Anti-CD20 monoclonal antibody combined with adenovirus vector-mediated IL-10 regulates spleen CD4+/CD8+ T cells and T-bet/GATA-3 expression in NOD mice

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Abstract. Type 1 diabetes (T1D) is an autoimmune disease characterized by a selective destruction of insulin-secreting β-cells. Both T cells and B cells serve a crucial role in pathogenesis and development of T1D. CD20 is a specific membrane antigen of B lymphocytes, while interleukin (IL)-10 is an important cytokine secreted by T helper 2 cells and has a short half-life *in vivo*. The combined effect of anti-CD20 and IL-10 on immune function of mice with T1D remains unknown. In the present study, 30 non-obese diabetic (NOD) mice were treated with anti-CD20 and adenoviral vector-mediated interleukin-10 (Ad-mIL-10) therapy. Alterations in CD4+, CD8+, CD4+CD25+Foxp3+ T cells, T-box expressed in T-cells (T-bet), GATA-binding protein-3 (GATA-3) interferon-γ (IFN-γ) and IL-4 were detected by flow cytometry, reverse transcription-quantitative polymerase chain reaction in NOD mice spleen tissue. The present results suggested that anti-CD20 and IL-10 treatment in NOD mice can modulate the immune functions by upregulating GATA-3 and IL-4 expression as well as downregulating T-bet and IFN-γ expression, which are involved in the pathogenesis of T1D. The current findings may provide a potential method for T1D treatment and a novel preventive therapy for T1D. Combination of anti-CD20 and Ad-mIL-10 treatment had not only immune regulatory effects but also protective effects on islet β-cells in NOD mice with T1DM at the early stages, by regulating T-bet/GATA-3 expression and Th1/Th2 cell differentiation, which has the potential for diabetes prevention and therapy.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by selective destruction of insulin-secreting β-cells in genetically predisposed individuals (1). T1D has been demonstrated to be a T cell-mediated disease. According to the surface membrane expression of CD antigens, mature T lymphocytes are classified into two major subsets, CD4+ and CD8+ lymphocytes. The appropriate quantity and proportion of CD4+/CD8+ T cells serve an important role in immune regulation and maintaining a normal immune system (2). T lymphocytes can also be mainly divided into three subsets by function: T helper (Th) lymphocytes, cytotoxic T lymphocytes (CTL) and regulatory T lymphocytes (Tregs), while dysregulation of CD4+CD25+ regulatory T cells (Tregs) can lead to autoimmune disease (3). A previous study indicated that the quantity and function of Tregs are insufficient in non-obese diabetic (NOD) mice (4).

The CD4+ T cell clones are classified into Th1 and Th2 categories because of non-overlapping secreting patterns of cytokines. Th1 cells predominantly produce interleukin (IL)-2 (IL-2), interferon (IFN)-γ and tumor necrosis factor (TNF)-β, and Th2 cells release the principal cytokines that include IL-4, IL-5 and IL-10. Th1 and Th2 cells are derived from a naive Th precursor (Thp or Th0) cell. The Th0 cells develop into either the Th1 or Th2 subset under the control of antigens, cytokines and transcription factors. Dysfunction of Th cells and the resulting cytokine alterations serve an important role in immune pathogenesis of T1D. Meanwhile, the reversal of Th1/Th2 cell dysfunction can prevent the occurrence of diabetes (5). The transcription factors T-box expressed in T-cells (T-bet) and GATA-binding protein (GATA)-3 are specifically expressed in Th1 cells and Th2 cells, respectively, which are important determinants of Th cell differentiation and are related to immune status change (6,7). IFN-γ and IL-4 are characteristic cytokines of Th1 and Th2 cells, respectively. The secreting levels of IFN-γ and IL-4 can represent the direction of Th1/Th2 cell differentiation. The expression of T-bet/GATA-3 can reflect the relationship between Th1/Th2

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differentiation and the pathogenesis, development and prognosis of autoimmune disease (8).

Moreover, previous studies demonstrate that B cells serve a critical role in many T-lymphocyte-mediated diseases, including the pathogenesis and development of T1D (9-11). CD20 is one of specific membrane antigens of B lymphocytes, involved in B cell activation. B cell-deficient NOD mice are resistant to T1D and treatment with CD20-specific antibody, rituximab, prevents and reverses autoimmune diabetes in the NOD model (12,13). CD20 can regulate the proliferation and differentiation of B cells by modulating transmembrane flow of calcium, and is therefore regarded as a target molecule of therapeutic monoclonal antibodies. Anti-CD20 serves an important role in B cell depletion through the process of antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity and inducing B cell apoptosis (14-16).

IL-10 is an important cytokine secreted by Th2 cells and has a short half-life *in vivo*. IL-10 significantly increases the number of $CD4^+CD25^+$ Treg cells to protect the islet β-cells (17) and $SGAD65_{190-315}$ /IL-10 DNA vaccine had protective effects on T1D by upregulating auto-antigen reactive Tregs (18). Previous research of the authors involved construction of adenovirus-mediated plasmid of IL-10 and confirmed it is involved in preventing the genesis of diabetes at the cellular level and animal experiments (19). Both IL-10 or anti-CD20 have a therapeutic effect on T1D (9,20), however, the co-effect of IL-10 and anti-CD20 treatment remains unknown. C-peptide is a precursor of insulin and forkhead box P3 (Foxp3) is a transcription factor that is involved in the differentiation and function of Tregs. To investigate the combined effect of anti-CD20 and IL-10 on immune function of mice with T1D, NOD mice were treated with anti-CD20 and IL-10 therapy in the current study, serum level of C-peptide, alterations in $CD4^+$, $CD8^+$, $CD4^+CD25^+Foxp3^+$ T cells, T-bet, GATA-3, IFN-γ and IL-4 were detected in the NOD mice spleen tissue.

