UNIVERSITY OF CALIFORNIA SAN DIEGO

CD38 Inhibition Attenuates Monosodium Urate Crystal-induced Inflammation in Macrophages

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2021

The thesis of Tiffany C Yan is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

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ABSTRACT OF THE THESIS

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Gout is an inflammatory disease that is characterized by monosodium urate (MSU) crystal deposition in the joints, resulting in extreme pain and swelling. Although there are multiple anti-inflammatory therapies for gout, management of the disease can

become difficult, since some anti-inflammatory drugs present many side effects and may be detrimental to individuals with comorbidities. Therefore, the discovery of a new anti-inflammatory therapeutic for gout is greatly needed. Inflammatory conditions are associated with the decline in nicotinamide adenine dinucleotide (NAD+). NAD+ is a fundamental cofactor for energy metabolism and cell function. NAD+ depletion could be due to numerous factors, such as precursor deficiency as well as NADase activity. Cluster of differentiation 38 (CD38) has been established as a proinflammatory surface marker on immune cells with NADase activity, which is the leading cause of NAD+ decline in mammalian tissues. This study investigates the role of CD38 in regulating MSU crystal-induced inflammation in murine bone marrow-derived macrophages (BMDMs) in vitro. MSU crystals were found to increase CD38 expression, causing a decrease in NAD/NADH ratio in BMDMs. However, CD38 inhibition by apigenin or genetic knockout of CD38 both increased the NAD/NADH ratio and attenuated the production of inflammatory cytokines induced by MSU crystals in BMDMs. These findings suggest that CD38 inhibition has the potential to be a novel therapeutic strategy for gouty inflammation.

INTRODUCTION

Gout is an inflammatory disease that is characterized by monosodium urate (MSU) crystals deposition in and around the joints (Thottam et al., 2017). Risk factors for gout include excessive consumption of alcohol, meat, and seafood; in addition, comorbidities, such as diabetes, obesity, and renal disease, have been known to be associated with gout (Singh et al., 2011). Other studies have also revealed that gout was associated with poor quality of life (Roddy et al., 2007). In severe cases of gout, individuals with inflamed joints experience intense pain and swelling, with the risk of damage spreading to other cartilage and bones (Ragab et al., 2017). In 2007-2008, an estimated 8.3 million adults in the US had gout (Zhu et al., 2011). Gout prevalence increases with age, where the risk of gout is higher in men than women; however, the risk of gout is now increasing for women after menopause (Kuo et al., 2015). Additionally, gout prevalence in the US has been increasing along with its associated comorbidities in the most recent years (Elfishawi et al., 2018). In essence, gout has developed into a public health issue, which calls for more attention for research on treatments and cures for the disease (Liu et al., 2015).

A primary indicator for gout is hyperuricemia (Roddy & Choi, 2014). Hyperuricemia is a condition where there is excess serum uric acid (SUA) in the bloodstream (Chen et al., 2016). Hyperuricemia can be caused by genetic factors, excess production of uric acid by the body, or lack of uric acid excretion by the kidneys (Ragab et al., 2017). Uric acid is the natural byproduct of purine metabolism, where purines are essential for DNA, RNA, as well as other important biomolecules in the

body (Chen et al., 2016). In a hyperuricemic state, the excess SUA can crystallize into MSU crystals, which cause extreme inflammation (Chen et al., 2016).

The removal of MSU crystals from the joints is first facilitated by macrophages, one kind of phagocyte (Martin et al., 2009). Macrophages uptake the MSU crystals and initiate an inflammatory cascade (Cronstein & Sunkureddi, 2013). The interactions between the MSU crystals and residential macrophages in the joint activate the NOD-Like Receptor Family Pyrin Domain Containing-3 (NLRP3) inflammasome, leading to the release of interleukin 1 beta (IL-1β) (Cronstein & Terkeltaub, 2006). IL-1β is a pro-inflammatory cytokine that mediates systemic inflammation in many typical inflammatory diseases (Alberts et al., 2019); specifically, it has been established to be a distinctive cytokine in gout (Martinon et al., 2006). Both IL-1β and NLRP3 are pro-inflammatory markers associated with cellular stress (Jablonski et al., 2020). Additionally, IL-1β and NLRP3 induce the release of CXC-chemokine ligand 1 (CXCL1) during gout flares (Amaral et al., 2012). CXCL1 is the mouse homolog of human interleukin 8, the major chemokine for neutrophil recruitment (Mohsenin et al., 2007). In essence, sustained inflammation of the joints can lead to chronic pain and permanently damaged joints (Hainer et al., 2014) and, therefore, attenuating inflammation has been a focal point of gout research.

