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Original Article

A tolerogenic peptide hCDR1 aggravates lupus in chronic graft-versus-host model of systemic lupus erythematosus

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Abstract: The hCDR1 is a synthetic peptide based on the complementarity determining region 1 of an autoantibody and ameliorates serological and clinical manifestations of lupus in NZB/W F1 mice. This study aimed to determine the potential effects of hCDR1 in SLE-like chronic graft versus host disease (cGVHD) mouse model. We found that hCDR1 administrated by gavage had no significant effect on the disease progression in SLE-cGVHD mice. However, hCDR1 administrated by subcutaneous injection exacerbated the lupus-like disease manifested by earlier onset of proteinuria, elevated serum levels of autoantibodies, more immune complex deposition in the kidney and severe kidney injury in SLE-cGVHD mice. SLE-cGVHD mice that received hCDR1 by subcutaneous injection also exhibited significant increase of CD4+Treg cells proportion. This study demonstrated that hCDR1 administrated through subcutaneous injection but not gavages slightly aggravated the disease in SLE-cGVHD.

Keywords: hCDR1, chronic graft-versus-host disease, systemic lupus erythematosus, CD4+Treg cells

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease involving multi organs characterized by the presentation of autoantibodies against nuclear antigens [1]. The classical therapy regimens of SLE are mainly based on the suppression of the whole immune system and thus have significant adverse effects [2]. To overcome the shortcomings of conventional therapy, recent studies have focused on the antigen-specific treatments of SLE. The hCDR1, a peptide based on the complementarity determining region (CDR) 1 of a human monoclonal anti-DNA autoantibody that bears the 16/6 idiotype (16/6Id) was designed and synthetized as a potential candidate for the antigen specific treatment for SLE [3, 4]. It was well demonstrated that weekly subcutaneous injections of hCDR1 (50 μg/mouse) could significantly ameliorate the manifestations in (NZB × NZW) F1 mice [3-5].

(NZB × NZW) F1, one of the oldest spontaneous lupus models, has been widely used to evaluate the efficacy of new drugs for SLE. However, (NZB × NZW) F1 model could only partially reflect human SLE due to the complex pathologies of human SLE [6, 7]. The SLE-like chronic graft-versus-host-disease model (cGVHD) characterized by autoantibody production, immunocomplex deposition and proteinuria is a classical induced model for lupus [6-9]. Unlike (NZB × NZW) F1 mice which are intrinsically 'autoimmune' and develop lupus-like manifestations spontaneously, the SLE-cGVHD model is induced in normal (C57BL/6 × DBA/2) F1 mice by the inoculation of homozygous parental DBA/2 lymphocytes cells. The pathogenesis of SLE-like cGVHD model involves the cognate recognition of foreign MHC class II of host B cells by alloreactive CD4+T cells from the donor [10-12]. The different pathological mechanism of NZB/W F1 and SLE-like cGVHD models means that we could complement these models to investigate new treatments for SLE.

It has been well established that hCDR1 could restore immune tolerance to lupus associated immune responses and ameliorate the clinical manifestations in (NZB × NZW) F1 mice. The diminished production of pathogenic cytokines

and up-regulated CD4+regulatory T cells (Treg) have been shown to play important roles in the ameliorating effects of hCDR1 [5, 13-16]. However, up to now the effect of hCDR1 on inducible SLE-cGVHD model of SLE remains unclear. Therefore, this study aimed to investigate the impact of hCDR1 on SLE-cGVHD model of SLE.

In this study, SLE-cGVHD was induced in (C57BL/6 × DBA/2) F1 mice by the injection of parental DBA/2 lymphocytes cells. hCDR1 was administrated to cGVHD mice through gavage and subcutaneous injection respectively, and disease severity was evaluated based on proteinuria, serum antinuclear antibodies and renal pathology. To elucidate the mechanisms underlying the effects of hCDR1 on SLE manifestations in SLE-cGVHD mice, the proportion of CD4+IFN-γ+ cells, CD4+IL-10+ cells and CD4+CD25+Foxp3+ cells in splenic lymphocytes were further analyzed by flow cytometry.

Materials and methods

Experimental animals

Female DBA/2 and (C57BL/6 × DBA/2) F1 mice (6-8 weeks old) were purchased from VitalRiver Experimental Animal Center (Beijing, China). Mice were housed in specific pathogen-free conditions (12 h light-dark cycle, 22 ± 1°C, and 55 ± 5% humidity). All animal procedures were approved by the institutional ethics committee for animal experiments of Xi'an Jiao tong University. All surgical and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of National Institutes of Health revised in 2011.