Materials and methods

Amplification, purification and titration of recombinant adenoviral vector. The adenoviral vector-mediated interleukin-10 (Ad-mIL-10) and recombinant adenovirus expressing enhanced green fluorescence protein (Ad-EGFP), used as a control virus, were successfully established by the authors' laboratory in their previous research (19). The Ad-mIL-10 and Ad-EGFP were amplified, purified and titer detected according to protocols. Briefly, the Ad-mIL-10 and Ad-EGFP were transfected into HEK293 cells using Lipofectamine 2,000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Transfected HEK293 cells (American Type Culture Collection, Manassas, VA, USA) were collected 7-10 days following transfection. The cells were lysed by freezing and thawing four times (alternating between -80°C and 37°C). Viral particles were purified by cesium chloride density gradient centrifugation. Viral particles were detected 1.0×10^{11} pfu/ml and stored at -80°C until thawed for use.

Animals and treatment. A total of 70 female NOD/Ltj mice aged 10 weeks old were obtained from Cavens Laboratory animal Co. Ltd. (Changzhou, China). All mice were kept in specific pathogen-free conditions in a 12-h dark/light cycle and housed in ventilated filter cages at 21-25°C and 56-60% humidity, with free access to food and drinking water. All mice were observed for polyphagia, polydipsia, polyuria, hair loss and decreased vitalities. Their body weight and blood glucose were measured weekly. Diabetes was confirmed by blood glucose measurement (≥13.9 mmol/l). Diabetic NOD mice were administered with daily subcutaneous insulin to keep the blood glucose under 25.0 mmol/l in order to maintain the mice in a hyperglycemic state but in relatively good general health. A total of 30 NOD mice aged 17-20 weeks old were randomly divided into an anti-CD20 group, Ad-mIL-10 group, anti-CD20 + Ad-mIL-10 group, Ad-EGFP group and normal saline (NS) group, with 6 mice in each group. In anti-CD20 group, 500 µg anti-CD20 monoclonal antibody (mAb) was injected through the tail vein on the first day from onset and 250 µg anti-CD20 was injected on days 3, 6 and 9, respectively. In the Ad-mIL-10 group, 0.1 ml Ad-mIL-10 was injected into the caudal vein of mice on days 1, 3, 6 and 9 of onset, respectively. For the Ad-mIL-10 + anti-CD20 group, the two drugs were injected into the caudal vein of mice on days 1, 3, 6 and 9, respectively. The doses of the two drugs were the same as above. In the Ad-EGFP group, 0.1 ml Ad-EGFP was injected into the caudal vein of mice on days 1, 3, 6 and 9 from the onset, respectively. In the NS group, 0.1 ml NS was injected into the caudal vein of mice on days 1, 3, 6 and 9, respectively. All studies were approved by the Institutional Animal Care and Use Committee of Qingdao University (Qingdao, China).

Specimen collection. At 6 weeks following treatment, an intraperitoneal injection of 0.1 ml 10% chloral hydrate was performed in NOD mice. A total of 0.8-1.2 ml whole blood was collected from bilateral eyes with a sterile operation then the serum was isolated and stored at -20°C for ELISA detection. Following blood collection, the mice were decapitated immediately. Part of the spleen tissues were sterilely obtained to prepare a single cell suspension with nylon mesh and then red blood cells were removed with fluorescence-activated cell sorting (FACS) lysis buffer (15 mM NH_4Cl, 1 mM $KHCO_3$, 0.01 mM $EDTA-Na_2$). Another part of the spleen tissues were fixed in 4% neutral formaldehyde for 24-48 h and then paraffin sections (4 µm per section) were obtained performed for immunohistochemistry (IHC). The remainder of the spleen was preserved at -80°C, which was prepared western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

IHC. The expression of CD20 and IL-10 in the spleen was detected by IHC staining with the anti-mouse CD20 monoclonal antibody (dilution, 1:150; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. SC-7735) and anti-mouse IL-10 monoclonal antibody (dilution, 1:300; Abcam, Cambridge, MA, USA; cat. no. Ab189392) as primary antibodies overnight at 4°C using the Biotin-streptavidin-peroxidase procedure (SP kit, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China, cat. no. ZLI-9033), IHC staining was performed. Following 3 extensive washes by PBS, secondary incubation was carried out using biotinylated antibodies for the CD20 monoclonal antibody (Invitrogen, Thermo Fisher

Table I. Comparison of body weight (g) of mice in different groups (mean ± standard deviation, n=6).

Group	At diagnosis	At 2 weeks following diagnosis	At 4 weeks following diagnosis	At 6 weeks following diagnosis
Anti-CD20	25.827±0.664	25.238±1.041	25.455±1.143	26.578±1.256
Anti-CD20+ Ad-mIL-10	25.772±1.100	26.165±1.786	26.457±2.002	26.720±2.201
Ad-mIL-10	25.328±1.176	24.935±1.414	25.673±1.447	26.557±1.478
Ad-EGFP	25.123±1.497	25.837±0.858	26.278±1.379	26.523±1.162
NS	25.295±1.142	24.920±0.637	25.173±1.109	26.578±1.256

P>0.05 vs. Ad-EGFP and NS groups. Ad-mIL-10, adenoviral vector-mediated interleukin-10; NS, normal saline; EGFP, enhanced green fluorescence protein.

Table II. Comparison of serum glucose level (mmol/l) of mice in different groups (mean ± standard deviation, n=6).

Group	At diagnosis	At 2 weeks following diagnosis	At 4 weeks following diagnosis	At 6 weeks following diagnosis
Anti-CD20	17.567±1.063	20.667±2.602[a]	28.167±2.884	29.567±2.421
Anti-CD20+ Ad-mIL-10	17.700±0.724	15.567±1.757[a]	21.350±3.968[a,b]	22.767±2.072[a,b]
Ad-mIL-10	18.133±1.011	21.467±2.032[a]	26.167±5.214	28.250±3.445
Ad-EGFP	17.567±0.985	27.533±1.341	28.550±2.321	29.767±1.344
NS	17.767±0.930	27.683±2.051	28.333±2.422	30.083±1.373

[a]P<0.05 vs. Ad-EGFP and NS groups, [b]P<0.05 vs. anti-CD20 and Ad-mIL-10 groups. Ad-mIL-10, adenoviral vector-mediated interleukin-10; NS, normal saline; EGFP, enhanced green fluorescence protein.

