# CD138 promotes the accumulation and activation of autoreactive T cells in autoimmune MRL/lpr mice

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**Abstract.** Autoreactive T cells, specifically CD138$^+$ (syndecan-1) T cells produced in Fas-deficient systemic lupus erythematosus (SLE) mouse models, were shown to significantly promote the generation of autoantibodies. In the present study, Murphy Roths Large lymphoproliferative (MRL/lpr) lupus mice were used to investigate the role of CD138 protein expression in T cells in the progression of SLE. Measurement of flow cytometry, immunofluorescence and Luminex were performed to determine the effect of CD138 on T cells in MRL/lpr mice. The results demonstrate that CD138$^+$ T cells induce apoptosis via a Fas-dependent pathway. CD138 protein expression in T cells of MRL/lpr mice significantly reduced T cell apoptosis and contributed to the accumulation of T cells and double negative (DN) T cells, whilst simultaneously promoting T cell activation in Fas-deficient lupus mice. CD138 protein expression in DN T cells also significantly increased the protein expression of Fas ligand to enhance the cytotoxicity of DN T cells. Furthermore, phorbol 12-myristate 13-acetate and ionomycin (PI) stimulation reduced CD138 protein expression in CD3$^+$ T cells and prevented CD138$^+$ T cell accumulation by inducing specific apoptosis. PI stimulation also activated T cells in MRL/lpr mice to increase CD69 protein expression. CD69 protein expression in CD138$^+$ T cells significantly increased the frequency of apoptotic CD138$^+$ T cells. In addition, results from the present study demonstrated that CD138$^-$ T cells of MRL/lpr lupus mice had an activation defect. CD138 protein expression in T cells significantly reversed the defective activation and activating T cells could significantly reduce CD138 protein expression in CD3$^+$ T cells of MRL/lpr mice. This suggests that CD138 protein expression in CD3$^+$CD138$^-$ T cells of MRL/lpr mice may be a consequence of the impaired activation in autoreactive T cells prior to exposure to self-antigens by the immune system. CD138 expression in autoreactive T cells has a central role in promoting the progression and development of autoimmune response in MRL/lpr mice.

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## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of multiple autoantibodies, including anti-nuclear antibodies and anti-double-stranded (ds) DNA antibodies (1,2). The generation of these autoantibodies was reported to have a detrimental effect on multiple tissues and organs, such as the skin, joints and kidney (3-5). Previous studies suggested that SLE is a complex disease involving the participation of both T and B cells in its progression (6-8).

Activated B cells differentiate into plasma cells to secrete antibodies in response to an antigen. CD138 is a marker of plasma cells (9,10). CD138$^+$ T cells, which express both CD3 and CD138 on their surface, were previously reported to be associated with plasmablastic B-cell neoplasms in clinical cases (11) and were also identified in SLE murine models (12-14). CD138$^+$ T cells were reported to significantly accumulate in Fas-deficient lupus mice (12-14). Furthermore, previous studies demonstrated that double negative (DN) T cells serve an important role in the progression of lupus and significantly contribute to tissue injury in SLE (6,15,16). Plasma cell accumulation was reported to be a hallmark feature of SLE (17,18). Our previous study demonstrated that the majority of CD138$^+$ cells in SLE murine models were CD138$^+$ T cells (19). A previous study also reported that CD4$^+$CD138$^+$ T cells could significantly promote autoantibody production both *in vivo* and *in vitro* (12). This suggests that CD138$^+$ T cells may be autoreactive and accumulate in Murphy Roths Large lymphoproliferative (MRL/lpr) mice to induce an autoimmune response. Therefore, CD138$^+$ T cells may aid in elucidating the underlying mechanism of SLE progression. In the present study, the mechanism by which CD138$^+$ T cells are involved in the progression of SLE in MRL/lpr mice was evaluated.

Phorbol 12-myristate 13-acetate (PMA) and ionomycin (PI) are commonly used to induce *in vitro* cellular activation (20,21). A previous study also demonstrated that T cell

receptor (TCR) activation negatively regulates CD138 protein expression in DN T cells (14). PMA and PI are commonly used to activate T cells and promote cytokine secretion in T cells (22,23). In the present study, the effect of PI stimulation on $CD138^+$ T cell accumulation in splenocytes of MRL/lpr mice was assessed. Furthermore, the function of CD138 protein expression in $CD3^+CD138^-$ T cells and its resulting accumulation in MRL/lpr mice was evaluated.

## Materials and methods

*Experimental animals*. Female Murphy Roths Large ($MRL/MPJ\text{-}Fas^{+/+}$; n=8; age, 4-week-old; weight, 20-25 g) and Murphy Roths Large lymphoproliferative ($MRL/lpr\text{-}Fas^{-/-}$; n=8; age, 4 weeks; weight, 20-25 g) mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Mice were housed at 22±1˚C with a relative humidity of 50-60% and a 12-h light/dark cycle. The mice were allowed free access to water and food. All animal experiments were compliant with the institutional ethical guidelines of Beijing Institute of Chinese Medicine (Beijing, China).

*Cell culture*. At 17-18 weeks of age, mice that did not undergo intervention or treatment were anesthetized using an intraperitoneal injection of 1% sodium pentobarbital (80 mg/kg) to harvest blood for serum samples. Mice were then euthanized by cervical dislocation and subsequently the spleen was harvested. Single-cell suspensions of splenocytes were then obtained via filtration through a 70-$\mu$m cell strainer (BD Biosciences). Splenocytes with 5x10^6 cells in each experiment were then cultured in RPMI-1640 medium (HyClone; Cytiva) with 10% fetal bovine serum, (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% $CO_2$ or in the presence of PMA (50 ng/ml; Thermo Fisher Scientific, Inc.) and PI (1 $\mu$g/ml; Thermo Fisher Scientific, Inc.) to stimulate the splenocytes (24-26).

*Measurement of serum cytokine levels using the Luminex™ platform*. Serum levels of IFN-γ, IFN-α, tumor necrosis factor, IL-6, IL-10, IL-17, IL-21 and IL-2 were measured using 8-Plex ProcartaPlex Panel (cat. no. PPX-08; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Diluted serum samples with universal assay buffer (from the Luminex kit) were added onto 96-well plates coated with magnetic beads and incubated at room temperature for 120 min after vortexing. The beads were then washed with wash buffer, and the 50X detection antibody mixture was diluted to 1X with detection antibody diluent, and then added and incubated for an additional 30 min at room temperature. After incubation and plate washing with wash buffer, the samples were analyzed on the Luminex 200 platform.

*Flow cytometry*. Splenocytes were incubated on ice with CD16/CD32 monoclonal antibodies (cat. no. 14-0161-85; eBioscience™; Thermo Fisher Scientific, Inc.) for 15 min, and then red blood cells were lysed using lysis buffer (BD Biosciences). Cells were subsequently incubated with the following antibodies (100 $\mu$l/test as recommended by the manufacturer for flow cytometry) at room temperature for 30 min for flow cytometric analysis: Anti-CD3 phycoerythrin (PE)-cyanine7 (cy7) (cat. no. 25-0032-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-CD3 allophycocyanin (APC)-cy7 (cat. no. 47-0032-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-CD4 fluorescein isothiocyante (FITC) (cat. no. 53-0041-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-CD8 peridinin-chlorophyll-protein (PerCP) (cat. no. 45-0081-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-CD8 APC (cat. no. 17-0081-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-CD19 APC-cy7 (cat. no. 47-0193-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-CD138 PE (cat. no. 142504; BioLegend, Inc.); anti-CD69 PE (cat. no. 104508; BioLegend, Inc.); anti-CD69 APC (cat. no. 17-0691-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-CD25 APC (cat. no. 17-0251-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-FasL APC (cat. no. 17-5911-82; eBioscience; Thermo Fisher Scientific, Inc.); anti-B220 PerCP (cat. no. 45-0452-82; eBioscience; Thermo Fisher Scientific, Inc.); and anti-B220 PE-cy7 (cat. no. 25-0452-82; eBioscience; Thermo Fisher Scientific, Inc.). Annexin V-FITC and 7-aminoactinomycin D (7-AAD) PerCP (cat. no. 35-6410 KIT; Tonbo Biosciences, Inc.) were utilized for staining, performed according to the manufacturer's instructions. Cells were incubated with annexin V conjugate for 15 min at room temperature and 7-AAD staining solution was added and incubated at room temperature for 5 min before flow cytometric analysis. Stained cells were analyzed using BD FACSVerse (BD Biosciences). Data were analyzed using FlowJo software (version 10.6 for PC; Tree Star, Inc.).

