Glucocorticoid prevents CD138 expression in T cells of autoimmune MRL/lpr mice

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Abstract. CD138+ T cells, the majority of which are CD4 and CD8 double-negative (DN) T cells, contribute to the production of anti-dsDNA antibodies in a CD4 receptor-dependent way to promote the development of systemic lupus erythematosus (SLE). Accumulation of CD138+ T cells in the spleen of MRL/lpr mice was significantly reduced by prednisone. Reduced expression of CD138 in DN T cells induced by prednisone treatment alleviated the accumulation of DN T cells in MRL/lpr mice. The frequency of CD138+ cells in CD4+ T cells of prednisone-treated MRL/lpr mice was also significantly reduced, which subsequently contributed to reduced production of anti-dsDNA antibody in the prednisone-treated MRL/lpr mice. Additionally, prednisone significantly reduced serum IgG and IgG subsets and simultaneously increased IgM secretion in serum. This suggested that glucocorticoids played a protective role during SLE treatment in MRL/lpr mice by promoting the production of IgM. The present study provides new insights into the mechanism of glucocorticoid for the treatment of SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic and multi-system autoimmune disease that predominantly affects women, especially between puberty and menopause (1,2). However, the mechanisms of SLE are complex and undeciphered. Although B cells play a central role in adaptive immunity, recent studies on SLE suggest both T and B cells are involved in the progression of SLE (3-5). Fas (CD95) is a member of the tumor necrosis factor receptor family and interacts with Fas ligand (FasL).

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Female MRL/lpr mice have been used extensively as animal models for SLE. These mice show signs of lymphadenopathy and splenomegaly, lupus nephritis, and in vivo inflammation...
with increased levels of multiple cytokines. Furthermore, autoantibodies such as anti-dsDNA and anti-SM which are detected in SLE patients were also observed in MRL/lpr mouse models (21,28,29). Glucocorticoid treatment is the first-line treatment option and has shown a significant therapeutic effect for the clinical treatment of SLE (30-33). Glucocorticoids have been demonstrated to have a significant therapeutic effect for both SLE patients and SLE murine models by reducing in vivo autoantibody secretion, including anti-dsDNA antibodies (30-34). In the present study, we further investigated the underlying mechanism of glucocorticoid for the treatment of SLE. We investigated whether glucocorticoid could prevent CD138+ T cell accumulation and suppress CD138 expression in DN T cells to alleviate DN T cells accumulation in MRL/lpr mice.

Materials and methods

Animals. A total of 8 female MRL/MPJ mice and 16 female MRL/lpr lupus mice were purchased from the Slac Laboratory (Shanghai, China). Mice were housed at 22±1°C with a relative humidity of 50-60% with a 12-h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Beijing Institute of Chinese Medicine and were performed in accordance with Animal Research protocols for reporting of In Vivo Experiments (ARRIVE) guidelines (35,36) and institutional regulations.

Methods. The 4-week-old female MRL/MPJ mice (25-30 g) and 4-week-old female MRL/lpr lupus mice (25-30 g) were acclimatized for one week. Eight female MRL/MPJ mice were used as negative controls (ddH2O, n=8), while the remaining 16 MRL/lpr lupus mice were randomly divided into two groups, i.e., the vehicle group (ddH2O, n=8) and the prednisone (PNS) 5.0 mg/kg/day group (n=8). Oral administration was performed daily from 9 to 16 weeks of age. Body weight was recorded every week during the study (Fig. 1A). At the 17-18th week of age, mice were anesthetized using 1% sodium pentobarbital (80 mg/kg) for serum collection, and then euthanized by cervical dislocation under anesthesia. The following tissues were harvested: lymph nodes and spleen (isolated and weighed), and kidneys (for histology).

Histology. To observe renal pathologic changes in lupus-prone mice, the kidneys were harvested at 17-18 weeks of age and snap-frozen in OCT compound for frozen tissue sections or fixed in 4% paraformaldehyde. Paraformaldehyde-fixed kidneys were embedded in paraffin and then sectioned at 4-µm thickness. Hematoxylin and eosin (H&E), periodic acid Schiff (PAS), periodic acid-silver metheramine (PASM), and Masson trichrome staining was performed on the paraffin sections.