Scientific, Inc., cat. no. SP-9001) and the anti-mouse IL-10 monoclonal antibody (Invitrogen, Thermo Fisher Scientific, Inc., cat. no. PV-9003) respectively for 30 min at 37˚C and incubation with streptavidin conjugated peroxidase complex for 30 min. Finally, sections were visualized by diaminoben-zidine (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) and counterstained with hematoxylin for 2 min in each proce-dure. Simultaneously, negative controls were performed by substituting the primary antibody with a normal serum at the same dilution. The results were evaluated semi-quantitatively under a high-magnification (magnification, x400) light microscope.

Western blot analysis. Spleen tissues were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc.) and the proteins were extracted. The protein concentrations in the samples were determined using a bicin-choninic acid protein assay kit . Equal amounts of proteins (30 μg) from homogenates were resolved using 10% SDS-PAGE prior to being transferred to nitrocellulose membranes. Membranes were blocked in TBS containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk for 2 h at room temperature. Following washing with TBST, the membranes were incu-bated with primary antibodies for CD20 (dilution, 1:300; Santa Cruz Biotechnology, Inc., cat. no. SC-7735) or IL-10 (dilution, 1:1,000; Abcam; cat. no. Ab189392) at 4˚C overnight. Then the membranes were washed again and incubated with the appropriate horseradish peroxidase-conjugated secondary

antibodies (dilution, 1:3,000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., cat. nos. ZB-2306 and ZB-2305) for 1 h at room temperature. The membrane was further washed with TBST for 30 min and incubated with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). β-actin (dilution, 1:1,000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., cat. no. TA-09) was used as an internal control. Immunoreactive proteins were analyzed using ImageJ v1.48 (National Institutes of Health, Bethesda, MA, USA).

ELISA. The concentrations of C-peptide, IL-4 and IFN-γ in serum were measured by ELISA. A commercially avail-able ELISA kit was used to measure the concentrations of C-peptide (BioVision, Inc. San Francisco, CA, USA, cat. no. BIV-K4757-100), IL-4 (cat. no. M4000B) and IFN-γ (cat. no. MIF00) (both from R&D Systems, Inc. Minneapolis, MN, USA) in serum. Each serum sample was run in duplicate. A total of 10 μl serum (dilution, 1:10) were placed into the ELISA plate and incubated for 45 min at 37˚C. Then the plates were washed with buffer and incubated with detection antibodies for 45 min at 37˚C. Following washing four times, the plates were incubated with substrate solution for 15 min at room temperature, then the reaction was stopped and the plates were detected by spectrophotometer at 450 nm wavelength. A standard curve was generated with the provided standards and used to calculate the quantity of C-peptide, IL-4 and IFN-γ in each serum sample. The cytokine contents in the serum were expressed as pg/ml.

Flow cytometry. Single spleen cell suspensions from NOD mice were obtained by grinding with a sterile plunger of syringe and filtering with a 200-mesh nylon screen. The following conjugated antibodies were obtained from eBioscience (Thermo Fisher Scientific, Inc.) to identify the three T cell subsets: Anti-mouse CD3-phycoerythrin (PE) (cat. no. 12-0031-81), CD4-fluorescein isothiocyanate (FITC) (cat. no. 11-0042-81), CD8-FITC (cat. no. 11-0081-81), CD25-PE (cat. no. 12-0251-81) and Foxp3$^+$-Cy5 (cat. no. 15-5773-80). For CD4$^+$ T cells, 1x10^6 spleen cells were stained with CD4-FITC (500 μg/ml) and CD3-PE (200 μg/ml) for 30 min at 4°C, then washed using 0.1% PBS twice and suspended in PBS again for flow cytometry analysis. For CD8$^+$ T cells, 1x10^6 spleen cells were stained with CD8-FITC (500 μg/ml) and CD3-PE (200 μg/ml) for 30 min at 4°C, then washed and suspended for flow cytometry analysis. For CD4+CD25+Foxp3+Tregs, 1x10^6 spleen cells were stained with CD4-FITC (500 μg/ml) and CD25-PE (200 μg/ml) for 30 min at 4°C, then fixed and permeabilized for 40 min, and then stained with Foxp3$^+$-Cy5 for 30 min at 4°C. They were finally washed and suspended for flow cytometry analysis. The stained spleen cells were analyzed with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and data was analyzed with FlowJo software (version, 7.6; FlowJo, LLC, Ashland, OR, USA).

RT-qPCR. RT-PCR was carried out to identify the changes of GATA-3, IL-10, T-bet and IFN-γ mRNA transcription levels in spleen tissues. The primer sequences used were as follows: GATA-3, 5'-CCCATTACCACCTATCC-3' (forward) and 5'-GACTTACATCCGAACCC-3' (reverse); IL-4, 5'-TCCTGCTCTTCTTTCTCG-3' (forward) and 5'-TTCTCCTGTGACCTCGTT-3' (reverse); T-bet, 5'-CCCATTCCTGTCCTTCA-3' (forward) and 5'-GCTGCCTTCTGCCTTTC-3' (reverse), IFN-γ, 5'-CAACAACATAAGCGTCAT-3' (forward) and 5'-CAAACTTGGCAATACTCA-3' (reverse). Extraction of mRNA from spleen tissue was prepared by using the Takara Code D9108B kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Subsequently, RNA quantity and purity was determined NanoDrop 2,000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The PCR conditions were 95°C for 10 min, 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Following amplification, melting curve analysis was performed to determine the specificity of the PCR products. The relative quantitative results of GATA-3, IL-4, T-bet and IFN-γ genes were also calculated by the 2$^{-\Delta\Delta Cq}$ relative quantitative formulas (21).