Reagents

hCDR1 (GYYWSWIRQPPGKGEEWIG) was synthesized by Invitrogen (Xi'an, Shaanxi, China). FITC-conjugated goat anti-mouse IgG was from ZSGB-bio (Beijing, China). Calf thymus DNA, Histone from calf thymus and 3, 3', 5, 5-tetramethylbenzidine (TMB) were from Sigma (St. Louis, MO, USA). Goat anti-mouse IgG and HRP-conjugated goat anti-mouse IgG were purchased from Bioss (Beijing, China). Anti-Mouse CD25 APC, Anti-Mouse CD4 FITC, Anti-Foxp3 PE, Anti-Mouse IL-10 PerCP-Cyanine5, Anti-Mouse IFN-γ APC, Mouse IgG1 K Isotype Control PE, Rat IgG2b k Isotype PerCP-Cyanine5, Rat IgG1 k Isotype APC, IC Fixation Buffer, Permeabilization buffer, Foxp3/Transcription Factor Fixation/Permeabilization, staining buffer and Cells Stimulation Cocktail (plus protein transport inhibitors) (500X) were all purchased from eBioscience (San Diego, CA, USA).

Induction of SLE-like cGVHD and treatment with hCDR1

SLE-cGVHD was induced in (C57BL/6 × DBA/2) F1 mice by the injection of lymphocytes from DBA/2 mice. Briefly, the spleen and thymus of DBA/2 mice were removed sterilely and ground into suspension in PBS. The suspension was adjusted to cell concentration of 2.5×10^8/ml. On days 1, 4, 7, and 10, 5×10^7 lymphocytes from DBA/2 mice or 0.2 ml PBS were injected into (C57BL/6 × DBA/2) F1 mice through the tail vein. The seven (C57BL/6 × DBA/2) F1 mice receiving PBS injection served as normal control group. The 24 (C57BL/6 × DBA/2) F1 mice which had received lymphocytes injection were divided randomly into three groups (n=8): cGVHD group, oral hCDR1-GVHD group receiving hCDR1 through gavages, sub hCDR1-GVHD group receiving hCDR1 through subcutaneous injection. hCDR1 was dissolved in PBS and given to mice (50 μg/mouse) each week after the induction of cGVHD. The mice in normal control group and cGVHD group were given the same dose of PBS.

Proteinuria

Proteinuria was measured by a standard semi-quantitative test using an albustix kit from Global Biotech (Guangzhou, China) every week after the induction of cGVHD. Urinary protein level was scored according to the following criteria: 0, ≤10 mg/dl; 1+, 10 mg/dl-30 mg/dl; 2+, 30 mg/dl-100 mg/dl; 3+, 300 mg/dl-1000 mg/dl; and 4+, >1000 mg/dl. Severe glomerulonephritis was confirmed if the score was ≥3.

Measurement of serum autoantibodies and Igs

Serum was taken through angular vein before cGVHD induction and every three weeks after cGVHD induction. Anti-dsDNA antibody, anti-histone antibody and the total IgG were measured using ELISA. Briefly, microtiter plates were coated with calf thymus DNA, calf thymus

Figure 1. Effects of hCDR1 on proteinuria in SLE-like cGVHD mice. A. The incidence of proteinuria in different groups. B. Urinary protein level at 5th weeks after cGVHD induction. C. Urinary protein level at 8th week after cGVHD induction. D. Urinary protein level at 9th week after cGVHD induction. In B-D, each symbol represented one mouse.

histone or goat anti-mouse IgG, respectively. 3% BSA, diluted serum, HRP-conjugated goat anti-mouse IgG, TMB and 2 M H_2SO_4 were added in order. Then the plates were read at 450 nm in a Titertek Multiskan photometer. For anti-dsDNA antibody detection, the plates were pre-coated with 100 μg/ml Poly-D-Lysine. Mouse serum was diluted at 1:20 for anti-dsDNA antibody detection. For anti-histone antibody examination, the serum was diluted 1:40 and total IgG was diluted 1:40,000.

Renal histopathology

The mice were killed and kidneys were removed sterilely for histological evaluation. The left kidney was fixed in 4% formalin and then embedded in paraffin. 5 μm sections of the kidney were stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS). The damage of the kidney was assessed blindly using the following scale: 0, normal morphology; 1, moderate expansion of the glomerular matrix without glomerulonephritis; 2, mild glomerulonephritis and mesangial hypercellularity or segmental necrosis; 3, severe glomerulonephritis with extensive sclerosis. More than 30 glomeruli were assessed for each mouse and a mean score was calculated. The right kidneys were snap-frozen in liquid nitrogen and embedded using OCT. Frozen cryostat kidney sections (6 um) were incubated with FITC-conjugated goat anti-mouse IgG. Staining was visualized using a fluorescence microscope. The intensity of the immune complex depositions (ICDs) was graded as follow: 0, no ICDs; 1, ICDs could only be seen at high-power field; 2, ICDs could be seen at low-power field but not clear; 3, ICDs could be seen clearly at low-power field; 4, ICDs are bright even at the low-power field. ICD analysis was performed by an assessor blinded to the source of sample.

