anti-CD3 treated mice showed enhanced ability to induce IL-10-producing polyclonal (Figure 6C and 6D) or monoclonal
CD4 T cells (Figure 6E and 6F), in comparison with dendritic cells from control antibody treated mice.

Discussion

To further improve the therapeutic efficacy of B cell-directed therapy for type 1 diabetes, in this study 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 type 1 diabetes 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 type 1 diabetes 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 dendritic cells to promote an additional subset of IL-10\(^+\) Tr1 CD4 T cells that were FoxP3\(^-\) in the small intestine. Not only were these cells detectable in the pancreatic lymph nodes, but they were also 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\(^+\) Treg cells. Moreover,
the combined treatment improved the suppressor function of CD4\(^+\)Foxp3\(^+\) Treg.

Interestingly, the expansion of CD4\(^+\)Foxp3\(^+\) Treg was mainly induced by anti-hCD20 treatment, rather than oral administration of anti-CD3. Nevertheless, both the intravenous anti-CD20 and oral anti-CD3 treatment contributed to the enhanced function of CD4\(^+\)Foxp3\(^+\) Treg. The improved function of CD4\(^+\)Foxp3\(^+\) Treg was demonstrated by improved suppression of diabetogenic CD4 T cells \textit{in vitro} and further delay of disease development \textit{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 this study, however, we did not observe any difference in Foxp3 expression between control and treated Treg. Instead, we observed upregulation of CTLA-4 in CD4\(^+\)Foxp3\(^+\) Treg. CTLA-4 has been implicated in 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 (pancreatic lymph nodes and small intestinal lamina propria), rather than downregulation of Foxp3 expression (38). In this study, not only was upregulation of CTLA-4 detected in a proportion of CD4\(^+\)Foxp3\(^+\) Treg, but a population of CD4\(^+\)CTLA-4\(^-\)Foxp3\(^-\) cells were induced in anti-hCD20/oral anti-CD3 treated mice. It implies that not only is CTLA-4 playing a role in enhancing suppressive function of Foxp3\(^+\) Treg from treated mice but the CD4\(^+\)CTLA-4\(^-\)Foxp3\(^-\) cells may also contribute to the reestablishment of immune tolerance, although the latter needs further investigation. This treatment is clearly immunoregulatory and the enhancement of effects are 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 beta cells to recover function. There is no evidence thus far that this treatment stimulates beta cell replication/regeneration. The effects of the treatment are likely to be enhanced by further combination with therapy that could improve beta cell mass.

Oral anti-CD3 has been shown to be effective agent in the treatment of lupus in (NZB × SWR) F1 mice, as well as Streptozotocin-induced autoimmune diabetes in AKR/J male mice, through the induction of CD4⁺CD25⁻LAP⁺ regulatory T cells (8, 9). In the current study, however, we could not detect induction of CD4⁺CD25⁻LAP⁺ regulatory T cells in oral anti-CD3 treated hCD20/NOD mice. The discrepancy observed among these animal models might be due to the genetic background differences, since NOD mice have several defects in the regulation of immune tolerance (2). However, a subset of IL-10-producing CD4 T cells in small intestine was effectively induced by the anti-hCD20/oral anti-CD3 treatment. IL-10-producing CD4 T cells were also induced in spleen of treated mice, albeit to a lesser extent, in contrast to the control spleen. These IL-10 producing CD4 T cells 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. Interestingly, IL-10-producing CD4 T cells in Peyer’s patches could only be detected in groups that were treated with oral anti-CD3, combined either with anti-hCD20 or with 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 indicated that the induction of IL-10-producing CD4 T cells was due to oral anti-CD3 treatment rather than 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 as anti-CD3 injected intraperitoneally
induced IL-10-producing CD4$^+$ T cells and attenuated colitis (39). Taken together, our
findings suggest that oral anti-CD3 acts synergistically with anti-CD20 in the prevention
and reversal of type 1 diabetes by both enhancement of CD4$^+$Foxp3$^+$ Treg function and the
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.