Statistical analysis. Data was expressed as mean ± standard deviation. Statistical analysis was performed with SPSS software (version, 17.0; SPSS, Inc., Chicago, IL, USA). Statistically significant differences were determined by Student's t test and one-way analysis of variance followed by the LSD post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Body weight and blood glucose. The diabetic mice ate more food, drank more water and purged more urine. The body weight of mice in different groups at onset stage of the disease
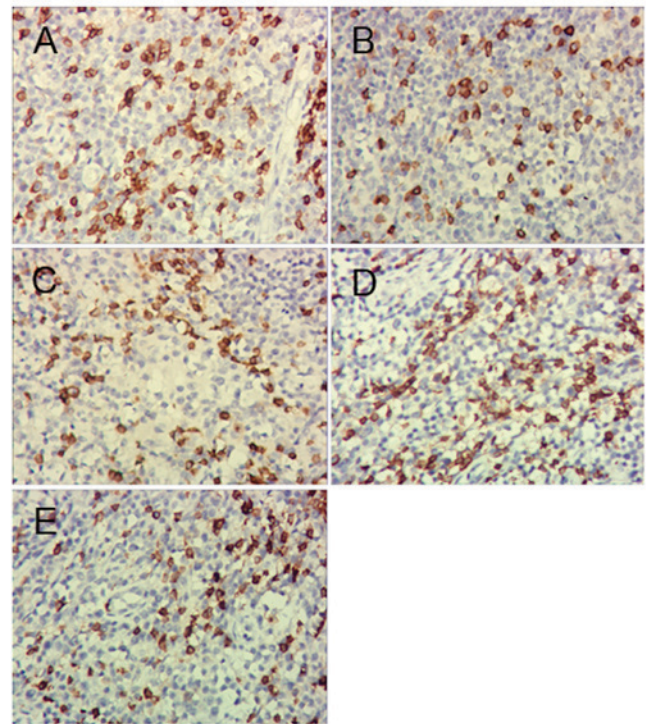

Figure 1. Immunohistochemistry results demonstrating CD20 expression in the spleen. (A) Anti-CD20 treatment, (B) anti-CD20 and Ad-mIL-10 treatment, (C) Ad-m IL-10 treatment, (D) NS treatment and (E) Ad-EGFP treatment (magnification, x400). The CD20 expression of splenocytes in mice from was examined by immunohistochemical staining to evaluate the B cell depletion effect of the anti-CD20 monoclonal antibody. The positive reactivity pattern of the CD20 was located in the cytoplasmic membrane, appearing as brown granules. The proportion of CD20 positive cells of different groups were calculated and compared. P>0.05 vs. Ad-EGFP and NS groups, P>0.05 vs. anti-CD20 and Ad-mIL-10 groups. Ad-mIL-10, adenoviral vector-mediated interleukin-10; IL-10, interleukin-10; NS, normal saline; EGFP, enhanced green fluorescence protein.

showed no significant difference (F=1.28, P=0.943). Their body weight increased slightly during 6 weeks following injection. Compared with mice in Ad-EGFP and NS control groups, mice in three treatment groups showed no significant difference in body weight at the end of 6 weeks (F=0.016, P=0.997; Table I).

Before injection, the average random blood glucose of mice in all groups was similar (F=0.436, P=0.729). Following injection, the average random blood glucose of mice in both anti-CD20 and Ad-mIL-10 group increased, but the blood glucose level was lower than that of mice in two control groups 2 weeks following diagnosis, and the blood glucose level of combined anti-CD20 + Ad-mIL-10 group decreased and was lower than that of mice in anti-CD20 and Ad-mIL-10 group (F=18.540, P<0.001). Following this point, the blood glucose of all mice increased. At 6 weeks following injection, compared with Ad-EGFP and NS groups, the blood glucose level reported no significant difference in mice of the anti-CD20 or Ad-mIL-10 groups. However, the blood glucose level of combined anti-CD20 + Ad-mIL-10 group was still lower than any other group (F=12.006, P<0.001; Table II).

The expression of CD20 in spleens. The CD20 expression of splenocytes in mice was examined by IHC staining

Table III. Comparison of levels of CD20 and IL-10 using immunohistochemistry (mean ± standard deviation, n=6).

Group	CD20 (%)	IL-10 (%)
Anti-CD20	25.67±7.12	25.83±2.32[a]
Anti-CD20 + Ad-mIL-10	24.33±5.13	38.83±3.25[a,b]
Ad-mIL-10	25.33±3.93	33.83±3.31[a]
Ad-EGFP	27.07±5.64	19.00±1.79
NS	26.50±6.38	19.00±2.90

P>0.05 compared with Ad-EGFP and NS groups; [a]P<0.05 vs. Ad-EGFP and NS groups; [b]P<0.05 vs. anti-CD20 and Ad-mIL-10 groups. IL, interleukin; EGFP, enhanced green fluorescence protein; Ad-mIL-10, adenoviral vector-mediated interleukin-10.
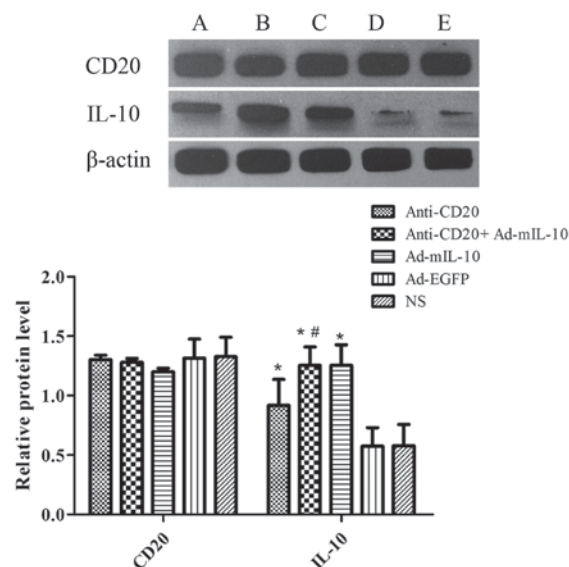

