Original Article
CD38 triggers inflammasome-mediated pyroptotic cell death in head and neck squamous cell carcinoma

Min-Juan Zhang[1*], Wei Gao[1*], Shuai Liu[1], Sharie Pui-Kei Siu[1], Min Yin[2], Judy Chun-Wai Ng[1], Velda Ling-Yu Chow[1], Jimmy Yu-Wai Chan[1], Thian-Sze Wong[1]

[1]Department of Surgery, LKS Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR, China; [2]Department of Otorhinolaryngology, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China. *Equal contributors.

Abstract: Background: Pyroptosis is a form of inflammatory cell death. Although it is recognized that NLRP3 (nucleotide-binding domain, leucine-rich repeat-containing family, pyrin domain-containing 3) inflammasome is involved in pyroptosis activation, the mechanism by which head and neck squamous cell carcinoma (HNSCC) inhibits pyroptotic cell death remains undefined. This study aims to delineate the role of calcium regulator CD38 in NLRP3 inflammasome-dependent pyroptosis in HNSCC. Methods: CD38 overexpressing HNSCC cell lines (SAS, CAL27, SNU899) were generated using lentiviral vectors. NLRP3 and gasdermin D (GSDMD) quantity were detected using Western blot. Caspase-1 activity changes were measured using the Caspase-Glo® 1 inflammasome assay. Cell death proportion was determined by flow cytometry analysis. Proliferation assay was performed using xCELLigence RTCA system. Mouse xenotransplantation was performed to evaluate the potential oncogenic or tumor-suppressive function of CD38. ChIP assay was conducted to verify whether transcription factor NFAT1-mediated NLRP3 expression. Results: Exogenous calcium treatment can lead to a significant increase in caspase-1 activity in HNSCC. This feature was also observed in HNSCC cells with stable CD38 overexpression. CD38-overexpressing cell lines showed a significant reduction in proliferation. Further, expression of NLRP3 protein level was significantly increased in CD38-overexpressing cell lines. The N-terminal effector domain of GSDMD was remarkably increased in the CD38-overexpressing HNSCC. ChIP assay indicated that calcium-sensitive transcription factor NFAT1 was possibly involved in the transcriptional upregulation of NLRP3 observed in CD38-overexpressing HNSCC. The pre-clinical xenograft model revealed that CD38 expression had an inhibiting function on HNSCC progression. Conclusion: In conclusion, our results suggested that activation of pyroptosis in HNSCC is a calcium-dependent process. Reduced expression of calcium ion regulator CD38 functions could prevent inflammasome-induced pyroptosis in HNSCC. CD38 may function as a tumor suppressor in HNSCC progression.

Keywords: CD38, NLRP3, calcium, pyroptosis, head and neck squamous cell carcinoma

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common histology form of cancers across the head and neck sites. HNSCC can be found in different anatomical sites along the upper respiratory tract. Despite the substantial advance in treatment regimes such as the introduction of target therapy and immunotherapy, HNSCC is still a poor prognostic disease. Overall, the 5-year overall survival rate remains at 40%-50% [1-4]. Hypopharyngeal cancers was particularly worst as compared with SCC in other head and neck sites. The

reported five-year age-standardised relative survival was 25% [5]. Molecular targets for HNSCC treatment remains limited. The approved target therapy drug (I.e. EGFR inhibitor) has limited efficacy. Thus, understanding the molecular biology is particular important for further development of effective treatment regimes.

Pyroptosis is a form of programmed cell death leading to gene-controlled destruction to the cells. During pyroptosis, membrane-damaging channels will be formed on the cell membrane of the cells. The resulting water flow into the cells lead to cell swelling and rupture. This fea-

ture has also been shared by necrotic cell death. The discharge of cellular content from cell rupture will amplify local or systematic inflammation [6]. In cancers, pyroptosis is suggested to be an autonomous tumor suppression mechanism with profound effects in suppressing tumor progression [7, 8]. Given that pyroptosis escape is favorable to cancer progression, inducing pyroptotic cell death or activating the relevant signaling pathways is suggested to be a new approach for cancer treatment [9].