*Immunofluorescence*. Frozen tissue sections (6 $\mu$m thick) were used for immunofluorescence assays. They were embedded in optimal cutting temperature compound (cat. no. 4583; Sakura Finetek), fixed with acetone cooled to 4˚C for 5 min, blocked with 5% donkey serum (cat. no. S9100; Solarbio Co., Ltd., China) at room temperature for 1 h and then stained with primary antibodies at room temperature for 1 h, namely anti-CD3 antibody (1:100 dilution; cat. no. ab33429; Abcam) and anti-CD138 antibody (10 $\mu$g/ml; cat. no. AF3190; R&D Systems), as previously described (27). Sections were visualized using donkey anti-goat IgG H&L (Alexa Fluor® 488) (1:200 dilution; cat. no. ab150129; Abcam) and donkey anti-rat IgG H&L (Alexa Fluor® 594) (1:200 dilution; cat. no. ab150156; Abcam) secondary antibodies at room temperature for 30 min. Immunofluorescence images were obtained and analyzed using ZEN Blue lite 2.3 software (Zeiss GmbH). Representative imaging data were obtained at identical settings with the ZEN Blue lite software and all assays included negative controls, where the primary antibodies were omitted (Fig. S1A).

*Polymerase chain reaction (PCR)*. To detect the genotype of the fas gene in MRL/lpr mice, total genomic DNA was extracted from the kidney tissue from all the eight MRL/MPJ and eight MRL/lpr mice using the TIANamp Genomic DNA Kit (cat. no. DP304; Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. The PCR containing 1.1X CataAmp Taq Plus PCR Mix (cat. no. C105; Beijing Catascis Biotech. Co. Ltd.) was performed according to genotyping protocols provided by the Jackson Laboratory (Jax Lab). The primer sequences for PCR were as follows: olMR1678/olMR1680 forward, 5'-GTAAATAATTGTGCT TCGTCAG-3'; olMR1678 reverse, 5'-TAGAAAGGTGCA

CGGGTGTG-3'; and olMR1680 reverse, 5'-CAAATCTAG GCATTAACAGTG-3'. The PCR protocol was executed as follows: 94˚C for 3 min for initial denaturation, then 94˚C for 10 sec for denaturation, 65˚C (initially, then decreasing by 0.5˚C per cycle) for 10 sec for annealing and 68˚C for 30 sec for extension, repeated for 10 cycles, next 72˚C for 10 sec, 60˚C for 10 sec and 72˚C for 30 sec for 28 cycles. This was followed by 72˚C for 60 sec for final extension and a hold at 10˚C. The PCR products were electrophoresed in a 3% agarose gel (cat. no. 1110GR100; BioFroxx) at a voltage of 80V. The agarose gel was stained with GelRed for 15 min. An image of the agarose gel was then obtained by Amersham ImageQuant 800 (Amersham Pharmacia Biotech, Inc.). The amplified products were analyzed and revealed a fragment length of 217 bp for the mutant and 179 bp for the wild-type (WT).

*Statistical analysis*. Data from all experiments are representative of 2-3 independent experiments showing reproducibility and are expressed as mean ± standard deviation. Data were analyzed using SPSS 17.0 (SPSS, Inc.). Comparisons between groups were performed using the Student's t-test, while comparisons among ≥3 groups were performed using one-way ANOVA followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*CD138 protein expression in T cells leads to defective apoptosis of T cells in MRL/lpr mice*. Results of PCR showed all MRL/MPJ mice had homozygous WT fas genes and all MRL/lpr mice had homozygous mutant fas gene (Fig. S1B). It demonstrated that the MRL/lpr mice we used were fas-deficient and SLE murine models were absolutely established in the present study (Fig. S1B). CD138 protein expression levels in $CD3^+$ T cells were negligible in splenocytes of MRL/MPJ mice, compared with the significant accumulation of abnormal ($CD138^+$) T cells in the splenocytes of Fas-deficient MRL/lpr mice (Figs. 1A and S1C). In addition, it was demonstrated that $CD138^+$ T cells infiltrated the kidneys of MRL/lpr mice, but is negligible in the kidneys of MRL/MPJ mice (Fig. 1B). $CD138^+$ T cells in MRL/MPJ mice also had significantly higher levels of apoptosis, compared with MRL/lpr mice, which were found to have low levels (Fig. 1C). This indicated that $CD138^+$ T cell apoptosis in mice was Fas-dependent and Fas deficiency in MRL/lpr mice led to the accumulation of $CD138^+$ T cells. However, it was demonstrated that $CD138^+$ T cells in the splenocytes of MRL/lpr mice were associated with a significant decrease in the number of apoptotic cells and a significant increase in the number of live cells compared with $CD3^+CD138^-$ T cells (Fig. 1D).

CD69 and CD25 are markers of early and late active T cells (12). In the present study, it was demonstrated that $CD138^+$ T cells in fresh splenocytes of MRL/lpr mice had a significant increase in $CD69^+$ cell frequency with a simultaneous significant decrease in $CD25^+$ cell frequency, compared with $CD138^-$ T cells in fresh splenocytes of MRL/lpr mice (Fig. 1E). Furthermore, CD138 protein expression significantly increased Fas ligand (FasL) protein expression in $CD3^+$ T cells of MRL/lpr mice, compared with $CD3^-$ T cells (Fig. 1E). CD138 protein expression also significantly increased FasL protein expression in $CD138^+$ DN T cells of MRL/lpr mice, compared with $CD138^-$ DN T cells (Fig. 1F). Therefore, it was concluded that CD138 protein expression in $CD3^+$ T cells led to defective apoptosis of $CD3^+$ T cells but prompted activation of $CD3^+$ T cells in Fas-deficiency MRL/lpr mice.

*$CD4^+$ T cells and DN T cells express CD138 in MRL/lpr mice*. The majority of $CD138^+$ T cells in MRL/lpr mice were demonstrated to be CD4 and CD8 double negative T cells, with a small proportion of $CD138^+$ T cells expressing CD4 and a negligible proportion of cells expressing CD8 (Fig. S1D). A significantly lower frequency of $CD138^+$ cells was demonstrated in $CD8^+$ T cells, compared with $CD4^+$ T cells and DN T cells (Fig. 1G). There was also a significantly greater frequency of $CD138^+$ cells in DN T cells than in $CD4^+$ T cells (Fig. 1G). This indicated that the majority of $CD138^+$ T cells in MRL/lpr mice were derived from $CD138^-$ DN T cells in MRL/lpr mice that had an increased accumulation of DN T cells. Additionally, it was demonstrated that $CD4^+$ T cells in MRL/lpr mice were comprised of two subsets, CD4 hi T cells and CD4 int T cells (Fig. 1H). CD4 int T cells had reduced expression of CD4 but increased expression of B220, compared with CD4 hi T cells (Fig. 1H). $CD4^+CD138^+$ T cells simultaneously expressed B220 with a significant downregulation of CD4 protein expression compared with $CD4^+CD138^-$ T cells (Fig. 1I and J).