Figure 2. Western blotting results of the spleen. (A) Anti-CD20 treatment, (B) anti-CD20 and Ad-mIL-10 treatment, (C) Ad-mIL-10 treatment, (D) NS treatment and (E) Ad-EGFP treatment. The CD20 and IL-10 expression of spleen cells from different groups was examined by western blotting and compared. *P<0.001 vs. Ad-EGFP and NS groups, #P<0.001 vs. anti-CD20 and Ad-mIL-10 groups. Ad-mIL-10, adenoviral vector-mediated interleukin-10; NS, normal saline; IL-10, interleukin-10; EGFP, enhanced green fluorescence protein.

and Western Blot to evaluate the B cell depletion effect of anti-CD20 mAb. As shown in Fig. 1, the IHC positive reactivity pattern of the CD20 was located in the cytoplasmic membrane, appearing as brown granules. After calculating the proportion of CD20 positive cells, there was no significant difference among the anti-CD20 group, Ad-mIL-10 group, anti-CD20 + Ad-mIL-10 group, Ad-EGFP group and NS group (F=1.274, P=0.301; Table III). The results of CD20 expression assayed by western blotting presented no significant difference between the five groups either (F=0.053, P=0.983; Fig. 2).

The expression of IL-10 in spleens. IL-10 expression of splenocytes in mice was also examined by IHC staining and western blotting to evaluate the transfection effect of Ad-mIL-10 *in vivo*. As presented in Fig. 3, the proportion of IL-10 positive spleen cells that present brown granules in the cytoplasm was calculated. Comparing to the control Ad-EGFP and NS group, IL-10 expression increased significantly, not only in the transfected Ad-mIL-10 group and anti-CD20 + Ad-mIL-10 group, but also in the non-transfected anti-CD20 group. In addition, the IL-10 level of anti-CD20 + Ad-mIL-10 group was significantly higher than that of the anti-CD20 group and Ad-mIL-10 group (F=96.292, P<0.001; Table III). No difference was observed between Ad-EGFP and NS groups (P=1.000). The result was also confirmed by the examination of western blotting (F=10.063, P<0.001; Fig. 2).

The proportion of CD4^{+}, CD8^{+} and CD4^{+}CD25^{+}Foxp3^{+} T cells in spleens. Three subsets of T cells from spleen in mice were identified by flow cytometry to evaluate the regulatory effects of the combined treatment group. The proportion of CD4^{+}, CD8^{+} and CD4^{+}CD25^{+}Foxp3^{+} T cells demonstrated a great increase in the spleens of treatment groups. In addition, the proportion of the three subsets of T cells in the combined treatment group increased significantly comparing to the two single treatment groups, which had a statistically significant difference. (F=23.71, P<0.001; F=41.806, P<0.001; F=8.386, P=0.001, respectively). No significant difference was identified between the anti-CD20 group and Ad-mIL-10 group (P>0.05). Furthermore, the ratio of CD4^{+}/CD8^{+} T cells in the treatment
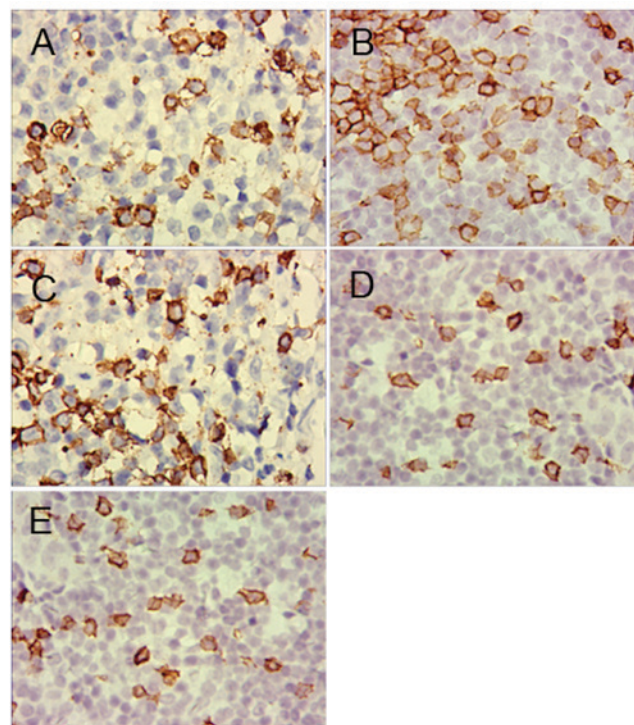

Figure 3. Immunohistochemistry results demonstrating IL-10 expression in the spleen. (A) Anti-CD20 treatment, (B) anti-CD20 and Ad-mIL-10 treatment, (C) Ad-mIL-10 treatment, (D) NS treatment and (E) Ad-EGFP treatment (magnification, x400). The IL-10 expression of splenocytes was examined by IHC staining to evaluate the transfection effect of Ad-mIL-10 *in vivo*. The IL-10 positive cells are represented by brown granules in the cytoplasm. The proportion of IL-10 positive spleen cells of different groups were calculated and compared. *P<0.001 vs. Ad-EGFP and NS groups, #P<0.001 vs. anti-CD20 and Ad-mIL-10 groups. IL-10, interleukin-10; Ad-mIL-10, adenoviral vector-mediated interleukin-10; EGFP, enhanced green fluorescence protein; NS, normal saline.

Table IV. Comparison of levels of serum IFN-γ, IL-4, IL-10 and C-p (mean ± standard deviation, n=6).