Translocation of gasdermin D (GSDMD) protein is an essential step for the formation of membrane pores during pyroptosis [10]. GSDMD is a lipid-binding protein that contains the N-terminal effector domain and the C-terminal inhibitory domain [11]. The canonical pathway for pyroptosis activation requires the enzymatic action of aspartate-specific cysteine protease, caspase-1. The importance of caspase-1 mediated pyroptosis was first recognized in immune defence, as a mechanism to eliminate infected immune cells [12]. Antigen released after pyroptosis may also aid adaptive immunity. Cleavage of GSDMD protein after Asp275 will generate the N-terminal cleavage product (GSDMD-NT). GSDMD-NT will then translocate to the plasma membrane and generate pores with an inner diameter ranged from 10-15 nm [11]. Thus, pyroptosis is sometime referred to gasdermin-mediated programmed necrotic cell death [10].

As a potent protease, caspase-1 is first synthesized as an inactive zymogen, which is activated after proteolytic cleavage. Caspase-1 activity is activated by the formation of NLRP3 inflammasome assembly. The inflammasome is a multi-protein complex that is initiated to assembly to activate caspase-1. The inflammasome complex contains NLRP3, adaptor ASC (an apoptotic speck-like protein containing caspase-recruitment domain) and pro-caspase-1 [13]. In immune cells and epithelial cells, activation of pattern-recognition receptors (PRRs) and particular transcription factors (e.g., NF-κB and AP-1) can trigger the assembly of NLRP3 inflammasomes. After self-oligomerization, the NLRP3 hexamers or heptamers in the inflammasome complex can induce autoactivation of pro-caspase-1 [13, 14]. Activated caspase-1 is involved in the maturation process of pro-

inflammatory cytokines such as proIL-1β. It is also the critical caspase for the generation of GSDMD-NT in pyroptosis.

NLRP3 inflammasome is activated in response to external stimulation such as bacteria or virus infection. Host factors such as extracellular adenosine triphosphate (ATP) & hyaluronan (released during injury) and extracellular glucose (metabolic stress) could also initiate NLRP3 inflammasome self-oligomerization [13]. In Schwann cells, extracellular ATP could trigger the mobilization and release of Ca^{2+} from intracellular stores [15, 16]. Activation of the calcium-sensing receptor with agonists can activate the NLRP3 inflammasome in condition without exogenous ATP [17]. It is recognized that both Ca^{2+} mobilization in endoplasmic reticulum (ER) through inositol triphosphate receptor (IP3R) and extracellular calcium influx through store-operated Ca^{2+} entry (SOCE) can activate NLRP3 inflammasome. Thus, it is reasonable to speculate that Ca^{2+} modulators in HNSCC are playing a part in inflammasome-mediated pyroptosis.

Cluster of differentiation 38 (CD38) is a type II transmembrane glycoprotein. CD38 functions as an ectoenzyme, which catalyzes the conversion of β-nicotinamide adenine dinucleotide (β-NAD$^+$) and nicotinamide adenine dinucleotide phosphate (NADP$^+$) into cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) [18]. Both cADPR and NAADP are Ca^{2+} mobilizing messengers, which signal the release of Ca^{2+} from ER intracellular stores or via membrane calcium channels. In general, CD38 can increase intracellular calcium level through cADPR and NAADP. In addition, Surface expression of CD38 is found on immune cells such as T cells, B cells and natural killer cells [19-21]. It is recognised as a marker for activated T/B cells, terminally differentiated plasma cells, and multiple myeloma [22, 23]. CD38 expression is also detected in bronchi, ductal epithelial, and prostatic epithelial cells [24, 25].

Here, we aimed to determine whether the functional role of CD38, a transmembrane glycoprotein with calcium modulating function at physiological condition, is involved in triggering inflammasome-mediated pyroptotic cell death in HNSCC.

Materials and methods

Cell culture and treatment

SAS and CAL27 are tongue squamous cell carcinoma cell lines obtained from JCRB cell bank and ATCC, respectively. SNU899 is a laryngeal squamous cell carcinoma cell line purchased from Korean Cell Line Bank. SAS was cultured in DMEM/F12 medium (Thermo-Fisher Scientific) with 10% fetal bovine serum (FBS, ThermoFisher Scientific) and Gibco® Antibiotic-Antimycotic (ThermoFisher Scientific). CAL27 and SNU899 were cultured in RPMI-1640 medium (ThermoFisher Scientific) with 10% fetal bovine serum and Gibco® Antibiotic-Antimycotic. Cell lines were incubated at 37°C in a humidified incubator with 5% CO_2. Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, E3889, Sigma-Aldrich) was used to treat HNSCC cells at the concentration of 1 or 2 mM. Cyclosporin A (ab120114, Abcam) was used to treat HNSCC cells at the concentration of 10 μM.