Flow cytometry

Spleens were harvested sterilely and ground into suspension in RMPI 1640 medium. Splenocytes (5×10^6 cells) were incubated with the

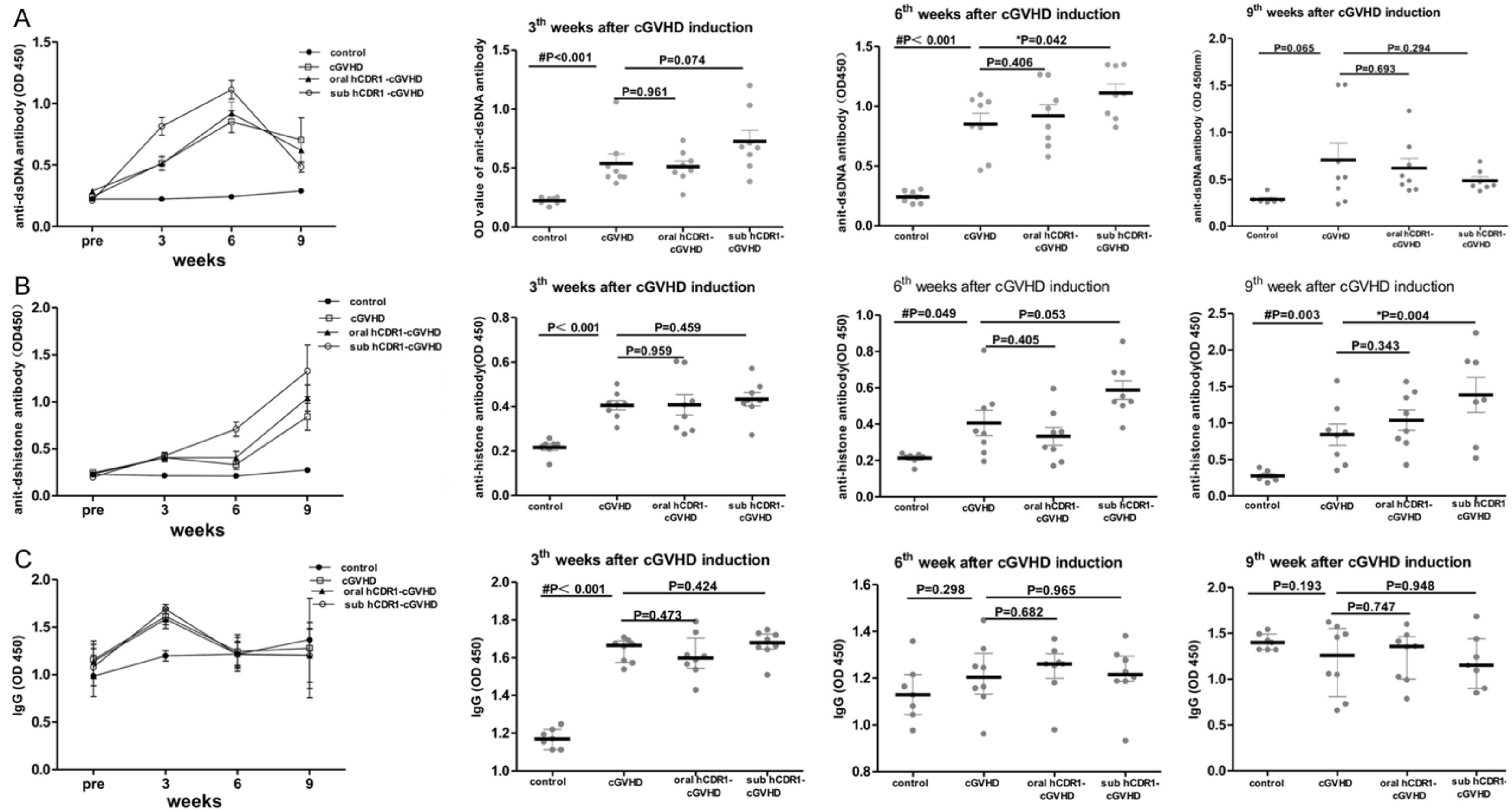

Figure 2. Effects of hCDR1 on the production of autoantibody in SLE-like cGVHD mice. A. OD value of anti-dsDNA antibody at different time points. B. OD value of anti-histone antibody at different time points. C. OD value of total IgG at different time points.

relevant Abs and analyzed by FACS. For Foxp3, IFN-γ, IL-10 staining, the cells were incubated with IC Fixation Buffer and then washed, and resuspended in permeabilization solution. Before IFN-γ and IL-10 staining the splenocytes were cultured with Cells Stimulation Cocktail (plus protein transport inhibitors) in RMPI 1640 medium for 5 h. Stained cells were resuspended in 400 μl Flow Cytometry Staining Buffer and acquire data on FACS. Lymphocytes were gated by forward and side scatter. For each sample, 10,000 events were collected.

Statistical analysis

Data were shown as means ± standard error of mean and analyzed by Student t test and unpaired non-parametric test (Mann-Whitney). $P<0.05$ was considered statistically significant. Statistical analysis was done with the SPSS 16.0 software package.