There are multiple anti-inflammatory drugs that attenuate MSU crystal-induced inflammation, such as nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine, and corticosteroids (Terkeltaub, 2009). NSAIDs are a traditionally preferred treatment for gouty inflammation (Hainer et al., 2014). NSAIDs are inexpensive compared to other gout treatments and substantially relieve pain and swelling (Cronstein & Terkeltaub,

2006). When NSAIDs are not recommended to a patient, colchicine or corticosteroids are used, as they have shown to be as successful (Bernal et al., 2016). Colchicine has been effective in treating pain and swelling in gout as well as other diseases (Terkeltaub, 2009). In addition, corticosteroids can be administered in several ways, such as orally and intra-articularly (Bernal et al., 2016). Corticosteroids as an intra-articular injection are commonly utilized and highly effective in patients with gouty arthritis in a few specific joints (Cronstein & Terkeltaub, 2006). However, these treatment options are limited by their side effects.

For instance, NSAIDs are typically taken at high doses, which may cause gastrointestinal issues (Cronstein & Terkeltaub, 2006). Additionally, NSAIDs have cardiovascular and renal side effects, which restrict its use on older individuals or patients with comorbidities (Bernal et al., 2016). Similarly, colchicine has also been found to have gastrointestinal side effects (Bernal et al., 2016). Colchicine has been known to cause nausea, vomiting, diarrhea, and abdominal pain (Hainer et al., 2014). In addition, colchicine toxicity and its interactions with other drugs are still being investigated (Terkeltaub, 2009). Colchicine can also be expensive and does not relieve pain (Hainer et al., 2014). Lastly, corticosteroids will occasionally result in recurrent gouty inflammation and cannot be used in patients with infected joints (Bernal et al., 2016). All these concerns call for new treatments and methods to address gout flares; therefore, more therapeutic approaches must be explored.

As previously mentioned, macrophages are known to initiate the inflammatory response to MSU crystals in gout (Martin et al., 2009). Continuous macrophage activation could result in chronic inflammation and, ultimately, tissue damage

(Jablonski et al., 2015). During an inflammatory response, macrophages and neutrophils upregulate the expression of cluster of differentiation 38 (CD38) on their cell surfaces (Partida-Sánchez et al., 2001, as cited by Partida-Sánchez et al., 2003). CD38 functions as a NADase, which was established to be the main cause for age-related NAD+ decline in mammalian tissues (Aksoy et al., 2006). NAD+ is involved in several energy metabolic processes, particularly glycolysis and oxidative phosphorylation (Minhas et al., 2019). Therefore, when there is overexpression of CD38, various metabolic disturbances occur (Camacho-Pereira et al., 2016). These disruptions include impairment in phagocytosis and inflammation attenuation by macrophages (Minhas et al., 2019). In addition to aging, NAD+ decline is also associated with metabolic diseases (Minhas et al., 2019). Because gout is an inflammatory disease where its prevalence increases with age (Kuo et al., 2015), NAD+ decline due to CD38 NADase activity should be considered when conducting gout research. Previous studies have shown that CD38 knockout mice had less NADase activity, resulting in more NAD+ in their tissues, compared to wildtype mice (Aksoy et al., 2006). CD38 has been shown to be robustly induced in human macrophages in inflammatory conditions (Amici et al., 2018) and in inflammatory macrophages in murine in vitro and in vivo models (Jablonski et al., 2015). Given the important role of macrophages in gouty inflammation, in the study, we carried out in vitro studies in mouse bone marrow-derived macrophages (BMDMs) to test the hypotheses that CD38 expression is induced by MSU crystals associated with reduced NAD+ availability and increased inflammatory cytokine production, and that inhibition of CD38 by its pharmacological

inhibitor or by CD38 genetic knockout prevents NAD+ decline and attenuates MSU crystal-induced inflammatory responses.

