Research Square

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Differential effects of calcineurin inhibitors on plasma cells: Potential therapy for antibody-mediated rejection

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Research Article

Keywords: cyclosporine, tacrolimus (FK506), antibody-mediated rejection, ER stress, plasma cell, CD138

Posted Date: September 12th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4843956/v1

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Additional Declarations: No competing interests reported.

Abstract

Calcineurin inhibitors, including cyclosporine and tacrolimus, are widely used to prevent postoperative rejection after solid organ transplantation and have successfully prolonged the survival of allografts since their introduction. The use of calcineurin inhibitors has dramatically reduced the rate of acute cellular rejection; however, the long-term survival of allografts is still compromised by the damage caused by alloantibodies and antibody-mediated rejection (AMR). Currently, AMR is the most important issue in controlling organ transplantation rejection. The pathophysiological mechanism of AMR is associated with organ damage after prolonged exposure to alloantibodies, which are synthesized and secreted by plasma cells. Therefore, targeting plasma cells to develop a treatment for AMR is an important issue. Since the introduction of tacrolimus (FK506) into the field of organ transplantation, FK506 has successfully suppressed the incidence of acute cellular rejection but is not satisfactory in terms of antibody-mediated rejection. In our study, we found that cyclosporine (CsA) induced endoplasmic reticulum (ER) stress in plasma cells, which was lower in the presence of FK506. The expression of CD138 in plasma cells can prolong the half-life of plasma cells; we found that ER stress in plasma cells induced by CsA could downregulate the protein expression of CD138, inhibit the p-STAT3 signaling and reduce cell survival, thus leading to cell death. Our findings offer an updated insight into the pharmacological effects of CsA on plasma cells, providing valuable options for tailoring treatment strategies in transplant patients undergoing treatment for AMR.

Introduction

Organ transplantation is one of the treatment options for end-stage organ failure. However, the alloimmune response to the allograft remains a significant challenge in preserving long-term allograft survival. Calcineurin inhibitors, including cyclosporine (CsA) and tacrolimus (FK506), are the most widely used immunosuppressive drugs for the prevention of acute rejection after organ transplantation. CsA and FK506 can block calcineurin activity and prevent the downstream protein nuclear factor of activated T cells (NFAT) from translocating into the nucleus in activated T cells, reducing the transcription of cytokines, such as interleukin 2 (IL-2), and inhibiting T-cell activation and proliferation. Nowadays, acute cellular rejection is well controlled with current immunosuppressive regimens (Qian 2015, Wood, Shankar et al. 2019), but subsequent findings reveal that the majority of organ transplant recipients receiving calcineurin inhibitors (CNIs) experience CNI-related renal toxicity years later. In vitro studies suggest that CsA poses a higher risk of renal toxicity compared to FK506 (Yilmaz, Kirschner et al. 2022). These study results have influenced the treatment strategies for organ transplant recipients. Over the past decade, healthcare professionals have leaned towards adopting FK506 as the immunosuppressive drug of choice, leading to a gradual increase in the usage of FK506 (Tsai, Cheng et al. 2012, Nelson, Alvey et al. 2022). One of the major challenges in clinical transplantation today is antibody-mediated rejection (AMR) due to donor-specific alloantibodies, which are highly associated with the loss of allografts (Svachova, Sekerkova et al. 2016, Valenzuela and Reed 2017, Kim and Brennan 2021). The current standard treatment for acute AMR includes plasmapheresis plus intravenous immunoglobulin (IVIG) (Wan, Ying et

al. 2018), unfortunately, the long-term results of AMR are not satisfactory. Therefore, more strategies are needed for the proper management of AMR.

The function of the endoplasmic reticulum (ER) is important for plasma cells to produce large amounts of antibodies. X-box binding protein 1 (XBP1), which is downstream of B-lymphocyte-induced maturation protein 1 (BLIMP1), is required for the differentiation of B cells into plasma cells and expansion of ER capacity for antibody synthesis. Knockout of XBP1 in mice leads to the absence of plasma cells and reduced levels of circulating antibodies but shows no effect on B-cell maturation or antibody isotype switching (Taubenheim, Tarlinton et al. 2012). Treatment of plasma cells with the proteasome inhibitors bortezomib or ONX0914 causes ER stress and leads to cell death (Neubert, Meister et al. 2008, Li, Koerner et al. 2019). ER stress involves the accumulation of misfolded or unfolded proteins in the ER, which activates the homeostatic process called the unfolded protein response (UPR). The UPR is activated by the actions of three ER transmembrane stress pathways: the PKR-like ER kinase (PERK) pathway, the activating transcription factor 6α (ATF6α) pathway, and the inositol-requiring enzyme 1α (IRE1α) pathway. In addition to activating the UPR, misfolded proteins also initiate the ER-associated degradation (ERAD) response, which transports excessive misfolded proteins out of the ER for degradation through the ubiquitin-proteasome pathway. When ER stress is prolonged and the UPR fails to alleviate it, cells undergo apoptosis (Lindholm, Korhonen et al. 2017). It has been reported that CsA induces ER stress and upregulates BiP and CHOP protein expression, which leads to the death of renal tubule cells (Han, Li et al. 2008, Pallet, Bouvier et al. 2008). Another study showed that increased expression of ER stress markers and chaperone proteins induced by CsA could induce phenotypic changes and the death of endothelial cells. However, the effect of FK506 on ER stress was mild (Bouvier, Flinois et al. 2009). Therefore, we hypothesize that CsA and FK506 may cause different ER stress responses in plasma cells.