Results

Earlier onset and higher incidence of proteinuria were detected in sub hCDR1-GVHD group

Proteinuria was first detected at 3th week and the incidence of proteinuria was 50% by the 8th week and reached 100% by the 9th week in cGVHD group (**Figure 1A**). Severe glomerulonephritis first appeared at the 6th week and half of the GVHD mice developed severe glomerulonephritis at the 9th week (**Figure 1D**). The mice in oral hCDR1-GVHD group showed similar proteinuria test results to cGVHD group. However, earlier onset and higher incidence of proteinuria were detected in sub hCDR1-GVHD group. In sub hCDR1-GVHD group, proteinuria was first detected at 2th week after cGVHD induction and the incidence of proteinuria reached 80% by 5th week (**Figure 1A**), severe glomerulonephritis was first detected at the 5th week and 80% mice developed severe proteinuria by the 9th week (**Figure 1D**). In contrast, proteinuria remained below 1+ in normal control mice during the whole observation period.

Subcutaneous injection of hCDR1 elevated serum level of autoantibodies in cGVHD model

The presence of high avidity IgG autoantibodies is a remarkable feature of SLE. Mice in oral hCDR1-GVHD group showed similar autoantibodies test results to cGVHD group. However, compared with cGVHD group, mice in sub hCDR1-GVHD group acquired higher OD value of dsDNA antibodies at 3th week (0.726 ± 0.094 vs. 0.515 ± 0.0563, P=0.074) and 6th week (1.11 ± 0.076 vs. 0.852 ± 0.088, P=0.042), but lower at 9th week (0.486 ± 0.041 vs. 0.704 ± 0.182, P=0.294) (**Figure 2A**). **Figure 2B** showed that mice in sub hCDR1-cGVHD group had higher anti-histone antibody level than cGVHD group at 6th week (0.683 ± 0.083 vs. 0.333 ± 0.050, P=0.053) and 9th week (1.329 ± 0.273 vs. 0.841 ± 0.144, P=0.004). For total IgG, there was only a transient increase at 3th week in cGVHD group compared with normal control group. No difference was detected between cGVHD group and sub hCDR1-GVHD group (**Figure 2C**).

The sub hCDR1-cGVHD mice exhibited more severe renal pathological changes and more IgG deposited on the glomerulus

HE and PAS staining of the kidney sections showed the proliferation of mesangial cells, the formation of glomerular crescent, glomerular sclerosis, renal tubules atrophy, protein casts filled in renal tubules, and renal interstitium infiltrated by inflammatory cells in cGVHD group. Compared with cGVHD group, mice in sub hCDR1-cGVHD group exhibited more severe pathological changes (**Figure 3A** and **3B**). Compared to cGVHD group, mice in sub hCDR1-cGVHD group had a slightly increased HE score (2.77 ± 0.70 vs. 2.23 ± 0.68, P=0.187). cGVHD group mice showed IgG fluorescent antibodies deposited along the glomerular basement membrane and the interstitial capillary walls, with a granular appearance. The sub hCDR1-GVHD group mice exhibited significantly increased glomerular intensity of the fluorescent antibody (**Figure 3C**). The median IgG deposition score was higher in sub hCDR1-GVHD group than in cGVHD group (3.23 vs. 2.50), but there was no significant difference (P=0.10) (**Figure 3E**).

The CD4+CD25+Foxp3+Treg in splenic lymphocytes were up-regulated in sub hCDR1-cGVHD mice

To elucidate the mechanisms underlying the aggravating effects of hCDR1 on SLE manifestations in cGVHD mice, splenic lymphocytes of the mice were further analyzed. Flow cytometry showed that the absolute number