*CD138 protein expression improves defective activation of $CD138^-$ T cells in MRL/lpr mice*. Multiple cytokines were found in the serum of MRL/lpr mice, of which interferon-γ, tumor necrosis factor (TNF), interleukin (IL)-6, IL-10, IL-17, and IL-2 were significantly increased compared with those in MRL/MPJ mice (Fig. 2A). However, there were no significant changes of IFN-α and IL-21 levels in the serum of MRL/lpr mice, compared with those in MRL/MPJ mice (Fig. 2A). This suggested that MRL/lpr mice were undergoing an inflammatory response (Fig. 2A). Unstimulated $CD138^-$ T cells derived from fresh splenocytes of MRL/lpr mice had a significantly increased frequency of $CD69^+$ cells compared with that in $CD138^-$ T cells from fresh splenocytes of MRL/MPJ mice (Fig. 2B). However, the frequency of $CD69^+$ cells in $CD138^-$ T cells of MRL/MPJ mice was significantly increased compared with that in $CD138^-$ T cells from MRL/lpr mice after 5 h of *in vitro* stimulation of the fresh splenocytes by PI (Fig. 2C). Furthermore, both CD69 and FasL protein expression levels in $CD138^-$ T cells were not significantly increased after *in vitro* stimulation of splenocytes with 2 or 4 h PI stimulation compared with the CD138- T cells without PI stimulation (Fig. 2D). These results demonstrated a defect in $CD138^-$ T cell activation in MRL/lpr mice.

$CD138^+$ T cells from fresh splenocytes of MRL/lpr mice had significantly increased $CD69^+$ cell frequencies (Figs. 1E and 2B) and FasL protein expression (Fig. 1E) compared with $CD138^-$ T cells. In addition, $CD138^+$ T cells showed a significantly increased $CD69^+$ cell frequency after *in vitro* 5 h PI stimulation, as well as significantly increased FasL protein expression compared with that in $CD138^-$ T cells in splenocytes from MRL/lpr mice (Fig. 2C and E). These results suggested that CD138 protein expression improved the defective activation of $CD138^-$ T cells in MRL/lpr mice.

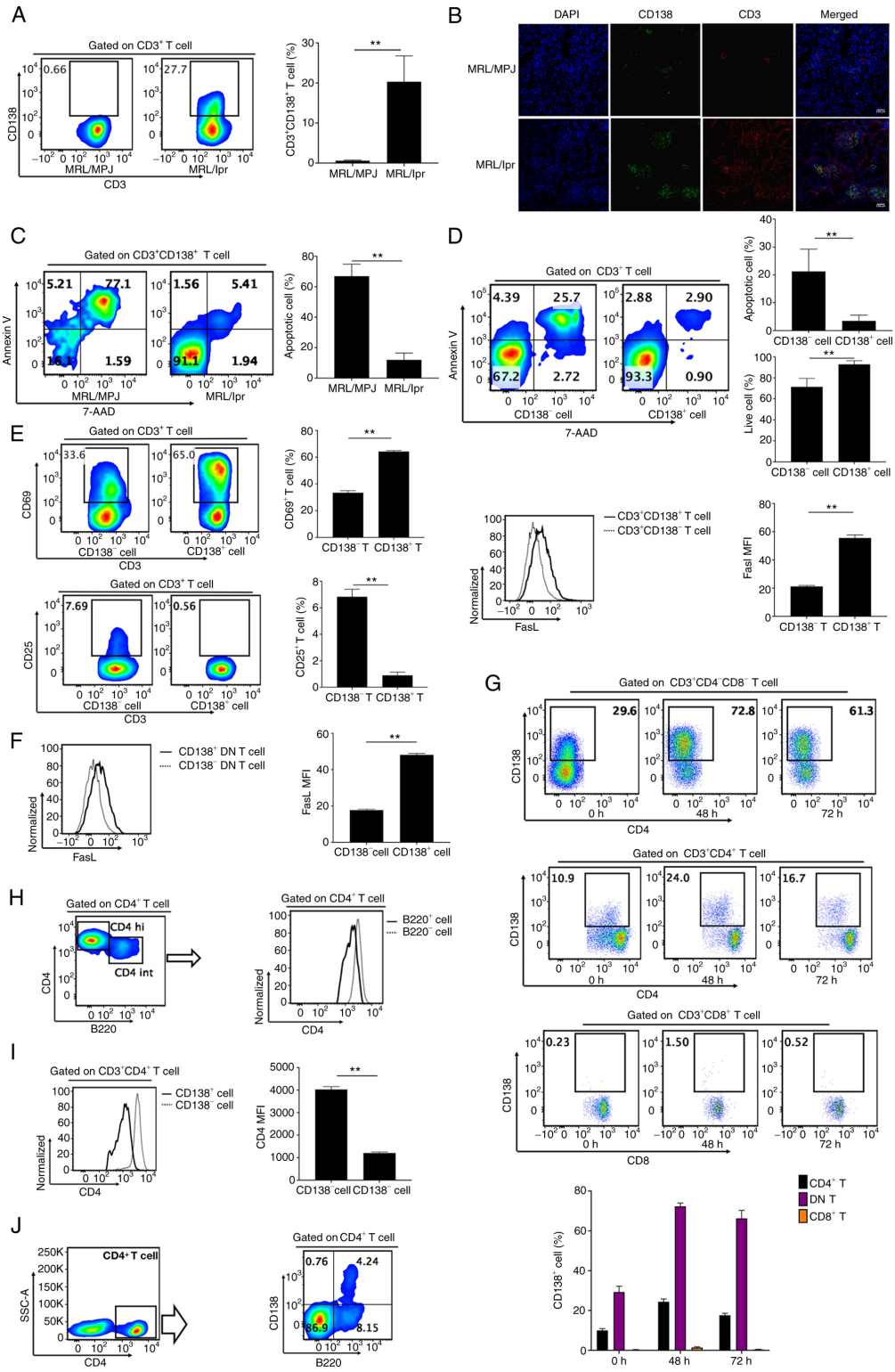


Figure 1. CD138 expression leads to defective apoptosis of T cells. (A) Flow cytometry analyses and bar chart showing the frequencies of CD138$^+$ cells in CD3$^+$ T cells from fresh splenocytes of MRL/MPJ and MRL/lpr mice aged 17-18 weeks. (B) Frozen kidney sections stained with CD3 (red), CD138 (green) and DAPI (blue) demonstrate CD138$^+$ T cell infiltration into the renal tissues of MRL/lpr mice (original magnification, x200). (C) Flow cytometry analyses and bar chart showing the frequency of apoptotic cells in CD3$^+$CD138$^+$ T cells of fresh splenocytes in MRL/MPJ and MRL/lpr mice. (D) Flow cytometry analyses and bar charts demonstrating the frequencies of apoptotic and live cells in CD3$^+$CD138$^-$ and CD3$^+$CD138$^+$ T cells in fresh splenocytes of MRL/lpr mice. (E) Flow cytometry analyses and bar charts showing CD69$^+$ and CD25$^+$ cell frequencies and FasL expression in CD3$^+$CD138$^-$ and CD3$^+$CD138$^+$ T cells in fresh splenocytes from MRL/lpr mice. (F) Flow cytometry analyses and bar charts demonstrating FasL expression in CD138$^-$ and CD138$^+$ DN T cells in fresh splenocytes from MRL/lpr mice. (G) Flow cytometry analyses and bar chart demonstrating CD138$^+$ cell frequencies of DN, CD4$^+$ and CD8$^+$ T cells in fresh splenocytes from MRL/lpr mice, and in fresh splenocytes from MRL/lpr mice that were cultured *in vitro* for 48 and 72 h with no stimulation. (H) Flow cytometric analyses of CD4 expression in CD4$^+$ T cell subsets. CD4$^+$ T cells of MRL/lpr mice were demonstrated to have two cell subsets, namely CD4 hi and CD4 int T cells. CD4 int T cells had significant downregulation of CD4 expression with simultaneous expression of B220. (I) Bar chart indicating CD4 expression in CD4$^+$CD138$^+$ and CD4$^+$CD138$^-$ T cells in MRL/lpr mice. (J) CD4$^+$CD138$^+$ T cells expressed B220 and were in the B220$^+$CD4 int T cell subsets. n=4-6 per group/experiment. $^{**}$P<0.01 by one-way analysis of variance. MRL/MPJ, Murphy Roths Large; MRL/lpr, Murphy Roths Large lymphoproliferative; FasL, Fas ligand; DN, double negative; 7-AAD, 7-aminoactinomycin D; MFI, mean or median fluorescence intensity.