Plasmid and transfection

CD38-overexpressing lentiviral vector (pLenti-GIII-CMV-GFP-2A-Puro) was purchased from Applied Biological Materials (abm) Inc. 293FT cells were transfected with CD38-overexpressing lentiviral vector or mock vector (System Biosciences) and packaging plasmids with Lipofectamine 3000 Transfection reagent (Thermo Fisher Scientific). Then, SAS, CAL27 and SNU899 were transduced with lentiviral particles using TransDux™ (System Biosciences).

RNA extraction and Real-time PCR

Total RNA was extracted from cells using TRIzol (Thermo Fisher Scientific) according to the manufacture's protocol. Then, 2 μg RNA was reverse-transcribed into cDNA using PrimeScriptTM RT reagent Kit with gDNA Eraser (RR047A, TaKaRa). Real-time PCR analysis was performed using a LightCycler® 480 Instrument II (Roche) with LightCycler® 480 Probes Master and hydrolysis probes (Roche). The amplification programme was at 95°C for 10 min followed by 45 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 1 sec and eventually cooling at 40°C for 10 sec. The amplification was conducted with the following primers: NLPR3 forward, TGATGTTCTGTGAAGTGCTGAA and reverse, CGCACTTTTTGTCTCATAATTGA; CD38 for-ward, CCTCACATGGTGTGGTGAAT and reverse, TTGCTGCAGTCCTTTCTCC; GAPDH forward, AGCCACATCGCTCAGACAC and reverse, GCCCAAT-ACGACCAAATCC.

Western blot

Proteins were extracted from the cells using RIPA lysis buffer supplemented with 0.01% phenylmethylsulfonyl fluoride, 0.02% protease inhibitor and 0.01% phosphatase inhibitor cocktail (Roche) and protein concentration was measured by BCA protein assay kit (Thermo Fisher Scientific). Proteins were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidine difluoride (PVDF) membranes (Merck Millipore). The PVDF membranes were blocked with 5% non-fat milk in TBS with 0.01% Tween-20 at room temperature for 1 h. The membranes were then incubated at 4°C overnight with primary antibodies: anti-NLRP3 antibody (1:500, D4D8T, Cell Signaling Technology), anti-caspase-1 antibody (1:1000, ab-207802, Abcam), anti-CD38 antibody (1:100, sc-374650, Santa Cruz Biotechnology), anti-GSDMD antibody (1:100, sc-81868, Santa Cruz Biotechnology), and anti-β-actin antibody (1:5000, ab8227, Abcam). Then, the membranes were incubated with horseradish peroxidase-labelled anti-rabbit or mouse secondary antibody for 1 h at room temperature and visualized using X-ray film with chemiluminescent western blotting (ECL Plus Western Blotting Detection system, GE Healthcare) according to the manufacturer's protocol. Image Studio™ Lite (LI-COR) was used for densitometry analysis.

Colony formation assay

Cells were seeded in 6-well plate with 500 cells in each well. The culture medium was changed every 3 days and cell growth was observed at the same time. Cells were fixed by 70% ethanol until cells have formed sufficient large clones and then stained with 0.5% crystal violet for 1 h. A colony is defined as a cluster of at least 50 cells that can be counted using stereomicroscope.

Real-time proliferation assay

The proliferation ability of cell lines was detected by RTCA DP instrument of xCELLigence Real-Time Cell Analyzer (Roche Applied Science).

Table 1. The forward and reverse primers for six binding sites

	Forward	Reverse
Site 1	AGAGGAGAGCAAGGTGAACC	AGAGCCTTCAGTTTGGAGGA
Site 2	ATTTGCCCAGTATCCCTCCC	GCTCTCCAGACAGCCCTATT
Site 3	AGAGGAGAGCAAGGTGAACC	GCCTCAGGAACAGCTAGAGA
Site 4	GCGAGGAGTAGATAGGCAGG	AGCACCTCAACCTAAAGCCA
Site 5	ATGGGGCTAGGAATAGGTGC	TGAGCCAAGATCACACCACT
Site 6	AGAGCCTTCAGTTTGGAGGA	AACAACCACTTCACGATGCC

Cells were seeded into the wells of the E-plate 16 which integrated microelectronic cell sensor arrays. The proliferation ability was monitored continuously through measuring the electronic impedance of these sensor electrodes and expressed as cell index (CI).