Group	IFN-γ (pg/ml)	IL-4 pg/ml	IL-10 (pg/ml)	C-p (pg/ml)
Anti-CD20	48.567±1.129[a]	47.718±1.150[a]	33.301±0.916[a]	9.207±0.326[a]
Anti-CD20 + Ad-mIL-10	45.784±0.920[a,b]	51.674±1.349[a,b]	35.814±0.887[a]	10.240±0.203[a,b]
Ad-mIL-10	50.139±0.498[a]	48.141±1.704[a]	35.519±1.008[a]	9.006±0.261[a]
Ad-EGFP	72.706±2.609	43.172±2.211	30.334±1.380	7.169±0.384
NS	72.297±2.053	43.693±2.392	30.288±1.088	7.269±1.059

[a]P<0.05 vs. Ad-EGFP and NS groups, [b]P<0.05 vs. anti-CD20 and Ad-mIL-10 groups. IFN-γ, interferon-γ; IL, interleukin; C-p, C-peptide; NS, normal saline; IL, interleukin; EGFP, enhanced green fluorescence protein; Ad-mIL-10, adenoviral vector-mediated interleukin-10.

Table V. Comparison of levels of T-bet, GATA-3, IFN-γ and IL-4 mRNA (mean ± standard deviation, n=6).

Group	T-bet	GATA-3	IFN-γ	IL-4
Anti-CD20	0.846±0.017[a]	1.386±0.028[a]	0.812±0.028[a]	1.339±0.019[a]
Anti-CD20 + Ad-mIL-10	0.706±0.028[a,b]	1.858±0.037[a,b]	0.602±0.016[a,b]	1.564±0.021[a,b]
Ad-mIL-10	0.807±0.018[a]	1.376±0.033[a]	0.730±0.021[a]	1.043±0.023[a]
Ad-EGFP	1.700±0.049	0.734±0.033	1.763±0.043	0.752±0.022
NS	1.708±0.038	0.722±0.025	1.768±0.040	0.740±0.046

[a]P<0.05 vs. Ad-EGFP and NS groups; [b]P<0.05 vs. anti-CD20 and Ad-mIL-10 groups. NS, normal saline; IL, interleukin; EGFP, enhanced green fluorescence protein; Ad-mIL-10, adenoviral vector-mediated interleukin-10; IFN-γ, interferon-γ; T-bet, T-box expressed in T-cells; GATA-3, GATA-binding protein-3.

groups significantly decreased, compared with the control Ad-EGFP and NS group (F=19.513, P<0.001). However, there was no significant difference among the single and combined treatment groups (P>0.05; Fig. 4).

The serum level of IFN-γ, IL-4, IL-10 and C-peptide. The levels of serum IFN-γ, IL-4, IL-10 and C-peptide in each group were measured by ELISA and compared. Compared with that of mice in Ad-EGFP and NS groups, the serum IFN-γ level substantially decreased in the treatment groups (Table IV). Meanwhile, the IFN-γ level in the anti-CD20 + Ad-mIL-10 combined treatment group was also significantly lower than the CD20 treatment group and IL-10 treatment group (F=125.003, P<0.001; Table IV). By contrast, IL-4 level in the anti-CD20 + Ad-mIL-10 treatment group was statistically higher than the single treatment groups and the levels of IL-4 in single treatment groups were also remarkably higher than the Ad-EGFP and NS groups (F=13.084, P<0.001; Table IV). In addition, serum IL-10 levels of mice in three treatment groups were significantly higher than that of mice either in Ad-EGFP or in NS group (F=27.976, P<0.001). There was no significant difference in serum IL-10 level between mice in Ad-mIL-10 group and anti-CD20 + Ad-mIL-10 group (P>0.05). Compared with the Ad-EGFP and NS groups, the serum C-peptide level substantially increased in the treatment groups (Table IV). Meanwhile, C-peptide level in the anti-CD20 + Ad-mIL-10 combined treatment group was also significantly higher than the CD20 treatment group and IL-10 treatment group (F=14.909, P<0.001; Table IV).

The mRNA expressions of T-bet, GATA-3, IFN-γ and IL-4. The mRNA levels of T-bet, GATA-3, IFN-γ and IL-4 were detected by RT-qPCR to reflect the differentiation of Th1/Th2 cell and compared. The results indicated that the treatment groups had a significantly lower expression level of T-bet mRNA than the Ad-EGFP and NS groups. Especially, the anti-CD20 + Ad-mIL-10 group decreased significantly compared to the single treatment groups (F=1990.329, P<0.001; Table V). All the treatment groups demonstrated higher mRNA expressions of GATA-3 than the Ad-EGFP and NS groups. GATA-3 mRNA in anti-CD20 + Ad-mIL-10 group was also higher than anti-20 group and Ad-mIL-10 group (F=48.813, P<0.001, Table V). It was also noticed that mRNA expression of IFN-γ was similar to the T-bet and presented a significant difference among treatment and control groups (F=2711.293, P<0.001, Table V). Additionally, the mRNA expression level of IL-4 was similar to GATA-3, which presented the statistical significance among the treatment and control groups (F=1339.450, P<0.001, Table V).