The CD38 inhibitor utilized in this study was apigenin. Apigenin is a non-toxic flavonoid found in many fruits and vegetables (Zhang et al., 2014). Flavonoids are compounds in foods that have anti-oxidative and anti-inflammatory properties (Ginwala et al., 2019). Apigenin has been recognized to inhibit CD38 in vitro and in vivo (Escande et al., 2013). In addition, previous studies have shown that inhibition of CD38 by apigenin results in more NAD+ in tissues (Escande et al., 2013). We discovered that MSU crystals induced an increase in CD38 expression, which caused the reduction of NAD/NADH ratio in BMDMs. However, CD38 inhibition by apigenin or genetic knockout of CD38 both increased the NAD/NADH ratio and attenuated the production of inflammatory cytokines induced by MSU crystals in BMDMs.

MATERIALS AND METHODS

Isolation of Murine Bone Marrow Cells

The mice (CD38 knockout (KO) and wildtype (WT) mice with C57BL/6 background) were sacrificed using a CO2 chamber. The femurs and tibia were cut at each end, placed in a 0.5-mL microfuge tube pierced at the bottom within a 1.5-mL tube, and centrifuged at maximum speed for 1 minute. The bones were discarded and the cells were resuspended in RPMI-1640 Medium (Sigma-Aldrich, St. Louis, MO). The resuspension was filtered through a 0.45 um cell strainer and counted. Subsequently, the cells were centrifuged at 1500 rpm for 5 minutes and the supernatant was removed.

Differentiation into Murine Bone Marrow-Derived Macrophages (BMDMs)

The cells were resuspended in RPMI-1640 Medium (Sigma-Aldrich, St. Louis, MO) with 10% FBS (Sigma-Aldrich, St. Louis, MO), 0.5% HEPES solution (Sigma-Aldrich, St. Louis, MO), 1% sodium pyruvate (Corning Inc., Corning, NY), 1% penicillin-streptomycin (Corning Inc., Corning, NY), and 20% L929 supernatant containing macrophage-stimulating factor. The cells were seeded at 7 million cells per well in 6-well plates and incubated at 37°C in 5% CO_2. The media was refreshed every three days, and the cells were treated 7 days after differentiation.

Drug Treatment of BMDMs

The medium was removed and the BMDMs were rinsed with DPBS (Sigma-Aldrich, St. Louis, MO). The cells were then pretreated with the control (vehicle) or apigenin (25 µM) diluted in RPMI-1640 Medium (Sigma-Aldrich, St. Louis, MO) with 1% FBS (Sigma-Aldrich, St. Louis, MO), 0.5% HEPES solution (Sigma-Aldrich, St. Louis, MO), 1% sodium pyruvate (Corning Inc., Corning, NY), and 1% penicillin-

streptomycin (Corning Inc., Corning, NY) for one hour. After one hour, monosodium urate (MSU) crystals were added to the appropriate wells at a concentration of 0.2 mg/ml, incubated for 6 hours or 24 hours at 37°C in 5% CO_2.

NAD/NADH Ratio

The intracellular NAD/NADH ratios were determined using the Amplite Fluorimetric NAD/NADH Ratio Assay Kit (AAT Bioquest, Inc., Sunnyvale, CA) on the cell pellet, according to the manufacturer's instructions. Fluorescence was read at Ex/Em = 540/590 nm (cutoff at 570 nm) after 1 hour.

Extraction and DNase Digestion of RNA

Total RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the RNA isolation protocol from RNA-STAT 60 Reagent (Amsbio). The RNA cleanup and DNase digestion steps were performed using the RNeasy Mini Kit by Qiagen, according to the manufacturer's instructions. The amount of RNA was determined using the NanoDrop OneC Microvolume UV-Vis Spectrophotometer with Wi-Fi (Thermo Fisher Scientific, Waltham, MA).

Synthesis of cDNA using RT-PCR

The extracted RNA were reverse transcribed into cDNA, using the Maxima H Minus cDNA Synthesis Master Mix with dsDNase (5X) (Thermo Fisher Scientific, Waltham, MA). 100 ng of RNA was used in the RT reaction. The RNA reaction mix was incubated as follows: heat lid to 110°C, 25°C for 10 min, 50°C for 15 min, 85°C for 5 min. The cDNA was subsequently used for qPCR.

qPCR

qPCR was performed using the PowerUp Sybr Green Master Mix (Thermo Fisher Scientific, Waltham, MA). Relative quantification was normalized using GAPDH as a housekeeping standard by the ΔΔCt method.