Measurement of total IgG, anti-dsDNA IgG, and anti-nuclear antibodies in the serum. Serum levels of total IgG, anti-dsDNA IgG, and anti-nuclear antibody (ANA) were measured using ELISA (total IgG ELISA kit, cat. no. 88-50400-22; Thermo Fisher Scientific, Inc.; anti-dsDNA IgG, cat. no. 5120 and ANA ELISA kit, cat. no. 5210; Alpha Diagnostic International), and were performed according to the manufacturers' instructions. Diluted serum samples were added onto coated 96-well plates and incubated for 1 h at room temperature. Afterward, the plates were washed with wash buffer and then incubated with anti-mouse IgG-HRP conjugate for 30 min at room temperature. Then, TMB solution was added and incubated at room temperature for 15 min. The reaction was terminated with a stop solution and the plates were read at 450 nm absorbance using a microplate reader.

Measurement of antibody isotypes in serum using the Luminex platform. Serum levels of multiple antibody subtypes were measured using the Luminex assay kits (Thermo Fisher Scientific, Inc.; cat. no. EPX070-20815-901). Measurements were performed according to the manufacturer's instructions. Diluted serum samples were added onto 96-well plates coated with magnetic beads and incubated for 120 min after vortexing. The beads were then washed, and the detection antibody mixture was added and incubated for 30 min at room temperature. After incubation and plate washing, the samples were analyzed on the Luminex™ platform (Thermo Fisher Scientific, Inc.).

Measurement of urine protein levels. Urine samples from individual 16-week-old mice were collected for 24 h. The concentration of proteins in the urine was determined using the Coomassie brilliant blue dye-binding assay kit (Biokits Tech. Inc.; cat. no. BCBU-027) and was performed according to the manufacturer's instructions (Biokits Tech. Inc.).

Flow cytometry. After harvesting the spleen, single-cell suspensions of splenocytes were obtained by filtering through a 70-µm cell strainer. Splenocytes were incubated on ice with CD16/CD32 monoclonal antibody (eBiosience/Thermo Fisher Scientific, Inc.; ready to use; cat. no. 14-0161-85) for 15 min, and then red blood cells were lysed using lysis buffer (BD Biosciences). The splenocytes were then fixed and permeabilized (Fixation/Permeabilization solution; BD Biosciences) before intracellular staining was performed. Cells were stained with the following ready to use antibodies for flow cytometry analysis: anti-CD3 PE-cy7 (eBiosience; Thermo Fisher Scientific, Inc.; cat. no. 25-0032-82), anti-CD4 FITC (eBiosience; Thermo Fisher Scientific, Inc.; cat. no. 11-0041-82), anti-CD8 APC (eBiosience; Thermo Fisher Scientific, Inc.; cat. no. 17-0081-82), anti-CD19 APC-cy7 (eBiosience; Thermo Fisher Scientific, Inc.; cat. no. 47-0193-82), anti-CD138 PE (Biolegend Inc.; cat. no. 142504), and anti-CD69 PE (Biolegend Inc.; cat. no. 104507). FACs data were analyzed using Flowjo software version 10.6 for PC (Tree Star, Inc.).

Cellular stimulation. CpGC (1 µM) or combined with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml ionomycin were used to stimulate and activate the cultured splenocytes from the mice.

Statistical analysis. Data from all experiments are expressed as mean ± SD and were analyzed using SPSS software 17.0 (SPSS, Inc.). Comparisons between the groups were performed for statistical significance using Mann-Whitney U test between two groups or one-way analysis of variance followed by Tukey's
Results

Successful construction of the murine SLE model and the significant therapeutic effects of prednisone. Compared to the control mice, significant enlargement of the spleen and lymph nodes, increased serum levels of anti-nuclear antibody (ANA) and anti-dsDNA IgG antibodies, and elevated urine protein levels in our murine lupus model were observed (Fig. 1B, C and E). Additionally, obvious renal injuries in the vehicle-treated lupus mice were observed and were characterized by hyaline deposits, interstitial and perivascular cellular inflammation infiltration, cellular crescent formation, glomerular fibrosis, glomerulosclerosis, and tubular cell necrosis (Fig. 1D). These results indicate that the murine lupus model was successfully constructed. As expected,
prednisone (PNS) treatment exhibited significant therapeutic effects on lupus mice by significantly alleviating the enlargement of the spleen and lymph nodes, decreasing serum levels of ANA and anti-dsDNA IgG antibodies, reducing urine protein levels, and improving histopathological injuries in the renal tissues (Fig. 1).