Dendritic cells play a role in induction of IL-10$^+$ CD4 T cells. In this study, we detected
upregulation of IL-27 and IL-10 in dendritic cells upon anti-hCD20/oral anti-CD3
treatment. When cocultured with sorted naïve CD4$^+$ T cells, these treated dendritic cells
showed enhanced ability to induce IL-10 in CD4$^+$ cells, in comparison with control
dendritic cells. Shiokawa and colleagues showed that IL-27 and IL-10 producing dendritic
cells enhanced IL-10 expression in T cells in an oral tolerance model (34). The induction
of IL-10 was further supported by the upregulation of transcription factors STAT3, STAT4,
GATA-3, 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 *Il-10* during Tr1 cell
differentiation (40). Our data further supported the notion that IL-27 from dendritic cells is
a growth and differentiation factor for Tr1 cells through the induction of the transcription
factor c-Maf (29, 33). The mechanism(s) by which anti-CD20 and oral anti-CD3 treatment
induces the expression of IL-27 and IL-10 in dendritic cells is 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 number and function of Foxp3+ Tregs and inducing IL-10+ CD4 Tr1 cells via the induction of IL-10 and IL-27-producing dendritic cells. These findings provide important preclinical evidence for the enhanced therapeutic efficacy of the combined treatment in comparison with anti-hCD20 monotherapy. We also identified mechanisms responsible for the improved prevention and reversal of T1D in NOD mice. These will guide the design and evaluation of novel combinatorial immunotherapy which 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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CY Hu designed and performed the experiments and wrote the manuscript. YD He and XJ Zhang performed the experiments. FS Wong wrote the manuscript. L Wen designed the experiments and wrote the manuscript. L Wen 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. The authors thank Jian Peng (Yale University) for genotyping of all the mice used in this study, Fangyong Li (Yale University) for
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transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol* 183:797-801.
Figure Legends

Figure 1. Anti-hCD20/oral anti-CD3 combined treatment prevents type 1 diabetes. Nine-week old pre-diabetic hCD20/NOD mice were treated with anti-hCD20/oral anti-CD3 or control combinations as described in the Materials and Methods. Glycosuria was monitored twice a week and diabetes was confirmed by blood glucose (≥250 mg/dL). Anti-hCD20 is designated as 2H7, oral anti-CD3 designated as 2C11, hamster IgG as H-IgG, and mouse IgG as mIgG. Statistical analysis was performed with logrank test. *P<0.05, **P<0.001, ***P<0.0001, n.s.: no significant difference.

Figure 2. Anti-hCD20/oral anti-CD3 combined treatment affects insulitis in hCD20/NOD mice. Mice were treated with anti-human CD20/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% and <75% infiltration; 3, >75% infiltration. Islets were examined from 5 euglycemic mice in each group and insulitis was scored in 53-205 islets. The statistical analysis was performed with $\chi^2$ test. *P<0.05, n.s.: not statistically significant.

Figure 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 Treg. Treg from combined 2H7 and 2C11 treated or control treated mice were cocultured with BDC2.5 CD4$^+$ T cells as responder cells at 1:5 and 1:2 ratios for 4 days. One representative experiment of two is
shown. The results are shown as stimulation index, which was calculated as 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 month post treatment, CD4⁺Foxp3⁺ Tregs were purified from splenocytes of anti-hCD20/oral anti-CD3 or control IgG treated mice and adoptively transferred into NOD/SCID mice together with splenocytes from diabetic NOD mice at 1:7 ratio. Diabetes development was monitored as described in Materials and Methods. N=6 each group. The statistical analysis was performed with logrank test. D) Sorted CD4⁺Foxp3⁻ T cells from FIR/NOD mice were adoptively transferred into anti-hCD20/oral anti-CD3 or control mouse IgG/oral hamster IgG treated hCD20/NOD recipients. 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’s t-test. *P<0.05, **P<0.001, n.s.: no significant difference.