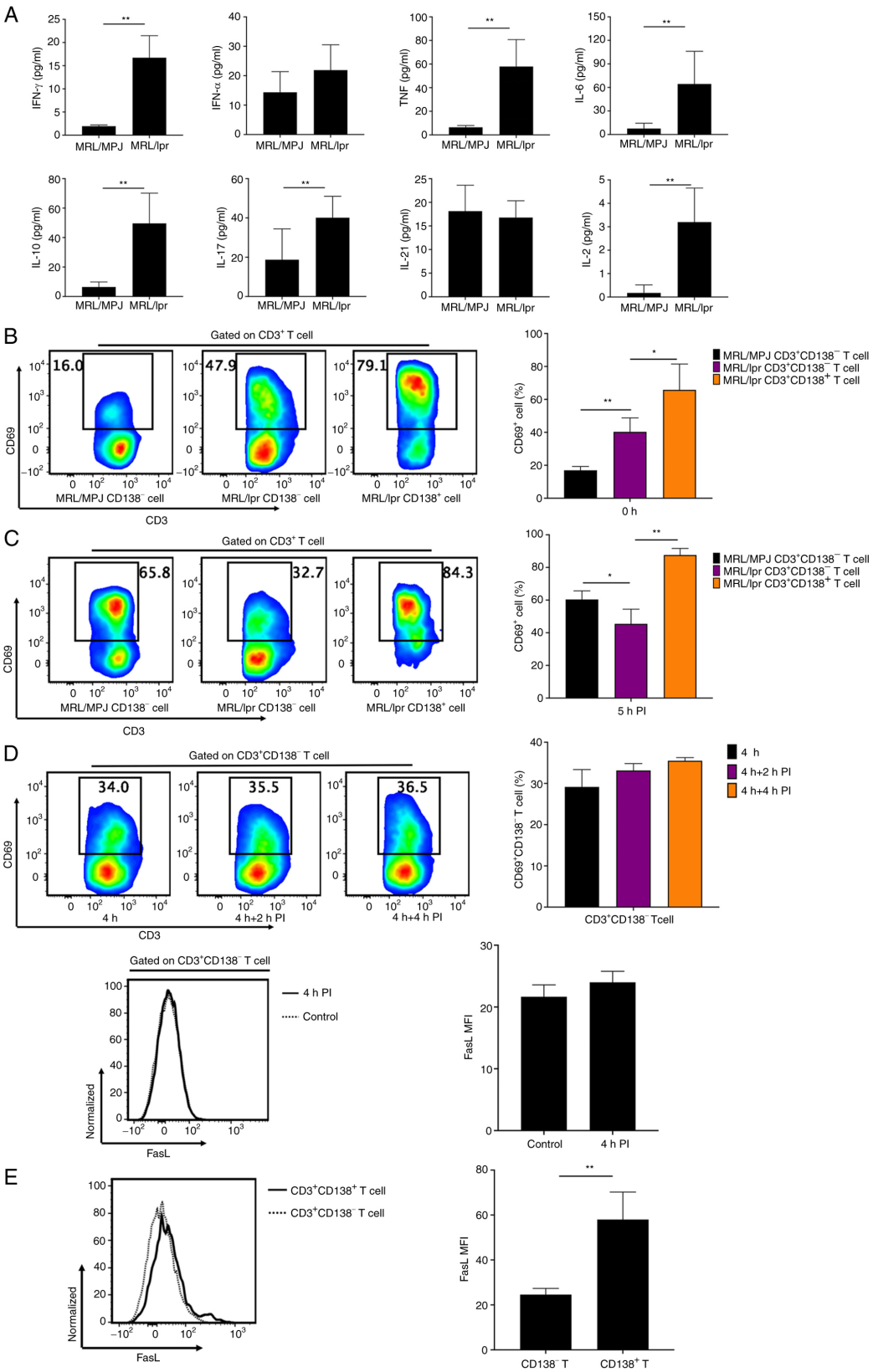


Figure 2. CD138 improves defective activation of CD138$^-$ T cells. (A) Bar charts showing serum levels of IFN-γ, IFN-α, TNF, IL-6, IL-10, IL-17, IL-21 and IL-2 in MRL/MPJ and MRL/lpr mice. (B) Flow cytometry analyses and bar chart demonstrating the frequencies of CD69$^+$ cells in CD138$^-$ T cells in fresh splenocytes from MRL/MPJ mice aged 17-18 weeks, and in CD138$^-$ and CD138$^+$ T cells in fresh splenocytes from MRL/lpr mice aged 17-18 weeks. (C) Flow cytometry analyses and bar chart demonstrating the frequency of CD69$^+$ cells in CD138$^-$ T cells in fresh splenocytes from MRL/MPJ mice aged 17-18 weeks, and in CD138$^-$ and CD138$^+$ T cells in fresh splenocytes from MRL/lpr mice aged 17-18 weeks after 5 h *in vitro* PI stimulation. (D) Flow cytometry analyses and bar charts demonstrating CD69$^+$ cell frequencies in CD138$^-$ T cells in splenocytes from MRL/lpr mice after 0, 2 and 4 h PI stimulation and FasL expression in CD138$^-$ T cells in splenocytes from MRL/lpr mice with or without 4 h PI stimulation. (E) Flow cytometry analyses and bar chart demonstrating FasL expression in CD3$^+$CD138$^+$ and CD3$^+$CD138$^-$ T cells from MRL/lpr mouse splenocytes with 4 h PI stimulation in. n=4-8 per group/experiment. $^*$P<0.05 and $^{**}$P<0.01 by one-way analysis of variance. IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; MRL/MPJ, Murphy Roths Large; MRL/lpr, Murphy Roths Large lymphoproliferative; FasL, Fas ligand; PI, ionomycin; MFI, mean or median fluorescence intensity.

*PI stimulation may significantly prevent CD138$^+$ T cell accumulation*. Splenocytes from MRL/lpr mice were stimulated by PI in order to demonstrate changes in CD138$^+$ T cell frequency. *In vitro* culture of unstimulated splenocytes from MRL/lpr mice for 4 h resulted in a significant increase in CD138$^+$ T cell frequency and CD138 protein expression in CD138$^+$ T cells, compared with that at 0 h (Fig. 3A and D). However, CD138$^+$ T cell frequencies progressively and significantly reduced after 2 and 4 h PI stimulation (Fig. 3A). PI stimulation (both 2 and 4 h) also significantly reduced CD138$^+$ cell frequencies in DN T cells and CD4$^+$ T cells compared with the 4 h DN T cells and CD4$^+$ T cells without PI stimulation (Fig. 3B and C). CD138 protein expression in CD138$^+$ T cells was also significantly and progressively downregulated after 2 and 4 h of PI stimulation, compared with the 4 h DN T cells and CD4$^+$ T cells without PI stimulation (Fig. 3D). These results demonstrated that PI stimulation prevented CD138$^+$ T cell accumulation in splenocytes from MRL/lpr mice.