The most essential factor in generating and maintaining the survival of plasma cells is IL-6 (Jourdan, Cren et al. 2014), and in IL-6-knockout mice, antigen-specific plasma cells were reduced (Manfred Kopf 1994). It has been reported that CD138 (syndecan-1) can bind to IL-6 and APRIL receptors to initiate signaling, which increases the expression of APRIL and IL-6 in plasma cells through positive feedback, enhances the presentation of the downstream survival factors Bcl2 and Mcl1, and protects plasma cells from premature apoptosis (McCarron, Park et al. 2017). CD138 is expressed in precursor B cells, but its expression disappears after these cells enter the circulation and mature; CD138 is then re-expressed in plasma cells after stimulation and differentiation (O'Connell, Pinkus et al. 2004). CD138 serves as a marker or receptor on the cell surface but does not directly facilitate signal transduction into the cell. It provides specific docking sites for growth factors or cytokines; it helps receptors amplify signal transmission and sensitivity and ultimately promotes cellular growth (Zemin Ren 2021). The study further revealed that myeloma cells exhibiting higher levels of CD138 expression demonstrated better survival status at starvation and more sensitivity to IL-6 signaling compared to those with lower CD138 expression levels (Akhmetzyanova, McCarron et al. 2020). Therefore, the expression levels of CD138 and IL-6 signaling have an interactive effect on the survival of plasma cells.

Since AMR remains a challenging issue in the field of organ transplantation, the effect of immunosuppressive drugs on plasma cells is worth studying. To understand the effect of calcineurin inhibitors on plasma cells, we treated plasma cell lines with CsA or FK506 and analyzed antibody production and cell viability. We found that antibody production was reduced in a dose-dependent manner, and the proportion of apoptotic cells was increased in plasma cells that were treated with CsA but not FK506. ER stress-related proteins were upregulated in plasma cells, and the expression of CD138 was reduced in cells that were treated with CsA. These results suggest that CsA may suppress the activity of plasma cells and have a beneficial effect on controlling AMR.

Materials and methods

Cell culture

The P3X63Ag8 cell line, which is a murine IgG1-secreting B lymphocyte cell line, was purchased from Bioresource Collection and Research Center (Hsinchu City, Taiwan). MF20 cells, a proprietary murine hybridoma IgG2b-secreting cell line from Developmental Studies Hybridoma Bank (DSHB), were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA), supplemented with 10%(v/v) FBS and2 mM L-glutamine (Thermo Fisher Scientific). Cells (4x 10^5 per well in 6-well plates) were cultured with or without clinical doses of CsA (0.25, 0.5, and 1 μM) and FK506 (5, 10, and 20 nM) for 4, 8, 12, and 24 hours. Tunicamycin (Tm) was used (2.5 or 0.1 μg/ml) as the ER stress-positive control. The ER stress inhibitor 4μ8C (IRE1αinhibitor, 5 μM) was used to treat cells in the presence or absence of CsA and FK506. CsA, FK506, Tm, and 4μ8C were purchased from Sigma–Aldrich (St. Louis, MO).

Reagents and antibodies

The following reagents and antibodies were used: RIPA buffer (AB156034; Abcam, Cambridge, UK), protease and phosphatase Inhibitor cocktail (AB156034; Abcam), anti-BiP antibody (610978; BD Bioscience), anti-CHOP antibody (#2895; Cell Signaling), anti-XBP1 antibody (ab220783; Abcam), anti-STAT3 (phosphor-Y705) antibody (AB76315; Abcam), anti-STAT3 antibody (sc-8019; Santa Cruz), anti-PERK antibody (IR135-538; iReal Biotechnology, Hsinchu City, Taiwan), anti-ATF6 antibody (IR132-533; iReal Biotechnology), Syndecan-1 (CD138; ab128936; Abcam), and Syndecan-4 (sc-12766; Santa Cruz).

Western blotting

Cells were lysed in RIPA buffer containing a protease and phosphatase Inhibitor cocktail, sonicated, and centrifuged to obtain protein lysates. Equal amounts of protein lysates (10–15 μg) were separated by SDS–PAGE and electro-transferred to PVDF membranes (Millipore, Billerica, MA). The membrane was blocked with TBST containing 0.2% Tween 20 and 5% nonfat milk for 1 h. The membranes were immunoblotted with specific primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies and developed with Immobilon Western HRP Chemiluminescent Substrate (GE

Healthcare, Chicago, IL). Then, the chemiluminescent signal was captured by Image Quant™ LAS 4000 (GE Healthcare).

RNA isolation and reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was purified using a GENEzol™ TriRNA Pure Kit (Geneaid, New Taipei City, Taiwan). For the RT experiment, 1 μg of RNA was used for reverse transcription with iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA). The PCR conditions were as follows: 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The RT–PCR products were resolved on agarose gels and detected with a UVP BioDoc-It®2 Imaging System (Analytik Jena, Jena, Germany). The primers for the amplification of specific transcripts were as follows:

XBP1

forward 5'-GTCTGCTGAGTCCGCA-3' and

reverse 5'-TCCTTCTGGGTAGACCTCTGGGAG-3'.

GAPDH

forward 5'-TGACTCCACTCACGGCAAAT-3' and

reverse 5'-GGCATGGACTGTGGTCATGAG-3'.

ELISA

The levels of IgG in the culture medium were analyzed using the Mouse IgG ELISA kit (501240; Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Briefly, 100 μl of each standard and diluted sample were added to a 96-well plate for one hour at room temperature with gentle shaking. After emptying and washing the wells, 100 μl of streptavidin–HRP solution was added to each well and incubated for one hour at room temperature. Then, the wells were emptied and washed, 100 μl of TMB substrate was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking, followed by the addition of 100 μl of stop solution. The absorbance was measured at 450 nm with a SpectraMax iD5 (Molecular Devices, San Jose, CA).

Cell death and apoptosis analysis

To analyze cell death at different times, plasma cells (10^4 cells per well in a 96-well plate) were treated with DMSO, CsA, FK506, or Tm and cultured in DMEM containing 1μM Hoechst 33342 (Thermo Fisher Scientific) and 1 μg/ml propidium iodide (PI; Sigma–Aldrich, St. Louis, MO.). Time-lapse images of plasma cells were captured by the ImageXpress® Nano Automated Imaging System (Molecular Devices, San Jose, CA). The image data were analyzed by MetaXpress software. Quantitative analysis of plasma cell apoptosis after treatment with DMSO, CsA, FK506, or Tm was assessed by annexin V and 7-AAD staining. Briefly, the cells were incubated with binding buffer containing CD138 antibodies, Annexin V-

FITC, and 7-Aminoactinomycin (7-AAD; BioLegend, San Diego, CA) for 15 minutes at 4°C. The proportion of apoptotic cells was analyzed by an LSRII flow cytometer (BD Bioscience), and the data were analyzed by FlowJo™ Software for Windows Version 10 (BD Bioscience).

siRNA interference for CD138

siRNA targeting the CD138 gene was purchased from Invitrogen (ID: s74642, Carlsbad, CA, USA), and negative control siRNA (siNC) was purchased from Biotools (BIOTOOLS Co., Ltd, Taiwan). All siRNA were used at 200 nM final concentration. $1x10^6$ P3X63Ag8 cells were transfected using the Neon Transfection System (Invitrogen) (1 pulse at 1500 V, 20 ms). After 24 hours, the cells were treated with DMSO, 10 μM CsA, 10 μM FK506, and 0.1 μg/ml Tm for 4 hours, then the cells were collected, and the apoptosis cells were analyzed by flow cytometry.