Flow cytometry analysis of cell death

SAS, CAL27 and SNU899 were seeded in 6-well culture plates and cultured in incubator with the stable condition of 5% CO_2 at 37°C overnight. Different concentrations of $CaCl_2$ were added into the medium for 24 hours. Then, cells were harvested in 0.5% trypsin and suspended in medium and centrifuged. Cells were washed twice with cold PBS and resuspended cells in 100 uL of 1× Binding buffer in 15 mL centrifuge tubes. Then, 5 µl of APC Annexin V (BD Biosciences, 550474) and 5 µl of 7-AAD (BD Biosciences, 559925) were added to cells. The mixture were incubated in the dark for 15 min at room temperature (25°C). Finally, 400 µl of 1× Binding Buffer were added into each tube. Analysis was conducted on BD FACSCantoII Analyzer and the data were analyzed by FlowJo 7.6.1.

Measurement of caspase-1 activity

The detection of caspase-1 activity were carried out using Caspase-Glo® 1 Inflammasome Assay kit (Promega, G9951). According to the protocol for measuring caspase-1 activity in cultured cells, 100 µl of SAS (4000), CAL27 (5000) and SNU899 (5000) cells were seeded in white opaque-walled 96-well plate (triplicated for each condition) and were cultured at 37°C with 5% CO_2 for 24 hours. The following day, cells were treated with different concentration of $CaCl_2$ for 6 hours. Then, the 96-well plate was removed from the incubator and equilibrated at room temperature for 5 minutes. Then, 100 µl of Caspase-Glo® 1 Reagent was added to the 96-well plate containing 100 µl of blank reaction (cell culture medium without cells), negative control cells or calcium treated cells in culture medium. The contents of the wells were gently mixed using a plate shaker at 300-500 rpm for 30 seconds. Finally, 96-well plate was incubated at room temperature for at least 1 hour to allow the luminescent signal to stabilize before measuring luminescence using Varioskan LUX Multimode Microplate Reader (Thermo Scientific).

Chromatin Immunoprecipitation (ChIP) assay

Cells were cross-linked by formaldehyde and sonicated into 200-1000 bp DNA-protein fragments. The sheared DNA-protein fragments were incubated with antibodies against NFAT1 (ab2722, Abcam) or mouse IgG (Santa Cruz). The complex was co-precipitated and captured by Protein G Magnetic Beads (Cell Signalling Technology). Then, chromatin was eluted from the antibody/protein G beads complex. The DNA-protein cross-links were reversed and the DNA was purified. The enrichment of 6 putative NFAT1 binding sequences in NLRP3 promoter was detected by qPCR with primers (**Table 1**).

Animal experiment

Athymic nu/nu mice (4~5 weeks old, weight: 18~22 g) were subcutaneously injected with the same amount of CD38-overexpressing SAS cells and mock SAS cells into the right and left flank area, respectively. Tumor size was measured once every three days with a caliper and tumor volume was calculated with the formula: volume = shortest diameter^2 × longest diameter/2. After 28 days, the nude mice were sacrificed and tumors were excised and recorded the weight. The protocol for animal experiment was approved by the Institutional Committee on the Use of Live Animals in Teaching and Research (reference number: 4312-17) at the animal laboratory in the department of Surgery at the University of Hong Kong.

Statistical analysis

All statistical tests were performed using SPSS software version 20.0 (IBM Corp). Results were presented as means ± standard deviation (SD) from three or more independent experiments.

The difference between the experimental and control groups were assessed by student's t-test. P<0.05 was considered as statistically significant.

Results

CD38 functioned as a tumor suppressor gene in HNSCC

Data from TCGA head and neck cancer dataset indicated that patients with high CD38 expression had better overall survival and disease-free survival (**Figure 1A**). To test the functional implication of CD38 in HNSCC, CD38-overexpressing SAS, CAL27 and SNU899 cell lines were developed using lentiviral vectors. Results from qPCR and western blot analysis suggested that CD38-overexpressing cell lines were successfully generated (**Figure 1B**, **1C**). Stable CD38-overexpressing cells showed a significant reduction in colony-forming ability and proliferation propensity as compared with the mock cells (**Figure 1D**, **1E**). These data revealed that CD38 had potential tumor suppressing functions in HNSCC.