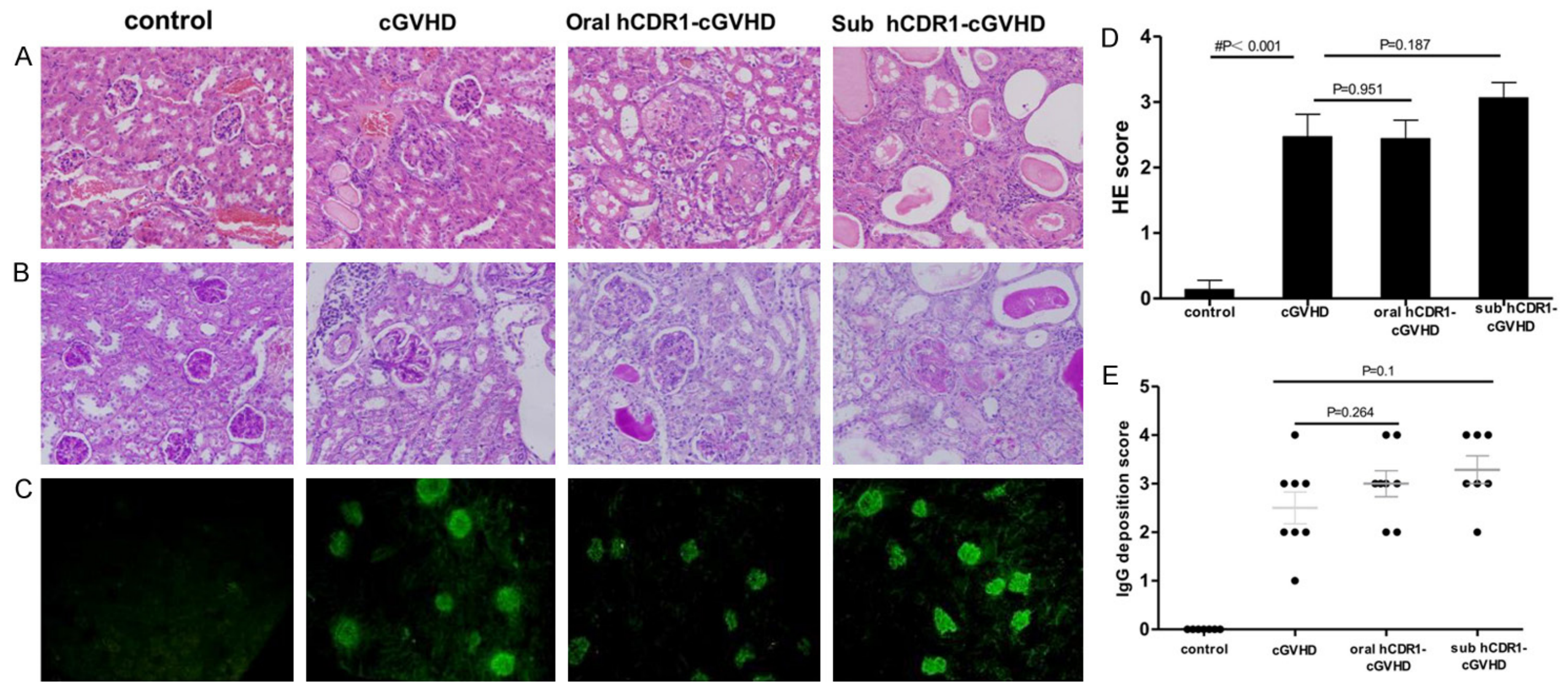

Figure 3. Effects of hCDR1 on renal pathological changes and IgG deposition in cGVHD model mice. A. Representative images of HE staining of kidney section (400 ×). B. Representative images of PAS staining of kidney section (400 ×). C. Representative images of kidney section stained with FITC-conjugated IgG antibody (200 ×). D. Scores of glomerular alterations. E. IgG deposition scores. Bars indicated mean ± standard error of the mean (SEM).