Mitochondrial respiration assays

The cells were seeded at 5 $x10^4$ per well in a Seahorse 24-well plate coated with Cell-Tak™ (Corning #354240) and treated with DMSO, CsA, FK506, and Tm for 4 hrs. The oxygen consumption rate (OCR) was determined by a Seahorse XFe24 Extracellular Flux Analyzer (Agilent, Santa Clara, CA). Reagents were added at the following final concentrations: 1 μM oligomycin, 1 μM FCCP, and 0.5 μM rotenone/antimycin A (Salabei, Gibb et al. 2014).

Animals

BALB/c and C57BL/6 mice aged 8–10 weeks were kept at the Experimental Animal Center of National Taiwan University. The animal care and experimental protocol followed the guidelines set by the Institutional Animal Care and Use Committee. (approval numbers:20190125 and 20200157).

Immunization and drug administration

Whole blood was obtained from BALB/c mice via cardiac puncture. Approximately 0.1 ml of whole blood from BALB/c mice was transfused into C57BL/6 mice through the jugular vein. The mice were randomly separated into 3 groups: the control, CsA, and FK506 groups. CsA (Sigma–Aldrich; 0.2 mg/20 g BW/50 μl) and FK506 (Sigma–Aldrich; 0.004 mg/20 g BW/50 μl) were dissolved in olive oil and administered to C57BL/6 mice by oral gavage every day beginning on Day 7 of transfusion for a total of 14 days. The control group received olive oil by oral gavage for 14 days, the same as the other groups. The mice were sacrificed on Day 20, and the spleen was collected for the subsequent analysis of plasma cells.

Analysis of CD138 expression in plasma cells.

This analytical approach is based on the article written by Wilmore JR et al. (Wilmore JR 2017) In brief, the spleen was collected from mice, washed, and resuspended at $1x10^7$solenocytes/100 μl in PBS with 2% FBS and was incubated with antibodies for 20 min at 4°C. After being stained, the cells were washed, fixed, and analyzed by an LSR II flow cytometer (BD Bioscience). In the previous step, the following antibody-conjugated fluorophores were used to assess the cellular phenotype: anti-CD4-PE-Cy7, anti-

CD8-PE-Cy7, anti-F4/80-PE-Cy7, anti-TER-119-PE-Cy7, anti-IgD-APC-Cy7, anti-Sca1-BV605, (BioLegend, San Diego, CA) and anti-SDC1-BV421 (BD Biosciences).

Statistical analysis

The data were analyzed and charted by GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and are presented as the means ± standard errors. Significance was determined by Student's two-tailed t-test, and one-way ANOVA was used to compare more than two groups. The P value less than 0.05 was considered to be significant.

Results

CsA induced an ER stress response through the IRE1α pathway and reduced IgG production in plasma cells

According to published reports, treating plasma cells with the proteasome inhibitor bortezomib or ONX0914 leads to cell death by causing ER stress (Neubert, Meister et al. 2008, Li, Koerner et al. 2019); therefore, we examined whether calcineurin inhibitors induced ER stress. First, we treated cells with CsA (0.25, 0.5, 1 μM) or FK506 (5, 10, 20 nM) and analyzed the expression of ER stress-related proteins by western blotting. As shown in Fig. 1A, the Bip, XBP1s, and CHOP protein levels were upregulated in a dose-dependent manner in CsA-treated cells but not in FK506-treated cells. The ER stress response is activated via three pathways: PERK, IRE1α, and ATF6. We observed an upregulation of XBP1s in cells treated with calcineurin inhibitors in time course (Fig. 1B, 1C), but PERK and ATF6 were not involved in the stress response induced by CsA or FK506 treatment (Fig. S1A, S1B), and the increase in XBP1s expression in CsA-treated plasma cells could be inhibited by the IRE1α inhibitor 4μ8C (Fig. 1D). These data show that CsA but not FK506 can induce plasma cell ER stress through the IRE1α-XBP1 pathway. To understand the effect of calcineurin inhibitors on antibody secretion by plasma cells, the cells were treated with calcineurin inhibitors for 24 hrs after serum starvation overnight, and then the culture medium was collected to analyze the concentration of IgG. The results showed that IgG production was decreased in a dose-dependent manner in the CsA-treated groups (Fig. 1E). IgG production in the FK506-treated group was similar to that in the DMSO-treated control group. We repeated the experiments with another antibody-producing cell line, MF20, and the expression profile of ER stress proteins (Fig. S2) and decreasing IgG production (Fig. S3) in CsA-treated MF20 cells was similar to that of P3X63Ag8 cells.

CsA treatment of plasma cells induced more apoptosis than FK506 treatment.

After determining the effect of CsA on IgG secretion, we investigated whether CsA caused apoptosis in plasma cells. We treated the cells with 1 μM CsA or 20 nM FK506 and analyzed the proportion of cell death using the ImageXpress Nano Automated Imaging System. The proportion of cell death was significantly increased in the CsA-treated group compared with the FK506-treated group at 12 to 24 hrs (Fig. 2A-B, Fig. S4A, S4B). To determine whether CsA-induced cell death could be attributed to apoptosis, we treated cells with 1μM CsA or 20nM FK506 for 4 and 8 hours, then analyzed apoptosis by flow

cytometry. The data showed that the proportion of apoptotic cells was increased in the CsA-treated group at 4 hrs and 8 hrs, but there was no difference in the FK506-treated group (Fig. 2C-E). To demonstrate the significantly different proportion of apoptotic cells, we treated plasma cells with 10 μM CsA and FK506 for 24 hrs and the data revealed that the proportion of apoptotic cells in the CsA-treated group was higher compared to the FK506-treated group (Fig. 2F-G). These data indicated that CsA treatment could induce cell apoptosis and resulting in decreased secretion of IgG.