**Prednisone reduces total IgG, IgG1, and IgG2a levels in the serum of the lupus mice, while simultaneously increasing IgM levels.** We found that the levels of total IgG, IgG1, IgG2a, IgG2b, IgG3, IgM, IgE, and IgA in the serum were significantly increased in lupus mice compared to the control mice (Fig. 2A-H). However, the levels of total IgG, IgG1, IgG2a, and IgG2b were significantly reduced in the MRL/lpr mice treated with PNS (Fig. 2A and C-E). In addition, we observed a further increase in IgM levels in the serum of the MRL/lpr mice (Fig. 2B). Our results indicate that PNS reduced the production of total IgG, IgG1, IgG2a, and IgG2b in the serum of the MRL/lpr mice, and simultaneously increased serum IgM levels in the MRL/lpr mice.

**Prednisone prevents activation of CD3+ T cells in the MRL/lpr mice.** We stimulated and activated splenocytes using PMA and ionomycin for 5 h. Activated T lymphocytes were stained with CD69 (34,37). Compared to the vehicle-treated MRL/lpr mice, T cells and T cell subsets including CD4+, CD8+ and DN T cells in the MRL/MPJ mice had significantly higher frequencies of CD69+ cells after 5 h of stimulation (Fig. 3A-C and E), suggesting that T cells and their subsets in MRL/MPJ mice were more easily activated. Consistent with these results, we observed that both CD4+ and CD8+ T cell frequencies in splenocytes of MRL/MPJ and MRL/lpr mice were equal before in vitro stimulation (Fig. 3D). But both CD4+ and CD8+ T cell frequencies in splenocytes of the MRL/MPJ mice were significantly higher...
than the frequencies in splenocytes of the MRL/lpr mice after 24 h of in vitro CpGC stimulation of splenocytes (Fig. 3D).

PNS showed significant effects on activation of T cells and T cell subsets. PNS significantly decreased CD69+ cell frequencies in CD3+ T cells and its subsets including CD4+, CD8+ and DN T cells in splenocytes of the MRL/lpr mice (Fig. 3A-C and E). The decrease in CD69+ cell frequency in CD8+ T cells of the PNS-treated mice was more significant than the frequencies in splenocytes of the MRL/lpr mice after 24 h of in vitro CpGC stimulation of splenocytes (Fig. 3D).

Figure 3. (A) Representative flow cytometry analyses and bar charts denote the frequency of CD69+ cells in CD3+ T cells in stimulated splenocytes. (B) Representative flow cytometry analyses and bar charts denote the frequency of CD69+ cells in CD4+ T cells in stimulated splenocytes. (C) Representative flow cytometry analyses and bar charts denote the frequency of CD69+ cells in CD8+ T cells in stimulated splenocytes. (D) Bar charts showing the frequency of CD4+ and CD8+ T cells before and after 24 h of CpGC stimulation in splenocytes. (E) Representative flow cytometry analyses and bar charts denote the frequency of CD69+ cells in double-negative (DN) T cells in stimulated splenocytes; Data are presented as mean ± SD; n=4-5 per group from two independent experiments; *P<0.05, **P<0.01 by one-way analysis of variance, vs. the vehicle-treated mice. PnS, prednisone.
compared with that in the CD4\(^+\) and DN T cells. However, PNS did not significantly reduce CD8\(^+\) T cell frequency in the splenocytes of the MRL/lpr mice (Fig. 3D). Contrarily, CD4\(^+\) T cell frequency in the splenocytes of the MRL/lpr mice with PNS treatment was significantly increased (Fig. 3D).

**Prednisone prevents CD138 expression in T cells of MRL/lpr mice.** Isolated splenocytes from the MRL/MPJ mice without stimulation had a near absence of CD138\(^+\) cells, whereas the splenocytes of Fas-deficiency MRL/lpr mice had a significant increase in CD138\(^+\) cell frequency compared with those of the MRL/MPJ mice (Fig. 4A). We next co-cultured and stimulated splenocytes with CpGC for 24 h. We also observed that CD138\(^+\) cells were accumulated in splenocytes of the MRL/lpr mice but not in the MRL/MPJ mice (Fig. 4A). Compared to the vehicle-treated lupus mice, the CD138\(^+\) cell frequencies in splenocytes of the PNS-treated lupus mice were significantly reduced both with and without 24 h of CpGC stimulation (Fig. 4A).