CD38 F: 5'- CTG TGG TGT GGT CCA AGT GA -3'

CD38 R: 5'- GGC CTG TAG TTA TCC ACG CA -3'

NLRP3 F: 5'- GAC CAG CCA GAG TGG AAT G -3'

NLRP3 R: 5'- ATG GAG ATG CGG GAG AGA TA -3'

IL-1β F: 5'- CTT CCA GGA TGA GGA CAT GAG -3'

IL-1β R: 5'- TCA CAC ACC AGC AGG TTA TC -3'

CXCL1 F: 5'- GCA CCC AAA CCG AAG TCA TAG -3'

CXCL1 R: 5'- TCT GAA CCA AGG GAG CTT CA -3'

GAPDH F: 5'- TGT GTC CGT CGT GGA TCT GA -3'

GAPDH R: 5'- CCT GCT TCA CCA CCT TCT TGA -3'

Cytokine Quantification

The release of IL-1β and CXCL1 in the conditioned media was analyzed using DuoSet ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± standard deviation of the mean. Two-way ANOVA and Tukey's multiple comparisons test were performed for statistical analyses. All statistical analyses were performed using GraphPad Prism 7.04 (GraphPad Software, San Diego, CA). p-values less than 0.05 were considered significant.

RESULTS

MSU crystals induced CD38 expression and reduced NAD/NADH ratio in BMDMs, which were reversed by CD38 inhibitor apigenin

We first examined the expression of CD38 and the NAD/NADH ratio in the presence or absence of apigenin. BMDMs were pretreated with or without apigenin (25 μM) for one hour and stimulated with MSU crystals for 5 hours for quantitative RT-PCR or 23 hours for NAD/NADH ratio assay. Quantitative RT-PCR was used to examine CD38 mRNA expression. The NAD/NADH Ratio Assay Kit was utilized to measure the concentrations of intracellular NAD, NADH, and NAD/NADH ratio. As shown in Figure 1A, MSU crystals significantly induced an increase in CD38 mRNA expression ($p=0.0008$), but this was attenuated by apigenin ($p=0.0015$). In parallel, MSU crystals reduced the NAD/NADH ratio (Figure 1B), which was prevented by apigenin ($p=0.0055$, Figure 1B). Notably, apigenin also increased basal levels of NAD/NADH ratio ($p=0.0004$, Figure 1B).

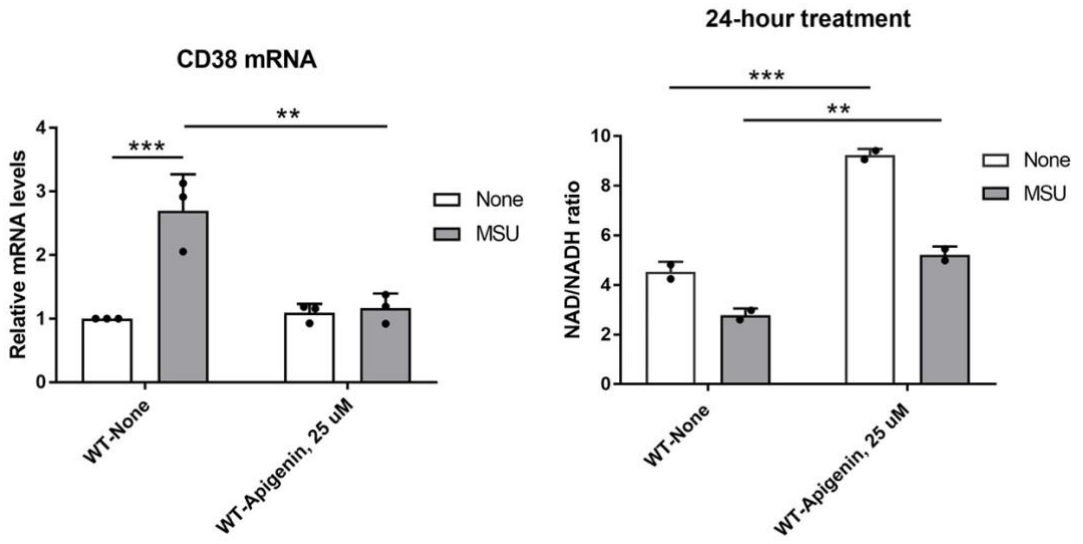

Figure 1. BMDMs were stimulated with MSU crystals (0.2 mg/ml) for 24 hours with and without pretreatment of apigenin (25 μM) for one hour. The cell pellets were subjected to both quantitative RT-PCR analysis (A) and the NAD/NADH ratio assay (B). Two-way ANOVA with Tukey's multiple comparisons test was used for the statistical analyses. (A) ***=0.0008, **p=0.0015. (B) ***p=0.0004. **p=0.0055.