*CD138 protein expression in T cells contributes to DN T cell accumulation in MRL/lpr mice*. Four-hour PI stimulation significantly reduced CD138$^+$ T cell frequency in splenocytes (Fig. 3A), as well as significantly reduced both CD3$^+$ T cell and DN T cell frequencies in splenocytes from MRL/lpr mice (Fig. 3E). However, 4-h PI stimulation failed to decrease CD4$^+$CD138$^-$ and CD8$^+$ T cell frequencies in splenocytes, conversely significantly increasing CD8$^+$ T cell frequencies (Fig. S1E). Splenocytes from MRL/lpr mice were then cultured without any stimulation for 24 h. CD138$^+$ T cell frequency in splenocytes was significantly increased after 24 h, compared with at 0 h (Fig. 3F). Simultaneously, CD3$^+$ T cell frequency and DN T cell accumulation were significantly increased at 24 h, compared with at 0 h (Fig. 3F). Conversely, 24-h *in vitro* culture without any stimulation significantly decreased the frequencies of CD4$^+$CD138$^-$ and CD8$^+$ T cells in splenocytes (Fig. S1F). These results demonstrated that CD138 protein expression significantly contributed to the increase in CD3$^+$ T cell frequency and DN T cell accumulation in splenocytes from MRL/lpr mice.

*Stimulation with PI induces specific apoptosis of CD138$^+$ T cells*. The mechanism by which PI stimulation prevented the accumulation of CD138$^+$ T cells in MRL/lpr mice was assessed. Splenocytes stimulated with 4-h PI demonstrated a significantly increased number of apoptotic cells and a decreased frequency of live cells in CD138$^+$ T cells, compared with controls (Fig. 4A). However, PI stimulation did not affect the number of apoptotic cells in CD3$^+$CD138$^-$ T cells, compared with controls (Fig. 4A). Conversely, although not significantly, PI stimulation even increased the frequency of live cells, compared with the controls (Fig. 4A). Furthermore, PI stimulation significantly decreased apoptotic cell numbers and simultaneously and significantly increased live cell numbers in CD3$^-$CD138$^+$ plasma cells, compared with the controls (Fig. 4B). This indicated that PI stimulation specifically induced cellular apoptosis in CD3$^+$CD138$^+$ T cells, but not in CD3$^+$CD138$^-$ T cells and CD3$^-$CD138$^+$ plasma cells. Moreover, the increased levels of apoptosis in CD138$^+$ T cells induced by PI stimulation were not caused by the cytotoxic effect of PI (28,29).

*Stimulation with PI promotes the activation of T cells with CD138 expression*. PI stimulation was demonstrated to have significantly increased the activation levels of CD3$^+$ T cells from MRL/lpr mice, with a significant increase in CD69$^+$ cell frequency and FasL protein expression, compared with controls (Fig. 4C). CD4$^+$ and DN T cells demonstrated significantly increased frequencies of CD69$^+$ cells after 4-h PI stimulation, compared with controls (Fig. 4D). CD69$^+$ cell frequency and FasL protein expression in CD138$^+$ T cells from MRL/lpr mice were also significantly increased after 4 h of PI stimulation, compared with the CD138$^+$ T cells without PI stimulation (Fig. 4E and F). Both CD138$^+$ DN and CD4$^+$CD138$^+$ T cells demonstrated significantly increased FasL protein expression after 4-h PI stimulation, compared with controls (Fig. 4F). However, PI stimulation failed to significantly promote the activation of CD138$^-$ T cells in MRL/lpr mice (Fig. 2D). This indicated that PI stimulation resulted in increased apoptosis levels of CD138$^+$ T cells, whilst simultaneously and significantly activating CD138$^+$ T cells but not CD138$^-$ T cells in splenocytes from MRL/lpr mice.

*The frequency of CD138$^+$ T cells is inversely associated with the activation levels of CD138$^+$ T cells*. Fresh splenocytes were isolated and cultured from MRL/lpr mice without any stimulation. CD138$^+$ T cell frequency significantly increased during the 48-h *in vitro* culture (Fig. 5A) and was accompanied by significantly reduced CD69$^+$ cell frequency in CD3$^+$ and CD138$^+$ T cells, when compared with those at 0 h (Fig. 5B-D). Subsequently, the CD138$^+$ T cell frequency significantly decreased (Fig. 5A) after 72 h of *in vitro* culture, accompanied by a significant increase in activation of CD3$^+$ and CD138$^+$ T cells, compared with that after 48 h (Fig. 5B-D). This indicated that CD138$^+$ T cell frequency was inversely associated with the activation levels of CD3$^+$ and CD138$^+$ T cells in MRL/lpr mice, suggesting that PI stimulation may have promoted the activation of CD138$^+$ T cells to induce specific apoptosis of CD138$^+$ T cells, which prevented CD138$^+$ T cell accumulation.

*CD69 protein expression in CD138$^+$ T cells results in a significant increase in apoptosis of CD138$^+$ T cells*. The authors hypothesized that the activation of CD138$^+$ T cells, stimulated by PI, induced their apoptosis. CD69$^+$ cells in CD138$^+$ T cells of MRL/lpr mice were demonstrated to have had a significant increase in apoptosis levels compared with CD69$^-$ cells in CD138$^+$ T cell populations (Fig. 5E). However, CD69 protein expression failed to significantly increase apoptosis levels of CD138$^-$ T cells in MRL/lpr mice (Fig. 5F). These results suggested that CD69 protein expression in CD138$^+$ T cells may have promoted its specific apoptosis. The increase in specific apoptosis in CD138$^+$ T cells was demonstrated to be due to the activation of CD138$^+$ T cells via the increase in CD69 protein expression.

## Discussion

The present study demonstrated that CD138$^+$ T cell apoptosis in MRL/MPJ mice operates via a Fas-dependent pathway. CD138$^+$ T cells were found to accumulate in MRL/lpr mice and CD138 protein expression in CD3$^+$ T cells significantly