CsA-treated plasma cells decreased the surface expression of CD138 and inhibited p-STAT3 following impacted cell viability.

Recent studies indicate that syndecan-1 (CD138; SDC1) is essential for prolonging the survival of differentiated plasma cells, and lower expression of CD138 in plasma cells reduces IL-6 signaling and increases apoptosis (McCarron, Park et al. 2017, Akhmetzyanova, McCarron et al. 2020). Syndecan-1-negative plasma cells are unable to respond to survival signals conveyed by IL-6 and a proliferation-induced ligand (APRIL) (Zemin Ren 2021).To understand whether CsA induces apoptosis in plasma cells by downregulating the expression of CD138, we analyzed CD138 expression in calcineurin inhibitor-treated plasma cells by western blotting. We found that treatment with CsA but not FK506 reduced the expression of CD138 in cells (Fig. 3A, 3B). CD138 is one of the members of the heparan sulfate proteoglycan (HSPG) family. The HS chain provides specific binding sites for various biomolecules, such as growth factors and cytokines, and induces multiple cellular effects (Yamaguchi 1991); we examined whether other HSPG proteins could be affected by CsA treatment similar to CD138. The western blot data showed that Syndecan-4 (SDC4) was not significantly different between plasma cells treated with CsA or FK506 (Fig. 3C, 3D). Then, to examine CD138 expression in cells undergoing apoptosis, we treated cells with calcineurin inhibitors for 24 hrs and analyzed the expression level of CD138 by flow cytometry. The expression of CD138 on cells undergoing apoptosis was lower than on healthy cells (Fig. 3E). The total MFI of CD138 expression level in cells was reduced in CsA treated group (Fig. 3F). To investigate the role of CD138 expression on plasma cells in CsA-induced apoptosis, we utilized siRNA transfection to knock down CD138 expression in the cells. Subsequently, the CD138 knockdown cells were treated with calcineurin inhibitors, and the apoptosis ratio was analyzed using flow cytometry. The results demonstrated that the proportions of CD138-knockdown plasma cells treated with CsA and FK506 were comparable (Fig. 3G, 3H). These data indicate that CD138 knockdown could eliminate the effects of the CsA-induced cell apoptosis. The phosphorylation of STAT3, a key signaling molecule in the IL-6 survival pathway, was found to be downregulated in CsA-treated cells at 24 hrs, as demonstrated in Fig. 3I, 3J. We further examined whether CsA would impact the expression of CD138 on plasma cells in vivo. We prepared all sensitized C57BL/6 mice by transfusing blood from BALB/c mice and then treated the mice with CsA or FK506 beginning on Day 7 for a total of 14 days. The expression of CD138 on plasma cells was analyzed by flow cytometry (Fig. 3K). In the CsA-treated group, the expression of CD138 on mouse plasma cells was lower than that in the other two groups, including the FK506-treated and control groups (Fig. 3L). These data showed that CsA significantly influenced the expression of CD138, and lower expression of CD138 could impact cell survival.

CsA treatment decreased the mitochondrial metabolism of plasma cells.

Metabolic activity is important for maintaining antibody production and survival in plasma cells, and the central site of nutrient usage in cells is mitochondria (Boothby, Brookens et al. 2022). In long-lived plasma cells, the research indicates that pyruvate-dependent respiration contributes to the survival of plasma cells. The T-dependent activation of B cells can induce the expression of oxidative phosphorylation mechanisms and enhance oxidative phosphorylation capacity, leading differentiated into plasma cells to exhibit higher oxidative metabolism to facilitate the secretion of antibodies (Price, Patterson et al. 2018). It has been reported that inhibiting the activity of ATP-citrate lyase (ACLY), which is related to the production of acetyl-CoA during B-cell differentiation, could reduce the expression of the plasma cell-specific markers CD138 (Dufort, Gumina et al. 2014). Based on these reports, we aimed to clarify whether mitochondrial function could be impacted by CsA treatment. We treated cells with 10 μM CsA and FK506 for 4 hrs and then utilized the Cell Mito Stress Test to analyze mitochondrial respiration. The data showed the oxygen consumption rate (OCR) was lower in CsA-treated cells than in FK506-treated (Fig. 4A). Basal respiration and mitochondrial ATP production were lower in CsA-treated plasma cells than in FK506-treated (Fig. 4B-C). Overall, mitochondrial function in plasma cells was inhibited by CsA, which might result in cell death.

CsA impacted the plasma cell viability through the ER stress, reducing expression of CD138, and inhibiting mitochondrial function.

The integrated chart shown in Fig. 5 demonstrates that CsA, but not FK506, induces ER stress in plasma cells by upregulating XBP1s, Bip, and CHOP expression. On the other hand, it downregulates the expression of CD138 and inhibits ATP production, thus inducing apoptosis in plasma cells.

Discussion

Calcineurin inhibitors are still the treatment of choice for controlling rejection in organ transplants (Wojciechowski and Wiseman 2021); however, the effect of calcineurin inhibitors on antibody-mediated rejection is still unclear. In cells, the ER plays an essential role in regulating protein folding, transport, and degradation; therefore, stable ER function may be crucial for plasma cells to continuously produce antibodies. In this study, we found that CsA but not FK506 could promote ER stress through the IRE1a-XBP1s pathway and thereby affect the survival of plasma cells and reduce the production of antibodies. We further found that ER stress induced by CsA treatment in plasma cells could downregulate the protein expression of CD138 (Fig. 3A, 3B), reduce the transmission of cell survival signals from IL-6 (Fig. 3I, 3J), and thus lead to cell death.