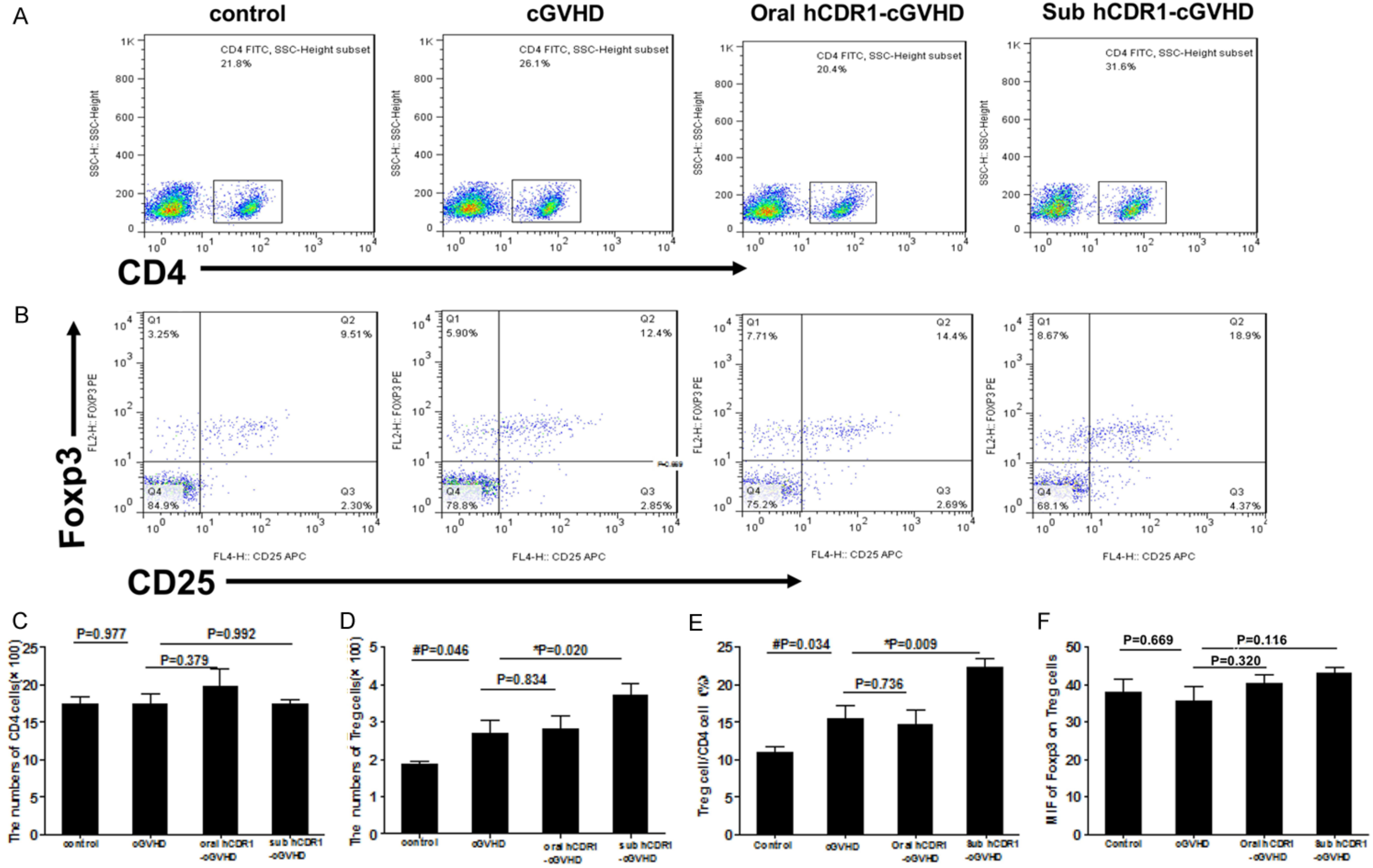

Figure 4. Effects of hCDR1 on the CD4+CD25+Foxp3+Treg cells in SLE-like cGVHD mice. A. Representative flow cytometry graphs of CD4+T cells. B. Representative flow cytometry graphs of CD4+CD25+Foxp3+T cells. C. The absolute number of CD4+T cells in each group. D. The absolute number of CD4+CD25+Foxp3+T cells in each group. E. The ratio of CD4+CD25+Foxp3+T cells/CD4+T cells in each group. F. The mean fluorescence intensity of PE-anti-Foxp3 antibody that combined with CD4+CD25+Treg cells in each group. Bars indicated mean ± standard error of the mean (SEM).

of CD4+T cells had no difference among all groups (**Figure 4C**). As shown in **Figure 4**, the CD4+CD25+Foxp3+Treg cells in cGVHD group increased to 268.38 ± 34.128 and represented 15.52% of CD4+spleen cells, whereas in sub hCDR1-cGVHD group a further upregulated proportion of CD4+CD25+Foxp3+Treg cells was detected (P=0.009). hCDR1 administrated by gavages had no influence on CD4+Treg cells. To evaluate the expression of Foxp3, the mean fluorescence intensity of PE-anti-Foxp3 antibody was measured and analyzed. It was showed that the expression of Foxp3 in CD4+ Treg cells had no difference among all groups (**Figure 4F**).

The synthesis of IFN-r and IL-10 was not influenced by hCDR1.

Considering the pathological role of IFN-γ and IL-10 in SLE, CD4+IFN-γ+ and CD4+IL-10+ cells in splenic lymphocytes were measured by flow cytometry. As showed in **Figure 5**, significant increases of CD4+IFN-γ+ cells and CD4+IL10+ cells were found in cGVHD group compared to normal control group. However, the CD4+IFN-γ+ cells and CD4+IL-10+ cells were not influenced by hCDR1.

Discussion

hCDR1 peptide was designed and synthesized as a potential candidate for the antigen-specific treatment of SLE. It was shown that hCDR1 given through subcutaneous at a dose of 50 μg/mouse could relieve SLE manifestations in (NZB × NZW) F1 mice [3-5, 17]. In this study, we have investigated the effects of hCDR1 in SLE-cGVHD mouse model. Compared with the therapeutic effects in NZB/W F1 mice, our data showed that hCDR1 by gavage had no significant influence on the disease progression in SLE-cGVHD mice. Notably, the same dose hCDR1 given to SLE-cGVHD mice through subcutaneous injection surprisingly exacerbated the lupus-like disease. In addition, subcutaneous injection of hCDR1 led to an increase of CD4+Treg cells in SLE-cGVHD, while the numbers of "pathogenic" CD4+IFN-γ+ cells and CD4+IL-10+ cells were not influenced by hCDR1.

Similar to human SLE and (NZB × NZW) F1 mouse model, SLE-cGVHD model is also characterized by the high level of serum anti-nuclear autoantibodies and immune complex mediated glomerulonephritis [6-9]. The anti-nuclear autoantibodies especially anti-dsDNA antibodies are not only the hallmark of SLE, but also related to the activity of the disease [18]. The anti-dsDNA antibodies were strongly implicated in immune complex-mediated glomerulonephritis [19]. In this study, mice in sub hCDR1-cGVHD group showed an elevated serum level of anti-dsDNA antibodies and anti-histone antibodies which indicated severe disease progression. Consistent with those elevated autoantibodies, the earlier onset of proteinuria, severe renal pathological changes and more immune complex deposition demonstrated more serious nephritis in sub hCDR1-cGVHD group.