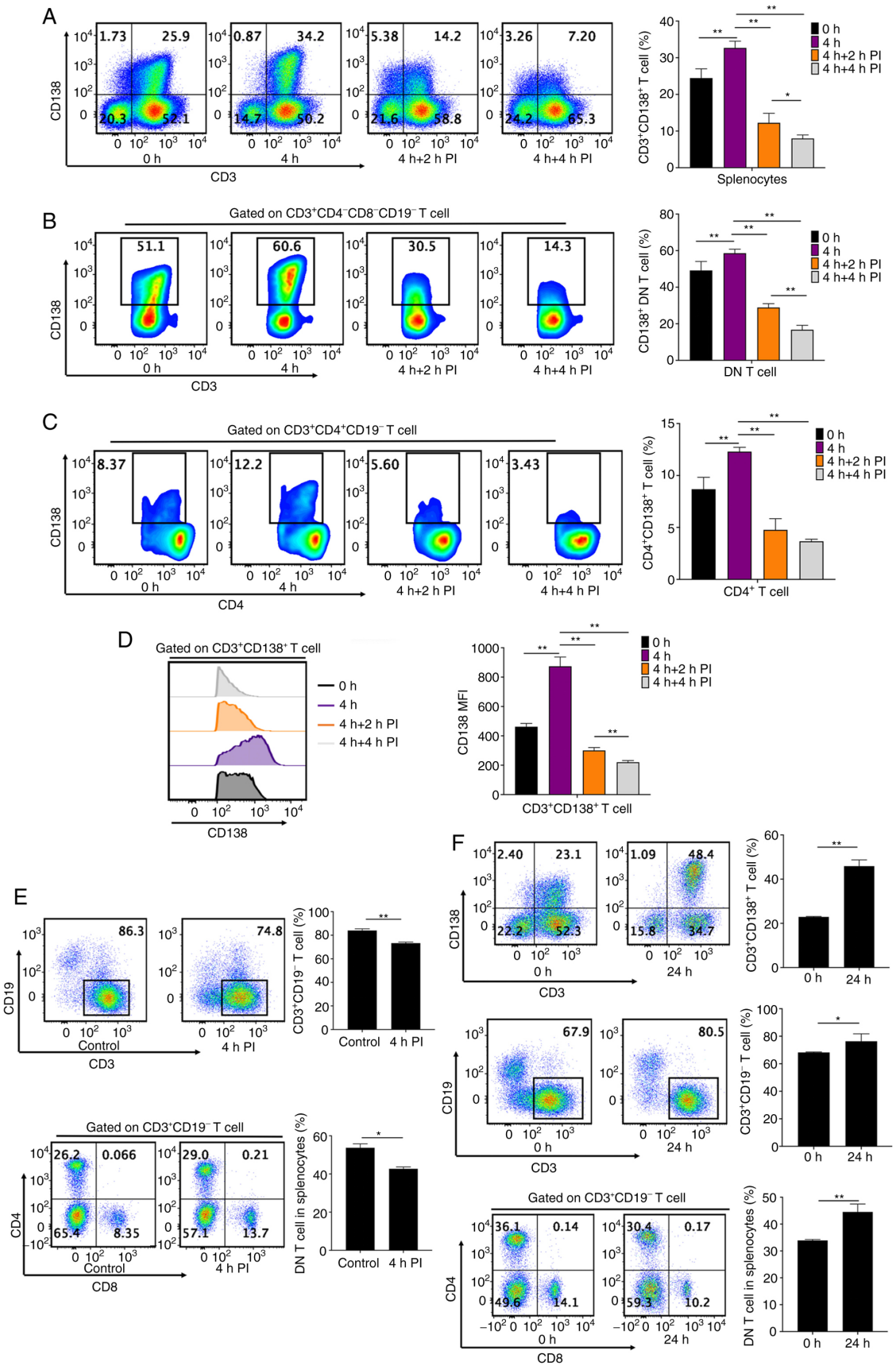


Figure 3. PI stimulation may significantly prevent CD138$^+$ T cell accumulation. Flow cytometry analyses and bar charts showing (A) CD138$^+$ T cell frequencies. (B) CD138$^+$ cell frequencies in DN T cells. (C) CD138$^+$ cell frequencies in CD4$^+$ T cells. (D) CD138 mean or median fluorescence intensity for CD138 expression in CD138$^+$ T cells of fresh splenocytes from MRL/lpr mice that were cultured *in vitro* for 0 h, 4 h, 4 h + 2 h PI stimulation and 4 h + 4 h PI stimulation. (E) Flow cytometry analyses and bar charts demonstrating frequencies of CD3$^+$ and DN T cells in splenocytes of MRL/lpr mice with or without 4 h *in vitro* PI stimulation. (F) Flow cytometry analyses and bar charts demonstrating frequencies of CD138$^+$, CD3$^+$, and DN T cells in splenocytes of MRL/lpr mice that were cultured *in vitro* for 0 and 24 h without stimulation. n=4-5 per group/experiment. $^*$P<0.05 and $^{**}$P<0.01 by one-way analysis of variance. MRL/lpr, Murphy Roths Large lymphoproliferative; PI, ionomycin; DN, double negative; MFI, mean or median fluorescence intensity.

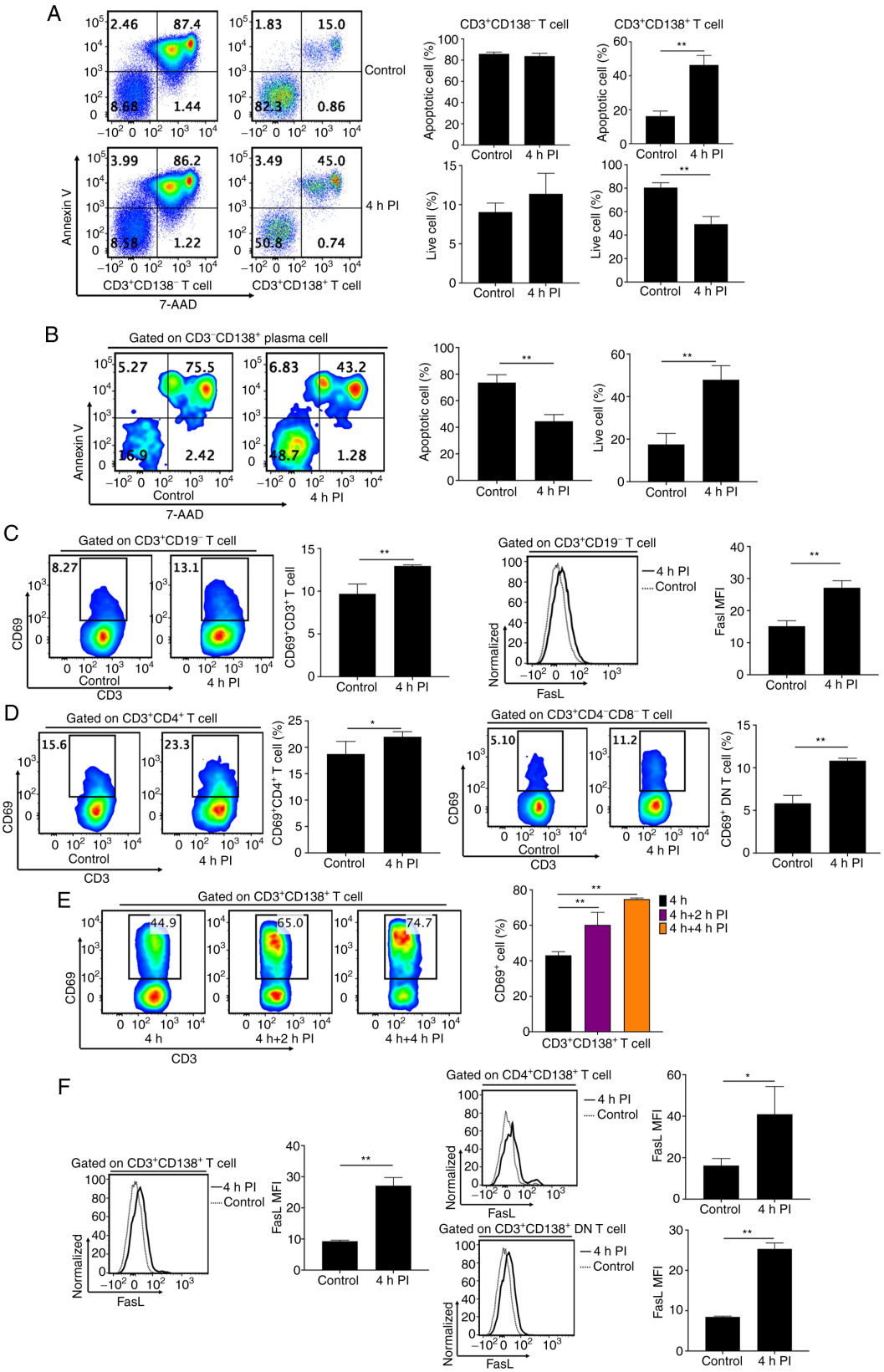


Figure 4. PI stimulation induces specific apoptosis of CD138$^+$ T cells. (A) Flow cytometry analyses and bar charts demonstrating frequencies of apoptotic and live cells in CD3$^+$CD138$^-$ and CD3$^+$CD138$^+$ T cells in splenocytes from MRL/lpr mice with or without 4 h PI stimulation. (B) Flow cytometry analyses and bar chart demonstrating the frequencies of apoptotic and live cells in CD138$^+$CD3$^-$ plasma cells in splenocytes from MRL/lpr mice with or without 4 h PI stimulation. (C) Flow cytometry analyses and bar charts demonstrating CD69$^+$ cell frequencies and FasL expression in CD3$^+$ T cells in splenocytes from MRL/lpr mice with or without 4 h PI stimulation. (D) Flow cytometry analyses and bar charts demonstrating CD69$^+$ cell frequencies in CD4$^+$ T cells and DN T cells of splenocytes from MRL/lpr mice with or without 4 h PI stimulation. (E) Flow cytometry analyses and bar charts demonstrating CD69$^+$ cell frequencies in CD138$^+$ T cells of splenocytes from MRL/lpr mice after 0, 2, and 4 h PI stimulation. (F) Flow cytometry analyses and bar charts demonstrating FasL expression in CD138$^+$, CD4$^+$CD138$^+$, and CD138$^+$ DN T cells of splenocytes from MRL/lpr mice with or without 4 h PI stimulation. n=4-5 per group/experiment. $^*$P<0.05 and $^{**}$P<0.01 by one-way analysis of variance. MRL/lpr, Murphy Roths Large lymphoproliferative; PI, ionomycin; FasL, Fas ligand; DN, double negative; 7-AAD, 7-aminoactinomycin D; MFI, mean or median fluorescence intensity.