In previous studies, CsA caused more damage to renal tubule cells than FK506 through the UPR. The proteinsXBP1s and CHOP were partially upregulated by PERK- and ATF6-dependent signaling pathways, following increased expression of cleaved caspase-3 and the promotion of cell apoptosis (Yilmaz, Kirschner et al. 2022). However, this phenomenon was not observed in our experiments. The use of the IRE1α RNase inhibitor 4μ8C effectively inhibited the splicing of XBP1, as

demonstrated in Figure 1D. This finding suggests that the induction of XBP1s expression by CsA in plasma cells may involve a distinct pathway compared to that observed in epithelial cells. A possible reason is that the IRE1a-XBP1 splicing pathway in plasma cells is related to maintaining antibody production, but the PERK and ATF6 pathways are less strongly affected (Li, Song et al. 2019). In future studies, it would be worthwhile to examine the detailed molecular mechanism of the CsA-induced ER stress response in plasma cells.

CD138, which is also known as syndecan-1, is a member of the syndecan family of transmembrane proteoglycans (PGs) located on the cell surface. The expression of CD138 is mainly in differentiated plasma cells during the development of B cells and CD138 is necessary to produce an effective long-term humoral response to produce antibodies. CD138 consists of core proteins where the growth factor-binding glycosaminoglycan (GAG) side chain is attached (S Saunders 1989, Szatmari and Dobra 2013). CD138 is involved in cell-cell adhesion and cell-matrix adhesion, which helps plasma cells adhere to the stroma in bone marrow (RC Ridley 1993) and allows cells to defend against apoptosis by binding to the insulin-like growth factor 1 receptor (IGF1R) and activating survival signaling in multiple myeloma (Beauvais, Jung et al. 2016). We found that CD138 expression in plasma cells was downregulated by treatment with CsA. Therefore, the downregulated expression of CD138 induced by CsA treatment may affect cell adhesion and antiapoptotic effects, which could lead to plasma cell death. In addition, CD138 can be cleaved extracellularly by matrix metalloproteinases (MMPs), such as MMP9, which are involved in angiogenesis in some tumor cells (Purushothaman, Uyama et al. 2010, Asuthkar S 2014). Furthermore, it has been reported that soluble CD138 may act as a regulator of plasma cell generation and autoreactive antibody production by binding to a proliferation-inducing ligand (APRIL) and enhancing the activation of B cells (Liu and Akkoyunlu 2021). APRIL plays an essential role in plasma cells by blocking caspase-12 activation induced by ER stress, which is caused by high antibody production (Cornelis, Chang et al. 2021). Furthermore, there are study have also reported that soluble CD138 in the serum of SLE patients is positively correlated with the progression of their disease. Therefore, the presence of soluble CD138 in the serum is a result of plasma cell expansion and activation (Minowa, Amano et al. 2011). We measured the expression of soluble CD138, and the level was decreased by CsA treatment (Fig. S5). It may be worth evaluating whether the reduction in soluble CD138 expression could influence antibody production and plasma cell survival in the future.

We sensitized C57BL/6 mice with blood from BALB/c mice to produce anti-BALB/c alloantibodies and fed C57BL/6 mice CsA or FK506 by oral gavage beginning on Day 7 after blood transfusion. We then sacrificed the mice and analyzed the levels of alloantibodies in serum on Day 21. However, we did not find significant differences between CsA and FK506 (data not shown). We think the half-life of the alloantibodies is long. Once the antibodies are produced in vivo, they will persist for a while. Although the drugs may induce apoptosis in plasma cells and prevent them from producing IgG, the time point of the evaluation in this study may be unable to clearly distinguish the difference between the effects of the two drugs. On the other hand, alloreactive plasma cells may account for only a small proportion and be mixed with the other existing plasma cells. We should design other experiments to analyze the small proportion of alloreactive plasma cells influenced by the drugs in the future.

Overall, our findings suggest that CsA induces apoptosis in plasma cells through ER stress and blocks the IL-6 survival signal by downregulating CD138 expression. Our research findings elucidate the pharmacological effects of CsA on plasma cells, potentially providing an alternative treatment strategy in managing transplant patients experiencing AMR in the future.

Declarations

Data availability statement:

The authors confirm that the data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements:

We thank the staff at the 7th Core Facility in the National Taiwan University Hospital for technical assistance. We thank the Immune and Animal Core Laboratory of the Department of Medical Research in the National Taiwan University Hospital for equipment and facility support.

Funding:

This work was supported by the National Taiwan University Hospital (NTUH109-M4628, NTUH110-M4970, NTUH 111-M0024, and NTUH 112-M0027 to C.-Y. Tsai and C.-Y. Lee) and the Ministry of Science and Technology (MOST) of Taiwan (109-2314-B-002-080 to C.-Y. Lee).

Authors' contributions:

C.K.C and C.Y.L supervised the project and manuscript revision. C.Y.T performed the experimental work, the data analysis and wrote manuscript. Y.L.L, T.Y.L. and J.H.C. provide support for research methodology.

Conflict of interest statement:

The authors certify that they comply with the ethical guidelines for authorship and declare no competing financial interests.