Despite similar manifestations, the pathological mechanisms of SLE-cGVHD model and (NZB × NZW) F1 model are quite different. The (NZB × NZW) F1 mice are intrinsically autoimmune and develop lupus-like manifestations spontaneously [6, 7]. While the pathogenesis of SLE-cGVHD model involves the cognate recognition of foreign MHC class II of host B cells by alloreactive CD4+T cells from the donor [10, 20]. The CD4+Treg cells which are generally considered as immunosuppressive cells are negatively correlated with the disease severity of (NZB × NZW) F1 mice. CD4+Treg cells play a complex role in SLE-cGVHD mice through regulating the function of CD8+T cells and CD4+T cells [21-23]. It has been demonstrated that hCDR1 could up-regulate CD4+Treg cells by 30-40% in (NZB × NZW) F1 mice and the up-regulated CD4+Treg cells was considered as one of the most important mechanisms for the immune tolerance induced by hCDR1 [13, 24]. Compared to the negative correlation between CD4+Treg cells and (NZB × NZW) F1 mice, the relationship between CD4+Treg cells and SLE-cGVHD mice was complex. It was reported that an elevation of CD4+Treg cells was detected in cGVHD patients and the deletion or dysfunction of CD4+Treg cells could prevent the development of lupus-like symptom in cGVHD mice [25-28]. However, other studies reported that co-transfer of CD4+Treg cells generated in vitro could reduce the mortality of SLE-cGVHD [29, 30].

To elucidate the mechanisms underlying the contrary effects of hCDR1 in SLE-like cGVHD mice and (NZB × NZW) F1 mice, we analyzed