The frequencies of CD138\(^+\) cells in CD3\(^+\) T cells of the MRL/MPJ mice were still negligible; however, CD138 was
abundantly expressed on CD3+ T cells in the MRL/lpr mice (Fig. 4B) both before and after 24 h of CpGC stimulation of splenocytes in vitro. CD138+ T cell frequencies in CD3+ T cells of the MRL/lpr mice with oral administration of PNS were significantly decreased compared to the vehicle-treated MRL/lpr mice both before and after 24 h of CpGC stimulation of splenocytes in vitro (Fig. 4B).

In addition, we observed that CD138 was also expressed in the CD4+ T cells in the MRL/lpr mice but not in the MRL/MPJ mice both with and without 24 h of CpGC stimulation of splenocytes in vitro (Fig. 4C). PNS also significantly prevented CD138 expression in CD4+ T cells. The CD138+ cell frequencies in the CD4+ T cells were significantly decreased in the MRL/lpr mice after prednisone treatment compared to the vehicle-treated mice both before and after CpGC stimulation of splenocytes in vitro (Fig. 4C).

Prednisone prevents DN T cell accumulation and CD138 expression in DN T cells of MRL/lpr mice. DN T cells strikingly accumulated in the splenocytes of the Fas-deficiency MRL/lpr mice but not in the MRL/MPJ mice (Fig. 5A). PNS significantly relieved DN T cell accumulation in the splenocytes of the MRL/lpr mice (Fig. 5A). Importantly, DN T cells in the MRL/lpr mice but not in the MRL/MPJ mice commonly expressed CD138 (Fig. 5B). However, PNS also prevented CD138 expression in DN T cells of the MRL/lpr mice. The frequency of CD138+ cells in DN T cells of the PNS-treated lupus mice was significantly reduced compared to the vehicle-treated mice (Fig. 5B). Furthermore, even after 24 h of CpGC stimulation of splenocytes in vitro, the CD138+ cell frequency in DN T cells of the PNS-treated MRL/lpr mice also showed a significant reduction compared to the vehicle-treated mice (Fig. 5B).

Discussion

The present study results demonstrated that prednisone (PNS) significantly relieved systemic lupus erythematosus (SLE) symptoms in MRL/lpr mice by alleviating enlargement of the spleen and lymph nodes, reducing the production of autoantibody in the serum including anti-double-stranded (anti-ds) DNA antibodies IgG antibody and anti-nuclear antibody (ANA), and ameliorating renal tissue injury and simultaneously preventing the accumulation of double negative (DN) T cells in splenocytes of MRL/lpr mice. In addition, it was demonstrated that PNS had a significant effect on CD138 expression in CD3+ T cells of the MRL/lpr mice. PNS prevented CD138+ T cell accumulation in the MRL/lpr mice and inhibited CD138 expression in both CD4+ and DN T cells of the MRL/lpr mice. In addition, PNS played a protective role
by significantly increasing levels of IgM secretion in the serum of the MRL/lpr mice. The results showed new insights into the mechanisms of glucocorticoid on SLE treatment. PNS, a glucocorticoid drug, exhibited significant therapeutic effects on MRL/lpr lupus mice in our study. We observed that PNS was able to significantly reduce the production of total IgG and multiple IgG antibody subsets such as IgG1, IgG2a, and IgG2b, and simultaneously increased IgM production in the serum of the MRL/lpr mice. Previous studies have demonstrated that in humans, IgA deficiency is associated with autoimmunity. However, this differs between SLE humans and SLE mice. IgM antibody has been demonstrated to be a protective antibody isotype in MRL/lpr mice (38-41). IgM deficiency was previously found to significantly contribute to accelerated development of lupus and elevated levels of IgG autoantibody secretion in MRL/lpr mice (38-41). This indicated that PNS could ameliorate lupus in MRL/lpr mice by increasing the production of IgM. This suggests that glucocorticoid ameliorates SLE by increasing the production of protective antibody subsets. Furthermore, PNS significantly decreased autoantibody production, including anti-dsDNA antibody in the MRL/lpr mice. Our results also showed that PNS significantly reduced CD4+CD138+ T cell frequency in CD4+ T cells of the MRL/lpr mice. A previous study demonstrated that CD138+ T cells contributed to the production of anti-dsDNA antibodies both in vivo and in vitro by a CD4 receptor-dependent mechanism. This suggested that CD4+CD138+ T cells were the autoreactive CD4+ T cells that promoted anti-dsDNA antibody production (21). This indicated that the decline in CD138 expression in CD4+ T cells induced by PNS prevented anti-dsDNA antibody production, which resulted in PNS decreasing anti-dsDNA autoantibody levels in the serum.