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Figures

Fig. 1

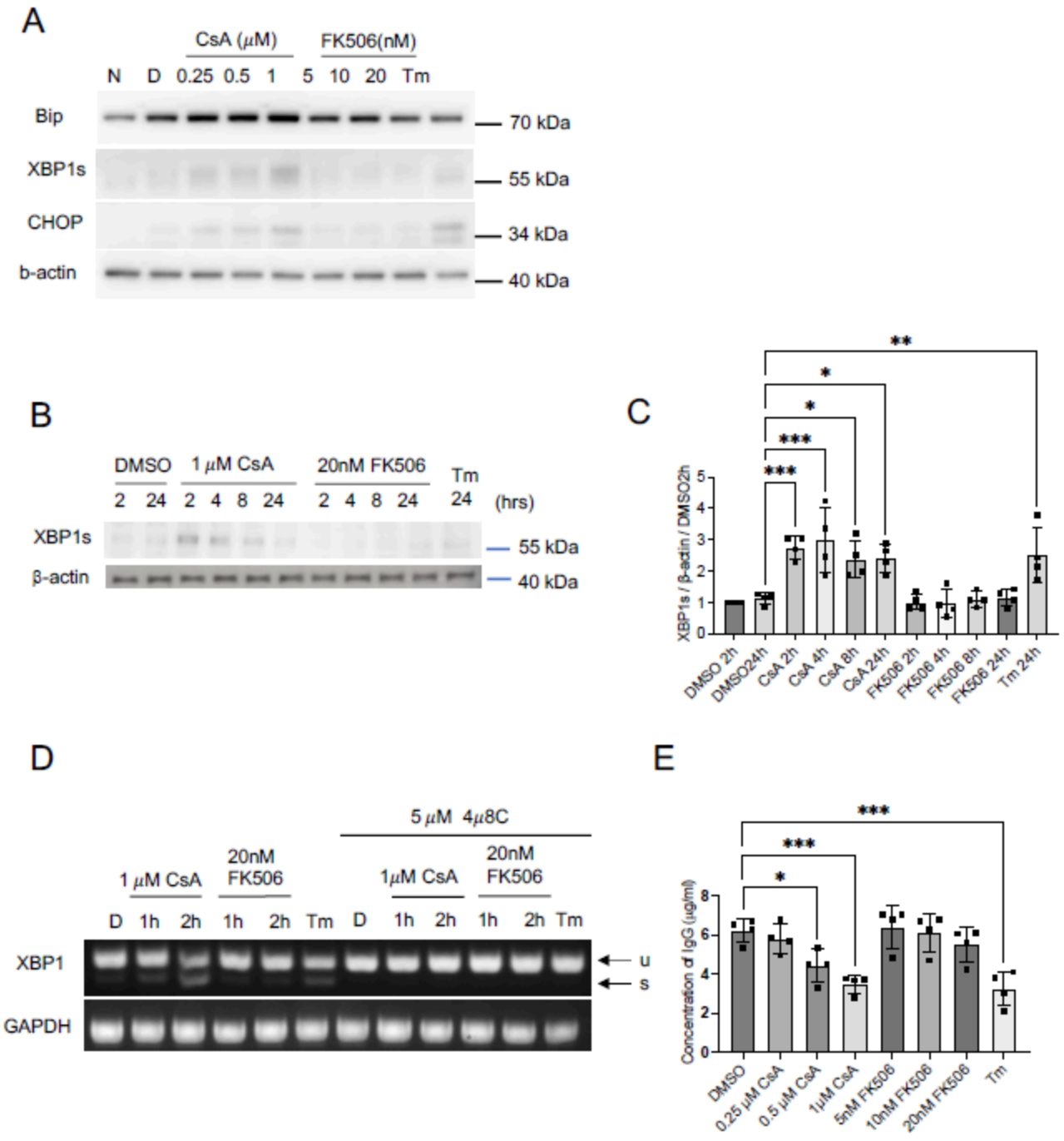


Figure 1

The expression of ER stress-related proteins was upregulated, and the expression of IgG was reduced in plasma cells treated with CsA.

A P3X63Ag8 cells were treated with DMSO, CsA (0.25, 0.5, and 1mM), FK506 (5, 10, and 20 nM), or Tm (tunicamycin, 0.1 mg/ml) for 24 hrs, and the expression of ER stress-related proteins was analyzed by western blotting. N, DMSO, and Tm are the normal control, negative control, and ER stress positive

control groups, respectively. The concentrations of CsA and FK506 mimic the plasma concentrations of drugs at clinical therapeutic doses in humans. **B, C** P3X63Ag8 cells were treated with DMSO, 1 mM CsA, or 20 nM FK506 for 2, 4, 8, and 24 hrs, and then the expression level of XBP1s was analyzed by western blotting. **D** P3X63Ag8 cells were treated with DMSO, 1 mM CsA, or 20 nM FK506, as well as by 5 mM 4m8C for 1hr and 2 hrs, and XBP1 mRNA expression was analyzed by RT-PCR. 4m8C is an RNase inhibitor of IRE1a. DMSO and Tm were used as negative controls and positive controls for ER stress, respectively. The D indicates DMSO, “u” indicates unsliced XBP1, and the “s” indicates spliced XBP1. **E** P3X63Ag8 cells were treated with DMSO, CsA, FK506, and Tm (0.1 mg/ml) for 24 hrs in the serum-free medium, and the medium was collected to analyze the expression of IgG by ELISA. DMSO and Tm were used as negative control, and ER stress positive control, respectively. * P = 0.01, ** P = 0.05, *** P < 0.001