Inhibition of CD38 either by apigenin or genetic knockout attenuated MSU crystal-induced gene expression of proinflammatory markers in BMDMs

Next, we tested whether CD38 inhibition via apigenin or genetic knockout attenuated MSU crystal-induced gene expression of proinflammatory cytokines such as IL-1β and CXCL1, as well as NLRP3, important inflammatory markers of gouty inflammation. BMDMs were pretreated with apigenin (25 μM) for one hour and stimulated with MSU for 5 hours. Total RNA was extracted and subjected to quantitative RT-PCR analysis of expression of NLRP3, IL-1β and CXCL1. As depicted in Figure 2, MSU crystals dramatically induced gene expression of NLRP3, IL-1β and CXCL1, which were significantly inhibited by apigenin or CD38 genetic knockout with the exception of CXCL1.

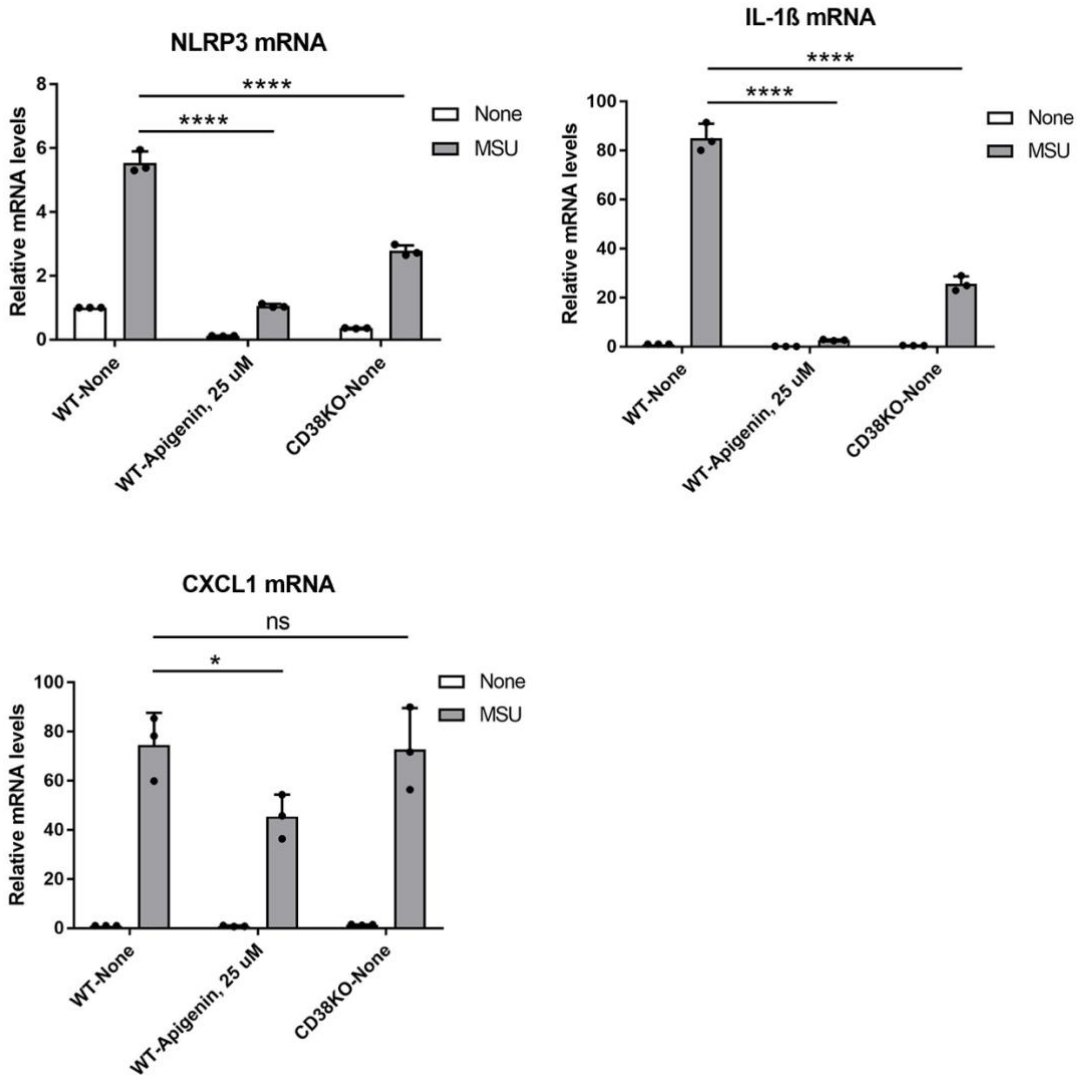

Figure 2. BMDMs were stimulated with MSU crystals (0.2 mg/ml) for 5 hours with and without pretreatment of apigenin (25 μM) for one hour. Total RNA was extracted and subjected to quantitative RT-PCR analysis of mRNA expression of NLRP3, IL-1β, and CXCL1 using GAPDH as a housekeeping control. Two-way ANOVA with Tukey's multiple comparisons test was used for the statistical analyses. (A) NLRP3. ****$p<0.0001$, (B) IL-1β. ****$p<0.0001$, (C) CXCL1. *$p=0.0253$, ns (not significant).