Ca^{2+} promoted cell death and caspase-1 activity in HNSCC cells

To address whether calcium induced cell death in HNSCC cells, cell lines were treated with escalating dose of $CaCl_2$ and stained with Annexin V and 7-AAD followed by flow cytometric analysis. $CaCl_2$ treatment could induce a significant upregulation of the percentage of death cells in a dose-dependent manner (**Figure 2A**). $CaCl_2$ treatment also dose-dependently promoted caspase-1 activity in HNSCC cells (**Figure 2B**).

CD38 triggered cell death via activating NLRP3 and caspase-1

In light of the regulatory role of CD38 on intracellular calcium release from intracellular calcium stores together with our observation that calcium induced cell death, we evaluated whether CD38 modulated cell death in HNSCC. Flow cytometric analysis demonstrated that the percentage of death cells was upregulated in CD38-overexpression cells compared to mock cells (**Figure 3A**). Furthermore, the expression levels of NLRP3, pro caspase-1 and cleaved caspase-1 were significantly upregulated in CD38-overexpressing cells (**Figure 3B**).

Taken together, these results implicated that CD38 triggered cell death via activating NLRP3 and caspase-1 expression.

CD38 enhanced NLRP3 expression through Ca^{2+}-NFAT signaling

To address the role of calcium in NLRP3 inflammasome activation, HNSCC cells (SAS, CAL27, SNU899) were incubated with different concentration of $CaCl_2$. Results from qPCR and western blot showed that exogenous Ca^{2+} treatment increased NLRP3 mRNA and protein expression level in a dose-dependent manner (**Figure 4A**, **4B**). In contrast, EGTA (extracellular Ca^{2+} chelator) treatment reduced NLRP3 mRNA expression level (**Figure 4C**). To explore whether $CaCl_2$ mediated LPS-induced NLRP3 inflammasome priming, HNSCC cells were treated by LPS in the presence or absence of EGTA and NLRP3 level was measured. LPS treatment induced NLRP3 expression (**Figure 4D**). EGTA treatment abrogated LPS-promoted NLRP3 activation (**Figure 4D**), indicating that $CaCl_2$ played a crucial role in LPS-induced NLRP3 inflammasome priming.

Then, we investigated the molecular mechanisms underlying CD38-Ca^{2+} induced NLRP3 expression. Nuclear Factor of Activated T cell (NFAT) is a transcriptional factor activated by Ca^{2+} signaling [26, 27]. Increased intracellular Ca^{2+} level activated phosphatase calcineurin which dephosphorylated NFAT, leading to translocation of NFAT from the cytoplasm to nucleus and activation of target gene expression [26, 27]. Given that Ca^{2+} enhanced NLRP3 expression, we suspected that CD38-Ca^{2+} promoted NLRP3 expression via activating NFAT. To validate our hypothesis, we performed an in silico analysis on NLRP3 promoter to find potential NFAT binding sequences (WGGAAA and TTTCCW). According to NCBI GeneBank database, there were 6 NLRP3 variants. Variants 3, 4 shared the same transcription start site while variants 1, 2, 5, 6 have the same transcription start site. Six putative NFAT binding motifs were found in the NLRP3 promoter (**Figure 4E**). ChIP assay indicated that NFAT1 bound to site 4 but not other sites in NLRP3 promoter in CD38-overexpressing HNSCC cells (**Figure 4E**, **4F**). Therefore, NFAT1 could regulate the expression of all the 6 variants of NLRP3 through binding to TTTCCA sequence. To further validate the regulatory role of NFAT1 on NLRP3 expression, HNSCC cells were treated by cyclosporin A