Figure 4. Anti-hCD20/oral anti-CD3 combined treatment induces TGF-β production. A) Sera were collected from anti-human CD20/oral anti-CD3 or control mouse IgG/oral hamster IgG treated hCD20/NOD mice 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’s t-test. *P<0.05, **P<0.001.
Figure 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 was detected by ELISA (BD Bioscience Inc). 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 Il-10 transcripts were detected by qPCR in purified CD4 T cells. E) c-Maf and AhR mRNA were detected in purified CD4 T cell 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’s t-test. *P<0.05, **P<0.001, ***P<0.0001.

Figure 6. Anti-hCD20/oral anti-CD3 combined treatment promotes IL-10 and IL-27-producing dendritic cells to induce IL-10 secreting CD4 T cells. A) Il-10 and Il-27 mRNA were determined by qPCR in dendritic cells from anti-hCD20/oral anti-CD3 or control mouse Ig/hamster Ig treated mice. B) IL-27 protein was detected by ELISA from sera of anti-hCD20/oral anti-CD3 or control IgG treated mice and supernatant of bone marrow-derived dendritic cell culture. Splenic dendritic cells from anti-hCD20/oral anti-CD3 or control IgG treated mice (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’s t-test. *p<0.05, **p<0.001, ***p<0.0001
Table 1. Therapeutic effect of anti-CD20/anti-CD3 on type 1 diabetes

Anti-hCD20/oral anti-CD3 combined treatment reverses diabetes in new onset diabetic mice. Newly diagnosed diabetic hCD20/NOD were treated with anti-hCD20/oral anti-CD3 or control IgGs within one week of disease diagnosis, using same regime as in the prevention study.

| Treatment groups            | Treated mice | Euglycemic mice (one month post treatment) | 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               |
Figure 2

- 15 days: n.s.
- 1 month: *
- 3 months: n.s.

Graph showing the percentage of insulitis over time for different groups:
- 2H72C11
- H1G+m16G
- 2H72C11
- H1G+m16G
- 2H72C11
- H1G+m16G

Legend:
- >75%
- <75%
- <25%
- 0
Figure 3

A. Spleen

- **: *p < 0.01
- *: *p < 0.05
- n.s.: not significant

| Treatment          | % of CD4+Foxp3+ in CD4+ cells |
|--------------------|-------------------------------|
| mlgG+IgG           | 10                            |
| 2C11+mlgG          | 20                            |
| 2H7+IgG            | 30                            |
| 2H7+2C11           | 40                            |

B. PLN

- **: *p < 0.01
- *: *p < 0.05
- n.s.: not significant

| Treatment          | % of CD4+Foxp3+ in CD4+ cells |
|--------------------|-------------------------------|
| mlgG+IgG           | 10                            |
| 2C11+mlgG          | 20                            |
| 2H7+IgG            | 30                            |
| 2H7+2C11           | 40                            |

B. B. Stimulation Index

- W/O Treg
- Control
- 2C11+mlgG
- 2H7+IgG
- 2H7+2C11

C. Days post transfer

- Diab-Spl
- Diab-Spl + IgGs-Treg
- Diab-Spl + 2H7/2C11-Treg

D. D. Control IgGs

- Spleen RFP
- PLN RFP

- 2H7+2C11

- Spleen RFP
- PLN RFP

- n.s.