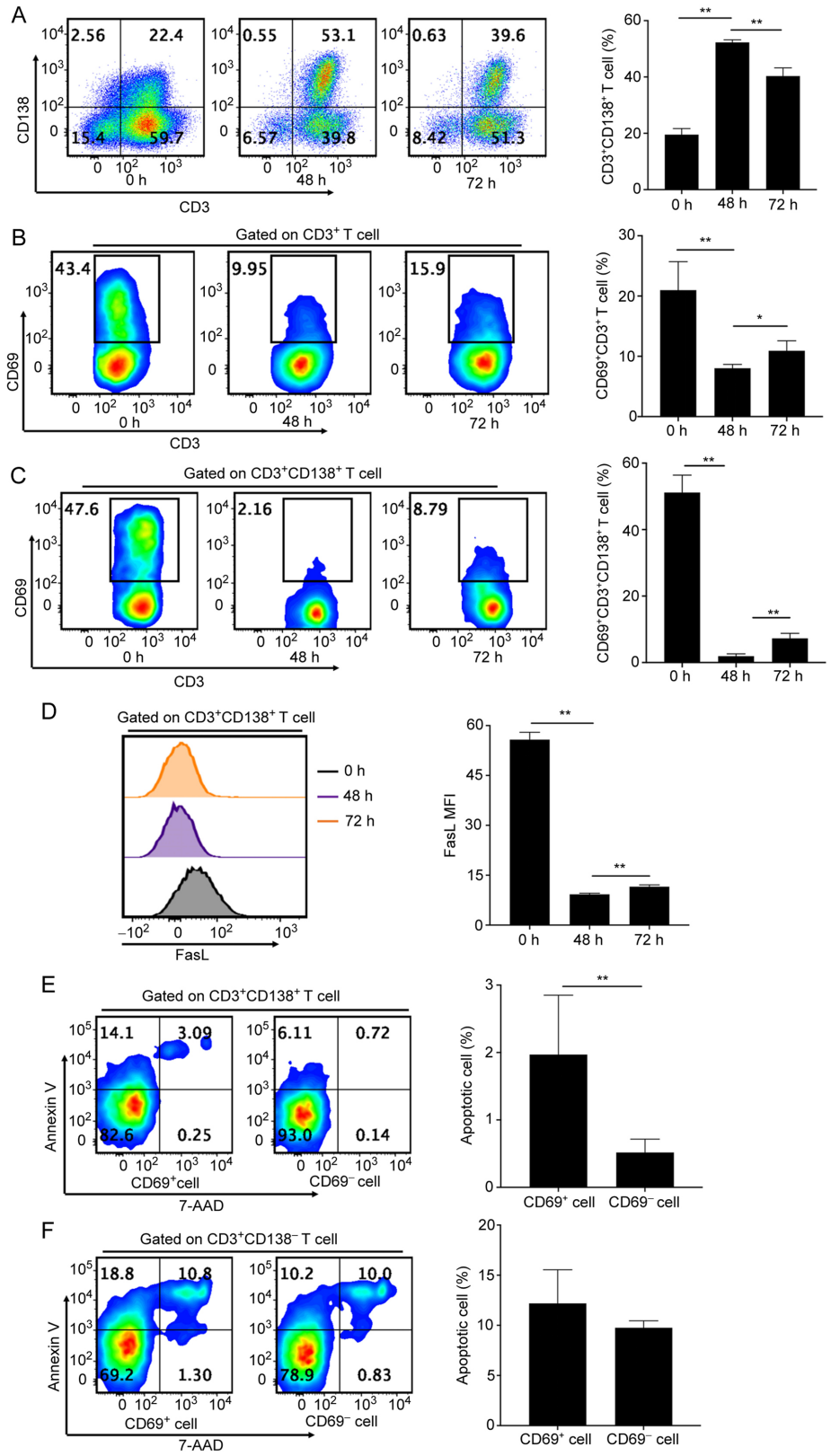


Figure 5. CD138$^+$ T cells are inversely associated with cellular activation. (A) Flow cytometry analyses and bar charts demonstrating frequencies of CD138$^+$ T cells in fresh splenocytes from MRL/lpr mice, in fresh splenocytes from MRL/lpr mice that were cultured *in vitro* for 48 and 72 h. (B) Flow cytometry analyses and bar chart demonstrating CD69$^+$ cell frequencies in CD3$^+$ T cells in fresh splenocytes from MRL/lpr mice, and in fresh splenocytes from MRL/lpr mice that were cultured *in vitro* for 48 and 72 h. (C) Flow cytometry analyses and bar chart demonstrating CD69$^+$ cell frequencies in CD138$^+$ T cells in fresh splenocytes from MRL/lpr mice, and in fresh splenocytes from MRL/lpr mice that were cultured *in vitro* for 48 and 72 h. (D) Flow cytometry analysis and bar chart demonstrating FasL mean or median fluorescence intensity for FasL expression in CD138$^+$ T cells in fresh splenocytes from MRL/lpr mice and in fresh splenocytes of MRL/lpr mice that were cultured *in vitro* for 48 and 72 h. (E) Flow cytometry analyses and bar charts demonstrating apoptotic cell frequencies of CD69$^+$ and CD69$^-$ cells in CD138$^+$ T cells of fresh splenocytes from MRL/lpr mice. (F) Flow cytometry analyses and bar charts demonstrating apoptotic cell frequencies of CD69$^+$ and CD69$^-$ cells in CD138$^-$ T cells of fresh splenocytes from MRL/lpr mice. n=4-6 per group/experiment. $^*$P<0.05 and $^{**}$P<0.01 by one-way analysis of variance. MRL/lpr, Murphy Roths Large lymphoproliferative; FasL, Fas ligand; MFI, mean or median fluorescence intensity; 7-AAD, 7-aminoactinomycin D.

prevented the apoptosis of $CD3^+$ T cells. This contributed to the accumulation of $CD3^+$ T and DN T cell subsets whilst simultaneously promoting $CD3^+$ T cell activation in Fas-deficient MRL/lpr mice.

CD138 protein expression may also have increased FasL protein expression in DN T cells to promote their cytotoxicity. PI stimulation prevented $CD138^+$ T cell accumulation and decreased $CD138^+$ cell frequencies in DN and $CD4^+$ T cells by inducing specific apoptosis of $CD138^+$ T cells. The specific apoptosis was caused by the activation of $CD138^+$ T cells via an increase in CD69 protein expression.

Fas (CD95) is a member of the TNF receptor family and interacts with FasL after TCR activation to induce apoptosis (16). Fas deficiency leads to DN T cell accumulation in MRL/lpr mice resulting in lymphadenectasis and splenomegaly (30,31). Results from the present study demonstrated that apoptosis of $CD138^+$ T cells was via a Fas-dependent pathway. A previous study reported that Fas deficiency in MRL/lpr mice led to the accumulation of $CD138^+$ T cells as they aged and lupus developed (12). Moreover, the majority of $CD138^+$ T cells were demonstrated to be $CD4^-$ and $CD8^-$ DN T cells in the present study. DN T cells had been commonly regarded as the abnormal T cells closely related to SLE. However, most of the DN T cells are also the CD138+ T cells. It is interesting that these two subjects are related to each other and suggests CD138 expression in T cells would implicate in SLE.