Fig. 2

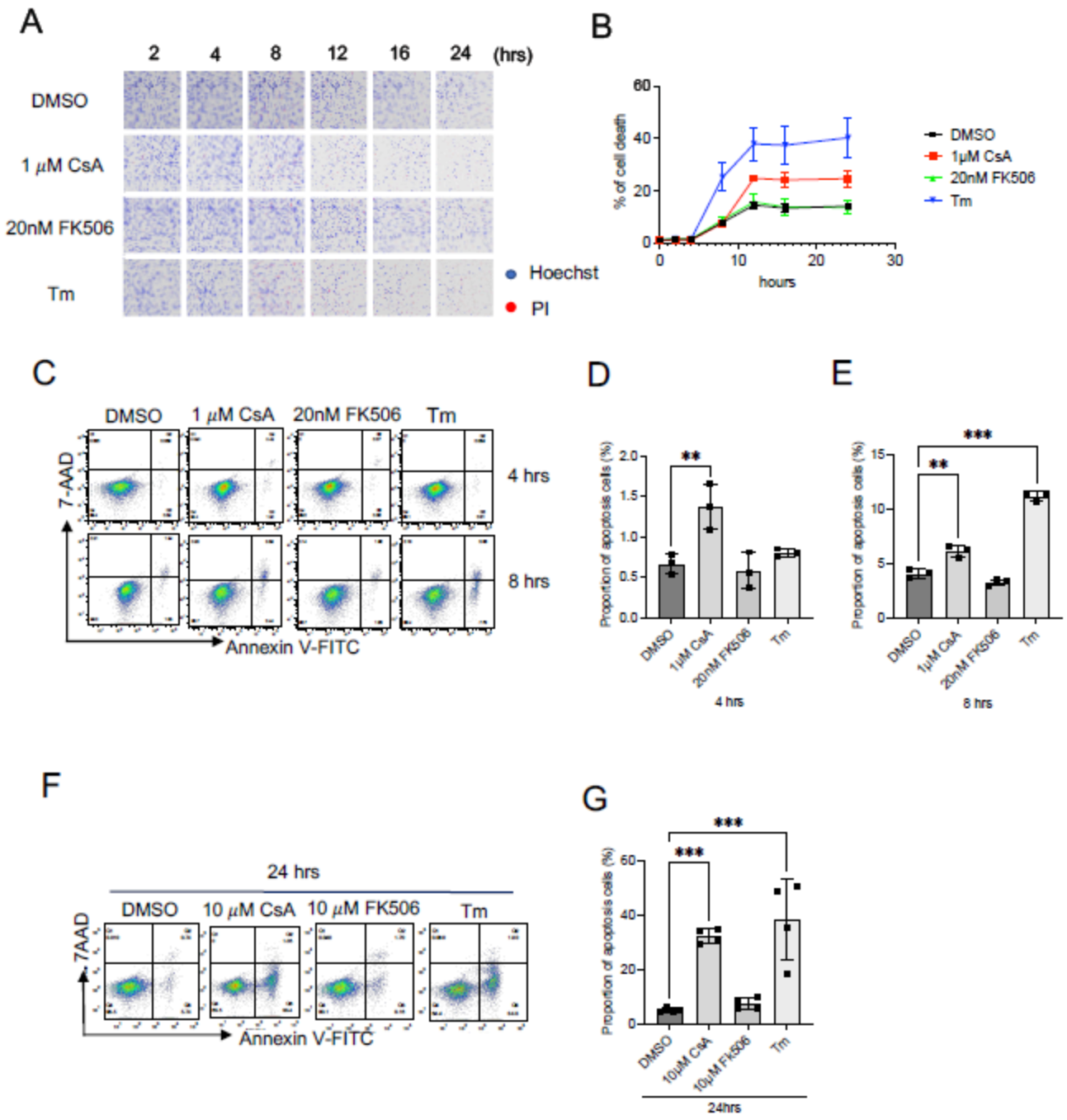


Figure 2

CsA treatment of P3X63Ag8 cells induced more apoptosis than FK506 treatment.

A, B P3X63Ag8 cells were treated with 1 mM CsA or 20 nM FK506 and stained with 1 mg/ml PI and 1 mM Hoechst 33342. In addition, an ImageXpress Nano Automated Imaging System captured the phase contrast and fluorescent image data for 24 hrs, and the percentage of cell death was quantified by MetaXpress software. **C** P3X63Ag8 cells were treated with 1 mM CsA and 20 nM FK506 for 4 hrs and 8

hrs and then reacted with Annexin V-FITC and 7-AAD for flow cytometry. DMSO was the negative control, and Tm was the positive control for ER stress. **D, E** The proportions of apoptosis cells at 4 hrs and 8 hrs were calculated by FlowJo software. **F, G** P3X63Ag8 cells were treated with 10 mM CsA and 10 mM FK506 for 24 hrs and then incubated with CD138-BV421 antibodies, Annexin V-FITC, and 7-AAD for flow cytometry analysis. ** P = 0.001, *** P < 0.001

Fig. 3

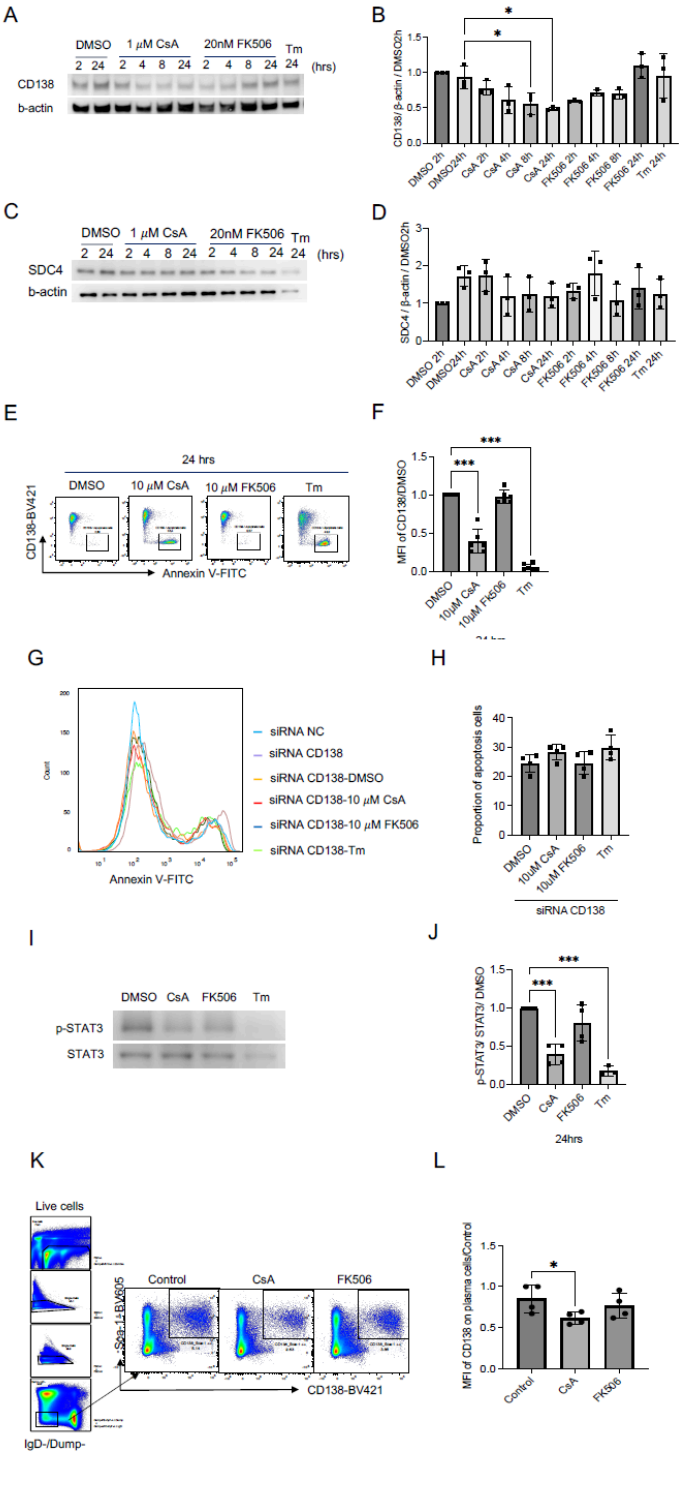


Figure 3

The expression of CD138 was downregulated in apoptotic plasma cells treated with CsA.