DN T cells have been shown to accumulate in the peripheral blood of SLE patients and the spleen of Fas-deficiency lupus mice (3,9,10). Our results showed that PNS significantly prevented DN T cell accumulation in splenocytes of the MRL/lpr mice. Previous research has demonstrated that DN T cells are involved in the development of systemic inflammation and tissue damage in lupus patients (3). Recent research has demonstrated that the adoptive transfer of DN T cells aggravates the pathology in young lupus mice, while significant infiltration of DN T cells was observed in both adult and pediatric lupus kidneys (3). However, our results showed that the majority of DN T cells in splenocytes of the MRL/lpr mice were CD138 positive. Previous research also showed that CD138+ T cells were associated with the production of anti-dsDNA antibodies both in vivo and in vitro, and demonstrated adoptive transfer of CD138+ T cells significantly contributed to renal tissue injuries in MRL/lpr lupus mice (21). These results demonstrated that CD138+ T cells are also strongly associated with the progression of lupus in MRL/lpr mice. Our recent study demonstrated that CD138 expression in CD3+ T cells strikingly prevented the apoptosis of CD3+ T cells and strikingly contributed to the accumulation of DN T cells in T cells of MRL/lpr mice (Xie T, Liu X and Li P; unpublished data). In the present study, our results also showed that DN T cells in the MRL/lpr mice had a high level of CD138 expression. PNS treatment significantly decreased CD138+ cell frequency in DN T cells of lupus mice. Our results demonstrated that reduced frequency of CD138+ cells in DN T cells contributed to reducing the accumulation of DN T cells in MRL/lpr mice after PNS administration. However, information and data regarding CD138 expression in T cells in MRL/lpr mice is still limited. The specific mechanism and signaling pathways involved in CD138 expression in CD3+ T cells of MRL/lpr mice are yet to be deciphered and have not been consistent between studies. Hence, more studies and data are needed to demonstrate the underlying mechanism and signaling pathways in the glucocorticoid-mediated prevention of CD138 expression in T cells of MRL/lpr mice.

CpG DNA, which includes CpGA, CpGB, and CpGC, are Toll-like receptor (TLR) agonists (42) that activate and induce interferon (IFN)-α secretion in plasmacytoid dendritic cells (pDCs) (43). CpGC stimulation was used in this study to mimic in vivo conditions in SLE patients (44), i.e., to induce the secretion of IFN-α to promote SLE development and aggravate tissue injury (43,45). Our present study showed that the frequencies of CD4+ and CD8+ T cells in fresh splenocytes of the control mice were nearly equal to the frequencies in the MRL/lpr mice. However, when splenocytes were stimulated by CpGC, CD4+ and CD8+ T cell frequencies in the control mice were significantly higher than the frequencies in the Fas-deficiency MRL/lpr mice. Furthermore, T cells in the control mice including CD4+ T cells and CD8+ T cells, were more likely to be activated in response to T cell stimulation compared to lupus mice. Our results indicated that both CD4+ and CD8+ T cells in the Fas-deficiency MRL/lpr mice had defective proliferation and activation.

In summary, PNS had a significant therapeutic effect on MRL/lpr lupus mice. PNS significantly prevented CD138+ T cell accumulation in the MRL/lpr mice. CD138 expression in DN T cells of the MRL/lpr mice was inhibited by PNS which contributed to PNS alleviating DN T cell accumulation. PNS also prevented CD138 expression in CD4+ T cells which significantly resulted in reduced anti-dsDNA antibody production in MRL/lpr mice after PNS treatment. In addition, PNS also decreased IgG and IgG subset production and simultaneously promoted IgM secretion which plays a preventive role in the progression of SLE treatment. Our results provide new insights into the therapeutic effects and mechanisms of glucocorticoid treatment for SLE.

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Availability of data and materials

The data generated in the present study are available from the corresponding author on reasonable request.
Authors' contributions

TX conceived the study and wrote the manuscript. TX and PL designed the experiments. TX and HL performed the laboratory work. TX and HL performed the data analysis. PL revised and edited the manuscript. TX and PL confirm the authenticity of all the raw data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work (including the data presented) are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Beijing Institute of Chinese Medicine and were performed in accordance with Animal Research protocols for reporting of In Vivo Experiments (ARRIVE) guidelines and institutional regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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