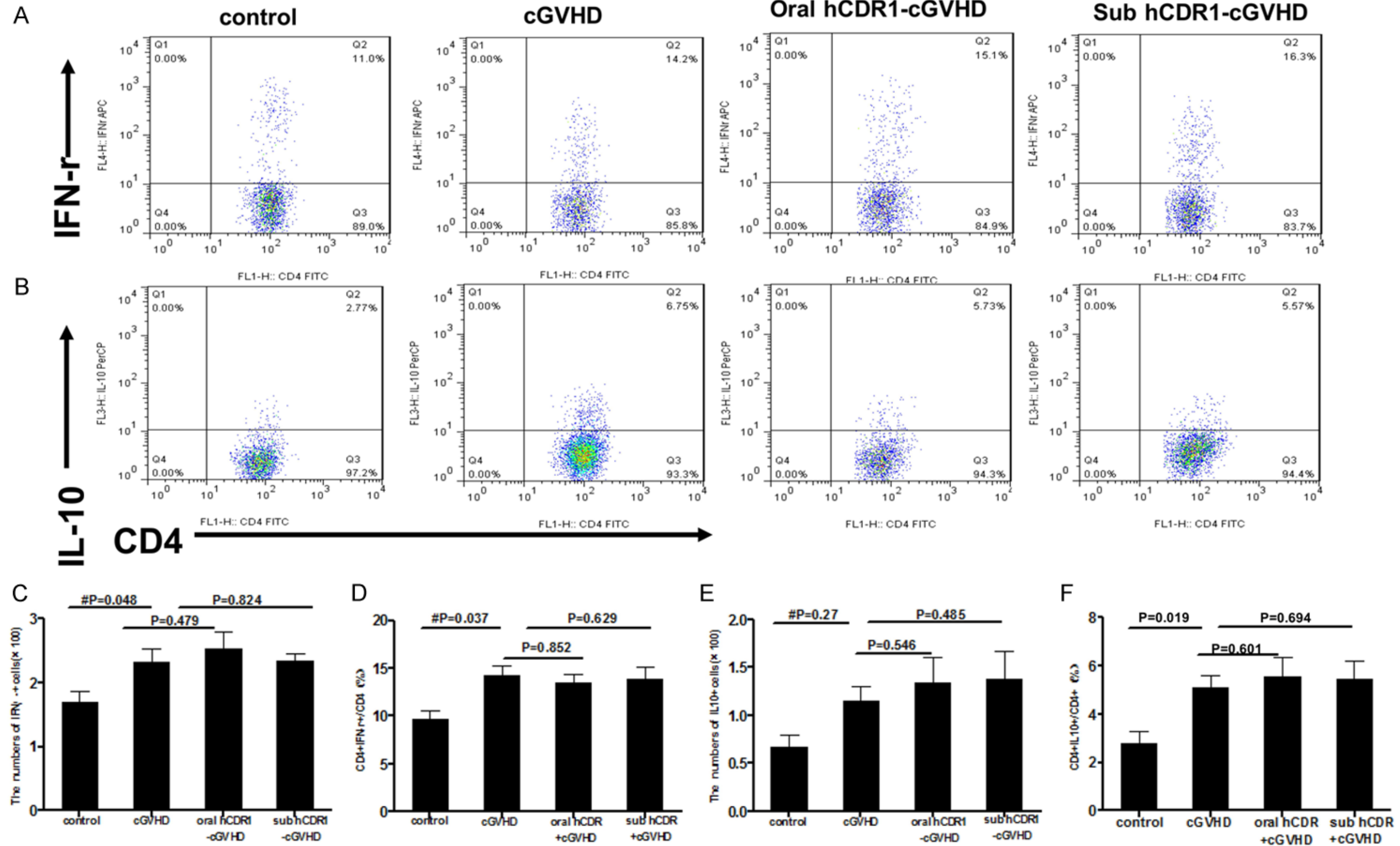

Figure 5. Effects of hCDR1 on the percentages of CD4+IFN-r+T cells and CD4+IL-10+T cells of splenocytes in SLE-like cGVHD mice. A. Representative flow cytometry graphs of CD4+IFN-r+T cells. B. Representative flow cytometry graphs of CD4+IL-10+T cells. C. The absolute number of CD4+IFN-r+T cells in each group. D. The ratio of CD4+IFN-r+T cells/CD4+ cells in each group. E. The absolute number of CD4+IL-10+T cells in each group. F. The ratio of CD4+IL10+T cells/CD4+T cells in each group. Bars indicated mean ± standard error of the mean (SEM).

the proportion of CD4+Treg cells in splenic lymphocytes. In agreement with the previous study that reported an increase of CD4+Treg cells in cGVHD patients, this study showed significant elevation of CD4+Treg in cGVHD mice. Consistent with the aggravated SLE manifestations, mice in sub hCDR1-cGVHD group showed a further increase of CD4+Treg cells compared with mice in cGVHD group. When the contrary effects of CD4+Treg cells in (NZB × NZW) F1 and SLE-like cGVHD mice were considered, the up-regulated proportion might be responsible for the opposite effects of hCDR1 in those two models.

Foxp3, a member of the fork-head/winged-helix family of transcription factors currently is the most reliable molecular marker for Treg cells [31]. It was reported that mutations in Foxp3 led to a fatal T cell-mediated autoimmune disease in mice characterized by the barely detected Treg cells. Besides, the Treg cells isolated from those mice also lacked suppressive activity [32]. Retroviral gene transfer of Foxp3 could convert CD4+CD25-T cells to Treg-like cells [31, 33]. As a master regulator of Treg cells, Foxp3 reflects the function of Treg cells in some degree. In this study the expression of Foxp3 on CD4+CD25+Treg cells was measured and no difference was found among those groups.

It was reported that IFN-γ and IL-10 play an important role in the abnormal immune regulation in SLE [34, 35]. The pathologic role of IL-10 in (NZB × NZW) F1 and SLE-cGVHD mice has been well established. The neutralization of IL-10 with IL-10 antibodies could delay the disease in (NZB × NZW) F1 mice and the same effect could be achieved by IFN-γ antibodies [36, 37]. However, conflicted results have been reported on the relationship between IFN-γ and cGVHD [38-40]. To explain the contrary effects of hCDR1 in those two models, we detected the percentages of IFN-γ+CD4 T cells and IL-10+CD4+T cells in splenic lymphocytes. We found significant increase of CD4+IFN-γ+ cells and CD4+IL10+ cells in cGVHD group than normal control group. In contrary to the down-regulated expression of IFN-γ and IL-10 in (NZB × NZW) F1 mice, CD4+IFN-γ+ cells and CD4+IL-10+ cells in cGVHD mice were not influenced by hCDR1.

Several limitations of the present study should be noticed as follows: First, although there was a trend of aggravated illness, some parameters showed no statistical significance between cGVHD group and sub hCDR1-cGVHD group. Higher dose of hCDR1 and less DBA/2 lymphocytes are needed to confirm the aggravating effects in cGVHD. Second, up-regulated proportion of CD4+Treg cells with normal expressed Foxp3 was detected in sub hCDR1-cGVHD group, but the detailed mechanism for the aggravating effects of hCDR1 in cGVHD still need to be further explored.

In summary, this study explored the implication of hCDR1 in experimentally inducible cGVHD model of SLE and demonstrated that hCDR1 administrated through subcutaneous injection but not gavages could slightly aggravate SLE manifestations and up-regulate CD4+Treg cells in SLE-like cGVHD. The slightly aggravated lupus manifestations in sub hCDR1-cGVHD mice indicate that the clinical use of hCDR1 might be not as effective as the use in (NZB × NZW) F1 mice, especial in those patients who share more similarity to SLE-like cGVHD.

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Disclosure of conflict of interest

None.

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