CD138 protein expression was demonstrated to have significantly increased FasL protein expression in $CD3^+$ T cells and their subsets, including DN T cells. However, DN T cells in MRL/lpr mice were reported to upregulate FasL and be strongly cytotoxic. This results in autoimmune injuries to multiple tissues and organs that express small amounts of the Fas receptor (6,16). In the present study, however, the results showed CD138 protein expression significantly increased the cytotoxicity of DN T cells, which in turn promotes lupus development and tissue injury in MRL/lpr mice.

The increase in $CD138^+$ T cells significantly decreased the number of apoptotic cells in the present study, whilst simultaneously increasing the number of live cells compared with $CD138^-$ T cells. This demonstrated that $CD138^+$ T cells had defective apoptosis in MRL/lpr mice. Moreover, previous studies reported that $CD138^+$ T cells had a lower proliferation compared to $CD138^-$ T cell subsets (12,14). Based on these results, it was concluded that $CD138^+$ T cell accumulation was mainly a result of their defective apoptosis. CD138 protein expression significantly contributed to the accumulation of T and DN T cells in MRL/lpr mice by reducing the number of apoptotic T and DN T cells.

Autoantibody production has a detrimental effect on multiple organs and serves a key role in SLE progression (32). Immature T cells undergo both positive and negative selection to become mature single positive T cells. During this process, T cells that recognize self-antigens are eliminated (33,34). Autoreactive T cells are usually eliminated by Fas-mediated apoptosis during negative selection in the thymus (35). Fas deficiency results in autoreactive T cells escaping negative selection (36,37). $CD138^+$ T cells commonly express B220, which is also expressed on autoreactive T cells, such as non-selected $CD8^+$ and DN T cells (35,38). $CD4^+CD138^+$ T cells were reported to be important for the generation of autoantibodies, such as anti-dsDNA and anti-smooth muscle antibodies (12). $CD138^+$ T cells were also reported to promote tissue injury when self-antigens are exposed to the immune system (7,12). Results from the present study demonstrated that $CD138^+$ T cells were autoreactive T cells that escaped Fas-dependent apoptosis during negative selection (12,14).

The present study demonstrated that CD138 protein expression in T cells served a key role in the progression of lupus in MRL/lpr mice. In addition to the significant decrease in apoptosis levels, $CD138^+$ T cells were demonstrated to be activated more easily than $CD138^-$ T cells. This indicated that CD138 protein expression could promote the activation of $CD138^-$ T cells in MRL/lpr mice. However, the mechanism by which CD138 protein is expressed in these abnormal T cells was not elucidated. The results from the present study demonstrated that $CD138^-$ T cells in MRL/MPJ mice were more easily activated compared to $CD138^-$ T cells in MRL/lpr mice. PI stimulation failed to significantly activate $CD3^+CD138^-$ T cells in MRL/lpr mice. These results indicated that $CD138^-$ T cells in MRL/lpr mice had defective activation. However, CD138 protein expression significantly reversed the defective activation of $CD3^+CD138^-$ T cells and promoted the activation of $CD3^+$ T cells in MRL/lpr mice.

PI stimulation could also significantly decrease CD138 protein expression in $CD138^+$ T cells. PI stimulation has been used to activate T cells and promote cytokines secretion in T cells (22,23). However, in the present study, PI stimulation was able to prevent $CD138^+$ T cell accumulation by inducing specific apoptosis of $CD138^+$ T cells. Furthermore, PI stimulation could significantly reduce the frequency of $CD138^+$ cells in the $CD3^+$ T cell population and its cell subsets by increasing the activation levels of $CD3^+$ T and $CD138^+$ T cells. A previous study demonstrated that TCR activation downregulated CD138 protein expression in DN T cells (14). The results from the present study indicated that CD138 protein expression in $CD3^+$ T cells could be prevented by PI stimulation to activate $CD3^+$ T cells.

CD69 and CD25 are broadly known as markers for early and late active T cells (12). Results from the present study demonstrated that $CD138^+$ T cells had a high level of CD69 protein expression and low levels of CD25 protein expression. In addition, it was demonstrated that $CD69^+$ cells in the $CD138^+$ T cell population had a significant increase in apoptosis levels compared to $CD69^-$ cells in the $CD138^+$ T cell population. However, both PI stimulation and TCR activation could significantly activate T cells and subsequently increase the number of $CD69^+$ cells in the T cell population (22,39). The present study demonstrated that the increased apoptosis of $CD138^+$ T cells and subsequent decreased accumulation of $CD138^+$ T cells were a result of the activation of $CD138^+$ T cells via the increase of CD69 protein expression.

A previous study also reported that $CD138^+$ T cells were autoreactive T cells that promoted autoantibody production when self-antigens were exposed to the immune system (12). The results from the present study demonstrated that $CD138^+$ T cell frequency was inversely associated with the activation levels of $CD3^+$ T and $CD138^+$ T cells. $CD3^+CD138^-$ T cells in MRL/lpr mice had defective activation, whilst CD138 protein expression significantly increased the activation of $CD3^+CD138^-$ T cells. PI stimulation activated $CD3^+$ T cells and simultaneously prevented

CD138 protein expression in $CD3^+CD138^-$ T cells. These results indicated that autoreactive $CD3^+CD138^-$ T cells in MRL/lpr mice failed to be activated in the absence of self-antigen exposure, which may have promoted the upregulation of CD138 protein expression. CD138 protein expression in these T cells therefore promoted the accumulation of autoreactive T cells and the autoreactive response in lupus.

The present study provided a novel insight into the mechanism of autoreactive T cell promotion in the progression of SLE. However, the findings were based on murine SLE models. Clinical studies are required to substantiate these findings to determine the specific marker expressed in human autoreactive T cells that serves a key role in promoting SLE development. In addition, the critical proteins that regulate CD138 protein expression in autoreactive T cells have remained elusive. In the future, more research needs to be conducted to explore the underlying mechanisms of the critical proteins regulating CD138 protein expression in autoreactive T cells.

In conclusion, CD138 protein expression in T cells was implicated in the progression of lupus in MRL/lpr mice. CD138 protein expression in $CD3^+$ T cells prevented apoptosis of T cells in MRL/lpr mice, simultaneously contributed to $CD3^+$ and DN T cell accumulation and promoted $CD3^+$ T cell activation. CD138 protein expression in DN T cells significantly increased their FasL protein expression, enhancing the cytotoxicity of the DN T cells. Defective apoptosis, induced by CD138 protein expression in $CD3^+$ T cells, resulted in $CD138^+$ T cell accumulation in the spleens of MRL/lpr mice. Results from the present study indicated that activation of T cells prevented $CD138^+$ T cell accumulation by increasing the specific apoptosis of $CD138^+$ T cells.

Furthermore, the results demonstrated that $CD138^-$ T cells in MRL/lpr mice had defective activation. Increased activation levels of $CD3^+$ T cells could prevent CD138 protein expression in $CD3^+$ T cells of MRL/lpr mice. The results also suggested that CD138 protein expression in $CD3^+$ T cells of Fas-deficiency MRL/lpr mice may be caused by the failure of activation in autoreactive T cells before self-antigen exposure to the immune system.

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**Availability of data and materials**

The datasets used and/or analyzed during the current 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 XL performed the laboratory work. TX and XL performed the data analysis. PL revised and edited the manuscript. All authors read and approved the final version of the manuscript. TX and XL confirm the authenticity of all the raw data.

**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 (approval no. 2021040202 and were performed according to institutional ethical guidelines.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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