MSU crystal-induced proinflammatory cytokine release was inhibited by apigenin or genetic knockout in BMDMs

Lastly, we assessed and compared the release of proinflammatory cytokines IL-1β and CXCL1 induced by MSU crystals in BMDMs with and without apigenin (25 μM) treatment or in CD38 KO BMDMs. BMDMs were stimulated with MSU crystals for 24

hours with and without pretreatment with apigenin (25 µM) for one hour. The conditioned media was used for ELISA analysis of IL-1β and CXCL1. As seen in Figure 3, MSU crystals notably induced release of IL-1β and CXCL1, which was significantly inhibited by both apigenin and CD38 KO.

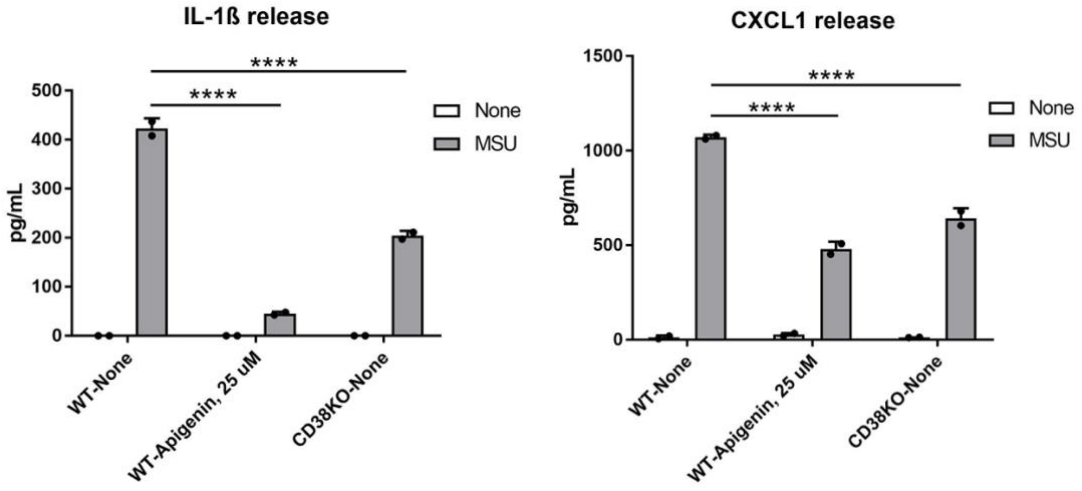

Figure 3. BMDMs were stimulated with MSU crystals (0.2 mg/ml) for 24 hours with and without one-hour pretreatment of apigenin (25 µM). The conditioned media was collected and subjected to ELISA analyses of the release of IL-1β and CXCL1. Two-way ANOVA with Tukey's multiple comparisons test was used for the statistical analyses. ****p<0.0001.