- % of Foxp3+ in CD4+ T cells
Figure 4

A

![Graph A showing TGF-β levels](image)

B

![Graph B showing IgG2a levels](image)
For Peer Review Only

Fig. 5

A

B

C

D

E

F

G

Diabetes
Figure 6

A) Gene expression (fold change)

B) IL-27 in supernatant (pg/ml)

C) IL-10 in sera (pg/ml)

D) % of IL-10+ in CD4+

E) % of IL-10+ in CD4+

F) % of IL-10+ in CD4+
Supplementary figure 1. Anti-hCD20/oral anti-CD3 treatment promotes CTLA4<sup>+</sup> Treg. A) Foxp3 and CTLA4 expression were detected by flow cytometry using a Foxp3 detection kit in splenic CD4<sup>+</sup> T cells of hCD20/NOD mice treated with anti-hCD20/oral anti-CD3 or control IgGs. B) Mean fluorescent intensity of CTLA-4 was measured in splenic CD4<sup>+</sup>CTLA4<sup>+</sup> T cells of hCD20/NOD mice treated with anti-hCD20/oral anti-CD3 or control IgGs (n=3 each group). The statistical analysis was performed with Student’s t-test. ** P<0.01.

Supplementary figure 2. A) Anti-hCD20/oral anti-CD3 treatment induces IL-10-producing CD4<sup>+</sup> T cells in both Peyer’s patches and small intestine luminal cells. Human CD20 transgenic NOD mice were treated with control IgGs, or anti-hCD20/oral anti-CD3, respectively. Peyer’s patches and small intestine luminal cells were collected to analyze IL-10-producing CD4 T cells by intracellular cytokine staining. B) IL-10-producing CD4 T cells were detected in spleens from anti-hCD20/oral anti-CD3 or control IgG treated mice by intracellular cytokine staining.
Supplementary Figure 1

A

Control IgGs

2H7+2C11

9.94 14.9

2.25

16.8 18.3

1.44

B

**

 CTLA-4 MFI

Control IgGs 2H7+2C11

0 50 100 150

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Supplementary Figure 2

A

Control IgGs

2H7+2C11

Peyer’s patch

Control IgGs

2H7+2C11

Small intestine Luminal cells

B

Control IgGs

2H7+2C11

Diabetes
We included online supplementary materials (2 figures and 2 tables) in our manuscript in order to better illustrate our findings and better answer reviewers’ questions.
Supplementary Table 1. Islet number in each group with different insulitis score.

| Insulitis score | Grade 0 | Grade 1 | Grade 2 | Grade 3 |
|----------------|---------|---------|---------|---------|
| 15 days mlIgG + H-IgG | 18 | 8 | 11 | 16 |
| Anti-CD20 + anti-CD3 | 26 | 11 | 14 | 7 |
| 1 month mlIgG + H-IgG | 30 | 17 | 22 | 19 |
| Anti-CD20 + anti-CD3 | 59 | 27 | 13 | 18 |
| 3 month mlIgG + H-IgG | 73 | 34 | 34 | 30 |
| Anti-CD20 + anti-CD3 | 88 | 29 | 38 | 50 |

Supplementary Table 2. Primer sets for qPCR.

| Primer name | Primer sequence |
|-------------|-----------------|
| IL-10-F     | GCAGTCCGCAGCTCTAGG |
| IL-10-R     | TGCTATGCTGCTGCTCTTTA |
| IL-27-F     | GAGGTTCAAGGCTATGTCCA |
| IL-27-R     | GTGGTAGCGAGGAAGCAGAG |
| Stat3-F     | TGTTGTACCTAGGGGCTCT |
| Stat3-R     | TTGGAATCTGAGTGCACTGG |
| Stat4-F     | CATCCCTGAAAACCCTCTGA |
| Stat4-R     | AGCAGACATATGCACCTTG |
| Gata3-F     | GTGCCCGAGTACAGCTCCGGA |
| Gata3-R     | ACCCATGGCGGTGACCATGCTG |
| C-Maf-F     | GAGGAGGGTGATCCGACTGAA |
| C-Maf-R     | TCTCCTGCTGAGGGTGCTCT |
| AhR-F       | AGCAGCTGTGCAATGGTG |
| AhR-R       | CTGAGCAGTCCCTGTAAGC |