A, B P3X63Ag8 cells were treated with 1 mM CsA or 20 nM FK506 for 2, 4, 8, and 24 hrs. Cells treated with DMSO for 2 and 24 hrs were used as the negative control group, and cells treated with Tm for 24 hrs were used as the positive control. Then, cell proteins were collected, and the expression levels of CD138 and SDC4 **(C, D)**were analyzed by western blotting. **E, F** P3X63Ag8 cells were treated with 10 mM CsA and FK506 for 24 hrs. The expression level of CD138 on plasma cells and the median fluorescence intensity (MFI) in the different treatment groups are shown. **G, H** P3X63Ag8 cells were transfected with 200 nM of CD138 targeting siRNA (siCD138) using the Neon Transfection System. After culturing for 24 hours, the cells were treated with DMSO, 10 mM CsA, 10 mM FK506, or Tm for 4 hours. Then, the cells were collected and stained with CD138-BV421 and Annexin V-FITC antibodies for flow cytometry analysis to compare the surface expression of CD138 on plasma cells and the proportion of apoptosis in CD138 knockdown cells. **I, J** The protein expression levels of p-STAT3 in plasma cells treated with 1 mM CsA or 20 nM FK506 for 2, 4, 8, and 24 hrs. **K** Blood-recipient mice received olive oil (control), CsA, or FK506 using oral gavage for 14 days, then the mice were sacrificed, and the spleens were collected. Splenocytes were stained with fluorochrome antibodies, and the proportion of plasma cells was determined by flow cytometry. **I** The comparison of median fluorescence intensity (MFI) of CD138 on plasma cells in different treatment groups was shown. * P < 0.05, *** P < 0.001

Fig. 4

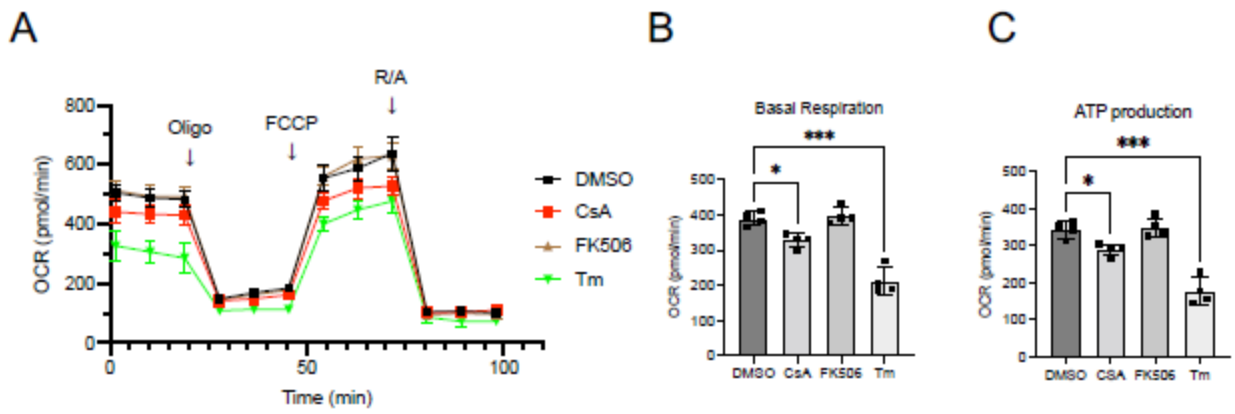


Figure 4

Mitochondrial ATP production was lower in CsA-treated plasma cells than in FK506-treated cells.

A The mitochondrial stress test was applied on the calcineurin inhibitors-treated plasma cells and analyzed the metabolism oxygen consumption through Seahorse XFe24. The mitochondrial oxygen consumption values were determined using Seahorse XF Cell Mito Stress Test Report Generators (Agilent, United States). The generated report includes measurements of basal respiration **(B)** and ATP

production **(C)**. Oligomycin (Oligo; 1 μM), FCCP (1 mM), and rotenone/antimycin A (R/A; 0.5mM) were injected in order. * $p < 0.05$, *** $p < 0.001$, compared with the DMSO group.

Fig. 5

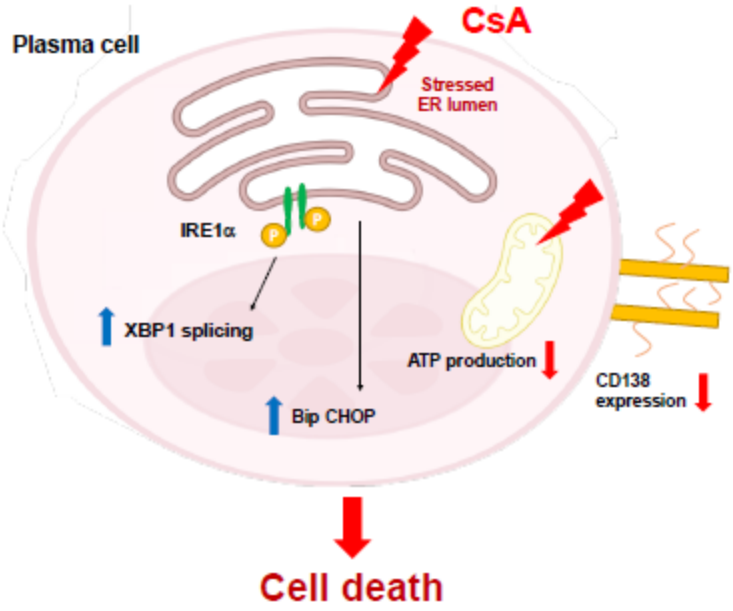


Figure 5

The integrated chart shows the induction of plasma cell apoptosis by CsA treatment. Created with BioRender.com

Supplementary Files

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- 202407SuppFigure.pdf