DISCUSSION

In this study, we investigated whether CD38 inhibition could be a potential therapeutic approach for MSU crystal-induced inflammation in murine bone marrow-derived macrophages (BMDMs). We observed that CD38 inhibitor apigenin (25 μM) increased the NAD/NADH ratio compared to the wildtype BMDMs control. We also found that apigenin (25 μM) and CD38 genetic knockout both reduced gene expression of NLRP3, IL-1β, CXCL1, and CD38. Lastly, we showed that apigenin (25 μM) and CD38 genetic knockout both decrease the release of IL-1β and CXCL1. Through utilizing NAD/NADH ratio assay, RT-qPCR, and ELISA, we discovered that CD38 inhibition by apigenin and genetic knockout of CD38 ameliorated gouty inflammation.

Gout is associated with resident macrophage infiltration and neutrophil recruitment in the joints (Mitroulis et al., 2013). Once macrophages and neutrophils encounter MSU crystals, they upregulate CD38 on their cell surfaces (Partida-Sánchez et al., 2001, as cited by Partida-Sánchez et al., 2003). CD38 exhibits NADase activity, which has been found to be the main cause for age-related NAD+ decline in murine tissues (Camacho-Pereira et al., 2016). It is well accepted that NAD+ decline is associated with aging (Hogan et al., 2019). Through inhibition of CD38, there is more available NAD+ in cells (Escande et al., 2013). Elevated levels of NAD+ have been shown to have protective effects against several metabolic diseases (Barbosa et al., 2007). In addition, many important proteins and pathways are dependent on NAD+ availability, such as sirtuins (Escande et al., 2013). Sirtuins utilize NAD+ to reduce metabolic stress and delay aging (Chang & Guarente, 2014). For example, SIRT1 is a deacetylase that maintains glucose homeostasis and has been found to suppress certain

cancers (Chang & Guarente, 2014). Additionally, SIRT3 has deacetylase activity and anti-oxidative properties (Ogura et al., 2020). Therefore, CD38 inhibition would be beneficial as it would increase the amount of available NAD+ for sirtuins to maintain homeostasis within the body.

Apigenin has been established to inhibit CD38 and increase NAD+ availability, which results in increased activity of sirtuins (Escande et al., 2013). Additionally, apigenin is a flavonoid, a natural compound found in many plants and vegetables (Ginwala et al., 2019). It has been shown to possess anti-inflammatory, antioxidative, and anti-cancer properties (Ginwala et al., 2019). Because of the many protective effects, apigenin has been explored in numerous diseases, such as cancer, inflammatory bowel disease, and neuroinflammation (Salehi et al., 2019). Given that MSU crystals increased the expression of CD38 on macrophages (Partida-Sánchez et al., 2001, as cited by Partida-Sánchez et al., 2003), apigenin was utilized in this study.

In addition to the inhibition of CD38, previous studies have shown that apigenin prevents the formation of the NLRP3 inflammasome, inhibiting the activation of caspase-1 (Zhang et al., 2014). Caspase-1 is responsible for the cleavage of pro-IL-1β into IL-1β (Cronstein & Terkeltaub, 2006). Because the formation of NLRP3 inflammasome is hindered, IL-1β production is also disrupted (Zhang et al., 2014). With the inhibition of NLRP3 inflammasome formation and IL-1β release, CXCL1 expression is lowered (Amaral et al., 2012), as CXCL1 has been found to work synergistically with NLRP3 inflammasome and IL-1β (Boro & Balaji, 2017). Therefore, along with CD38 inhibition by apigenin, there is overall decreased inflammatory

response. In essence, the anti-inflammatory effects of apigenin demonstrate that apigenin could be a potential treatment for MSU crystal-induced inflammation.

Although our findings demonstrated that apigenin is capable of addressing gouty inflammation, further studies on CD38 inhibition by apigenin in gout would be advantageous. Apigenin is not exclusively a CD38 inhibitor (Escande et al., 2013). Thus, more research is needed to fully elucidate its effects on other pathways. However, the CD38 inhibitor 78c has recently been established to be highly specific to CD38 (Tarragó et al., 2018). Therefore, additional studies on specific CD38 inhibitors or CD38 genetic knockout models should be conducted to address this concern.

Western blotting and in vivo application of apigenin in mice have already been conducted and the results are consistent with this study (Liu-Bryan et al., unpublished). Apigenin will eventually be used in anti-inflammatory treatment, but it has already been used as dietary supplements (Shukla and Gupta, 2010). In essence, apigenin and other CD388 inhibitors could be an alternate approach to many inflammatory diseases.

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