Discussion

CD20 is a specific marker of B cells and is expressed at various stages of B cells, except plasma cells. B lymphocytes promote autoimmunity and participate in T1D genesis through different pathways, such as producing a variety of autoantibodies, maintaining memory of CD4+ T cells, as well as secreting cytokines to regulate the development, proliferation and differentiation of Th1/Th2 cell subgroups (22). A previous study reported that
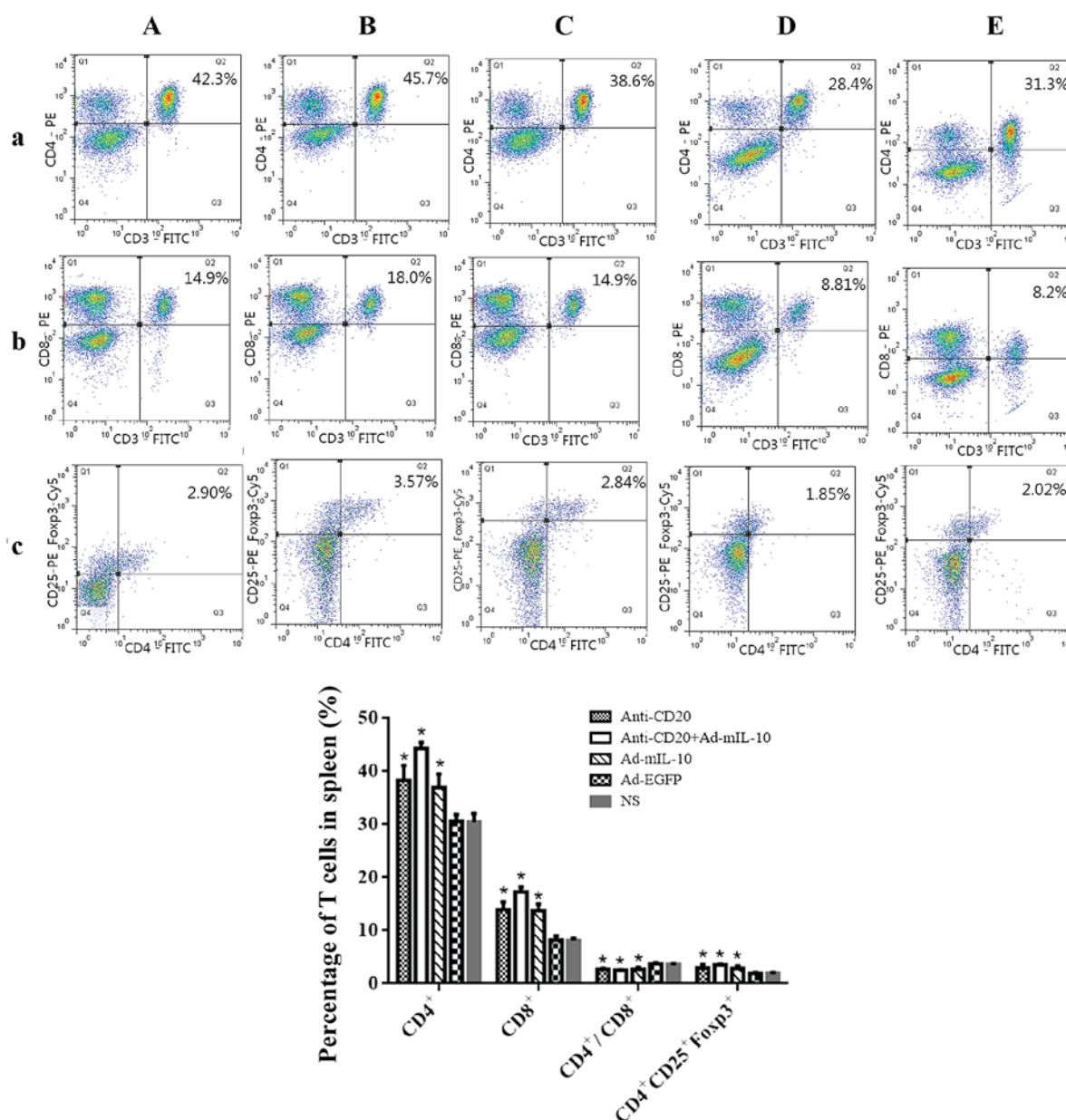
Figure 4. Flow cytometric analysis of CD4^{+}, CD8^{+} and CD4^{+}CD25^{+}Foxp3^{+} T cells in spleens. (A) The proportion of CD4^{+} T cells. (B) The proportion of CD8^{+} T cells. (C) The proportion of CD4^{+}CD25^{+}Foxp3^{+} T cells. (A) Anti-CD20 treatment, (B) anti-CD20 and Ad-mIL-10 treatment, (C) Ad-mIL-10 treatment, (D) NS treatment and (E) Ad-EGFP treatment The proportion of CD4^{+}, CD8^{+} and CD4^{+}CD25^{+}Foxp3^{+} T cells of spleen in mice was analyzed by flow cytometry to evaluate the regulatory effects of treatment group. Both the proportion of three T cell subsets and the ratio of CD4^{+}/CD8^{+} from different groups were compared. *P<0.001 vs. Ad-EGFP and NS groups. Ad-mIL-10, adenoviral vector-mediated interleukin-10; NS, normal saline; EGFP, enhanced green fluorescence protein; FITC, fluorescein isothiocyanate.

anti-CD20 treatment can lead to B cell depletion and reverse the process of hyperglycemia in mice (23). In the present study, a combination of anti-CD20 and Ad-mIL-10 was used to treat NOD mice and the expression CD20 was examined to evaluate the B cell depletion effect of anti-CD20 mAb, the results indicated that CD20 expression levels in mice spleen of treatment group was slightly lower than the NS group following 7 weeks treatment, but there was no statistical significance. The research of Xiang Y (23) demonstrated that the B cell depletion began to be seen in blood circulation on 2nd day and in the spleen on 7th day after anti-CD20 mAb therapy. The efficiency of B cell depletion was different in different issues such as peripheral blood, mesenteric lymph nodes, axillary

lymph nodes and bone marrow, and which was relatively low in the spleen. The shortest time before B cells began to appear and then gradually recovering to a normal level following depletion was about 21 days. The current results showing no difference in CD20 expression in treatment groups may be related to the gradual recovery of B lymphocytes following depletion.

In the present experiment, the level of IL-10 was very high both in the Ad-mIL-10 and in the combined treatment groups, which indicated that the adenovirus-mediated IL-10 gene was successfully introduced into NOD mice *in vivo*. IL-10 expression in the spleen was also increased in the anti-CD20 treatment group, which was possible a result of low levels

of B lymphocytes following B cell depletion. A previous study indicated that therapeutic B cell depletion resulted in CD4$^+$CD25$^+$Treg cell expansion, which could induced IL-10 expression (24). The present research reported that IL-10 expression in the spleen of the combined treatment group was significantly higher than the monotherapy groups, suggesting combination of anti-CD20 and IL-10 has a synergistic effect on increasing the local expression and function of IL-10. This is consistent with a previous study (24), which suggested that combination of anti-CD20 and anti-CD3 therapy can significantly increase serum IL-10 expression levels in new-onset diabetes in mice.

CD4$^+$CD25$^+$Treg cells are the most specific subset of Tregs, and Foxp3 is a transcription factor that is involved in the differentiation and function of Tregs. The present research indicated that the proportion of CD4$^+$CD25$^+$Foxp3 T cells was significantly increased in three treatment groups. B cell depletion was thought to be the main reason for Tregs increase in anti-CD20 treatment group, but how the depletion B cells lead to development of Tregs remains unknown (17). As for the IL-10 group, some researchers identified that IL-10 could protect β-cells by improving CD4$^+$CD25$^+$ Treg cell compartment quantitatively and qualitatively (17). The current study also suggested that combination therapy can improve the number of CD4$^+$CD25$^+$ Treg cells in the spleen of NOD mice, which was confirmed by another study and may be associated with local overexpression of IL-10 (17).

In the pathogenesis of T1D in NOD mice, although the spleen T cells are reduced in the insulitis stage and increased in the hyperglycemia stage, the ratios of CD4$^+$/CD8$^+$ continuously increased and there is always an imbalance of CD4$^+$/CD8$^+$ T lymphocytes (25). The current research demonstrated that CD4$^+$ and CD8$^+$ T cells increased in treatment groups but the ratios of CD4$^+$/CD8$^+$ decreased significantly. It is thought the increased proportion of CD8$^+$ is related to the overexpression of local IL-10, through which the immune system was suppressed by inducing and activating CD8$^+$CD28$^+$ T cells in other experiments (25). Although CD4$^+$ T cells increased, the ratios of CD4$^+$/CD8$^+$ decreased and the proportion of CD4$^+$CD25$^+$ Tregs relatively increased, which indicated that the tolerance immune pathway in favor of CD4$^+$/CD8$^+$ balance is enhanced in treated NOD mice.

The ratio of Th1/Th2 imbalance plays a role in tumor cells and autoimmune diseases, including in T1D pathogenesis. Reversing the Th1/Th2 imbalance can be beneficial to the prevention and treatment of T1D (26). T-bet is one of the susceptibility genes of T1D and variation in T-bet transcriptional activity may involve in the development of T1D, possibly through the effect on IFN-γ production in Th1 cells (27). IL-10 may have immunosuppressive activity and anti-inflammatory effects which can reduce expression of MHC-II, inhibit the production and release of IL-2, IFN-γ and other pro-inflammatory cytokines as well as suppress Th1 cells proliferation and cytokine production (28). In the current study, spleen mRNA levels of T-bet and IFN-γ mRNA significantly decreased while GATA-3 and IL-4 mRNA expressions significantly increased following anti-CD20 and Ad-mIL-10 treatment. Serum IFN-γ levels reduced while levels of IL-4 were increased and the difference was significant. These results indicated that there is an imbalance of Th1/Th2 and

Th1 cells are in predominance in NOD mice. The reducing IFN-γ levels and increased levels of IL-4 suggested that the imbalance of Th1/Th2 cells was reversed after anti-CD20 and Ad-mIL-10 therapy. Anti-CD20 and Ad-mIL-10 combination therapy can reduce the transcriptional activity of T-bet and inhibit the Th0 cells differentiating into Th1 cells then reverse the Th1 cells immune deviation due to IFN-γ gene activation and increased secretion, which is related to IL-10 overexpression in the spleen.

When the NOD mice diagnosed with diabetes, they had already had destructive insulitis for several weeks and most islet β-cells had been damaged (29). The diabetic mice presented hyperglycia, polyphagia, polydipsia and polyuria. Dynamic observation of blood glucose levels suggested that the blood glucose level of combined anti-CD20 + Ad-mIL-10 group decreased and was lower than that of mice in anti-CD20 and Ad-mIL-10 group, although the average random blood glucose of mice in both anti-CD20 and Ad-mIL-10 group increased, it was lower than that of mice in two control groups 2 weeks following injection (P<0.001). At 6 weeks following injection, only the blood glucose level of combined anti-CD20 + Ad-mIL-10 group was still lower than any other group and the blood glucose level showed no significant difference in mice of anti-CD20 or Ad-mIL-10 group (P<0.001), indicating that the combined anti-CD20 and Ad-mIL-10 intervention could not prevent or reverse but could alleviate the progression of diabetes in NOD mice. C-peptide is a precursor of insulin. The secretion level of C-peptide directly reflects the functions of pancreatic β-cells. In the current study, the serum C-peptide level was examined through ELISA and the C-peptide level of mice in combined treatment group was significantly elevated than anti-CD20 and IL-10 groups. In addition, the C-peptide level of mice anti-CD20 and Ad-mIL-10 groups was higher than that of control groups, suggesting that both anti-CD20 and IL-10 could protect pancreatic β cells and the combined therapy had enhanced protection efficacy.

In summary, the anti-CD20 and Ad-mIL-10 treatment enhanced the expression of IL-10 in spleen and serum, which improved the proportion of CD4$^+$CD25$^+$ Treg cells in spleen, up-regulated GATA-3 and IL-4 expression as well as downregulated T-bet and IFN-γ expression, and increased serum C-peptide level, thus rebalanced Th1/Th2 subsets and protected β-cells in NOD mice. Although the combined treatment could not reverse the diabetic process, it improved the blood glucose in NOD mice, which indicated its effect of alleviating the diabetic process. The current findings may provide a potential method for T1D treatment. In future studies, the authors intend to further investigate the clinical utility of the combined treatment by dynamically observing the regulatory effect of the combined therapy on islet β-cells in NOD mice.

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