A

Overall Survival

Disease Free Survival

B

C

D

E
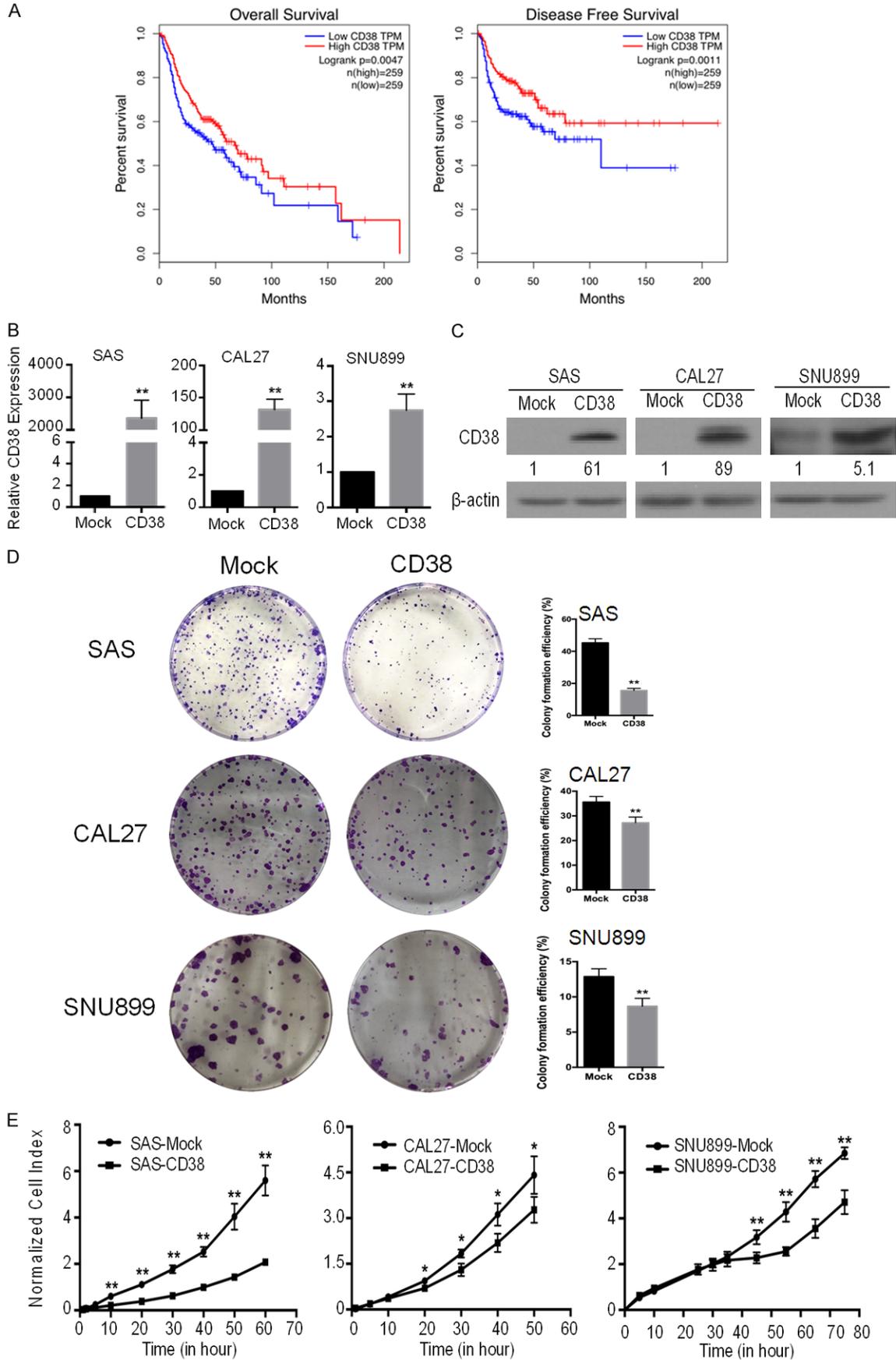
Figure 1. CD38 suppressed the colony-forming ability and proliferation rate of HNSCC. A. Data from TCGA head and neck cancer dataset showed overall survival and disease-free survival of patients with low or high CD38 expression. B. qPCR analysis of CD38 expression in CD38-overexpressing HNSCC cells and mock cells. C. Western blot analysis of CD38 expression in CD38-overexpressing HNSCC cells and mock cells. Densitometry values of bands were determined using Image Studio™ Lite software and were normalized to β-actin. Relative band intensities were displayed under each blot. D. The Representative images showing colony formation of CD38-overexpressing HNSCC cells and mock cells (left panel). Colony formation efficiency was compared between CD38-overexpressing HNSCC cells and mock cells (right panel). E. The proliferation rate was monitored by xCELLigence real-time cell analyzer in CD38-overexpressing HNSCC cells and mock cells. Data are expressed as mean ± SD (n=3). *P<0.05; **P<0.01.
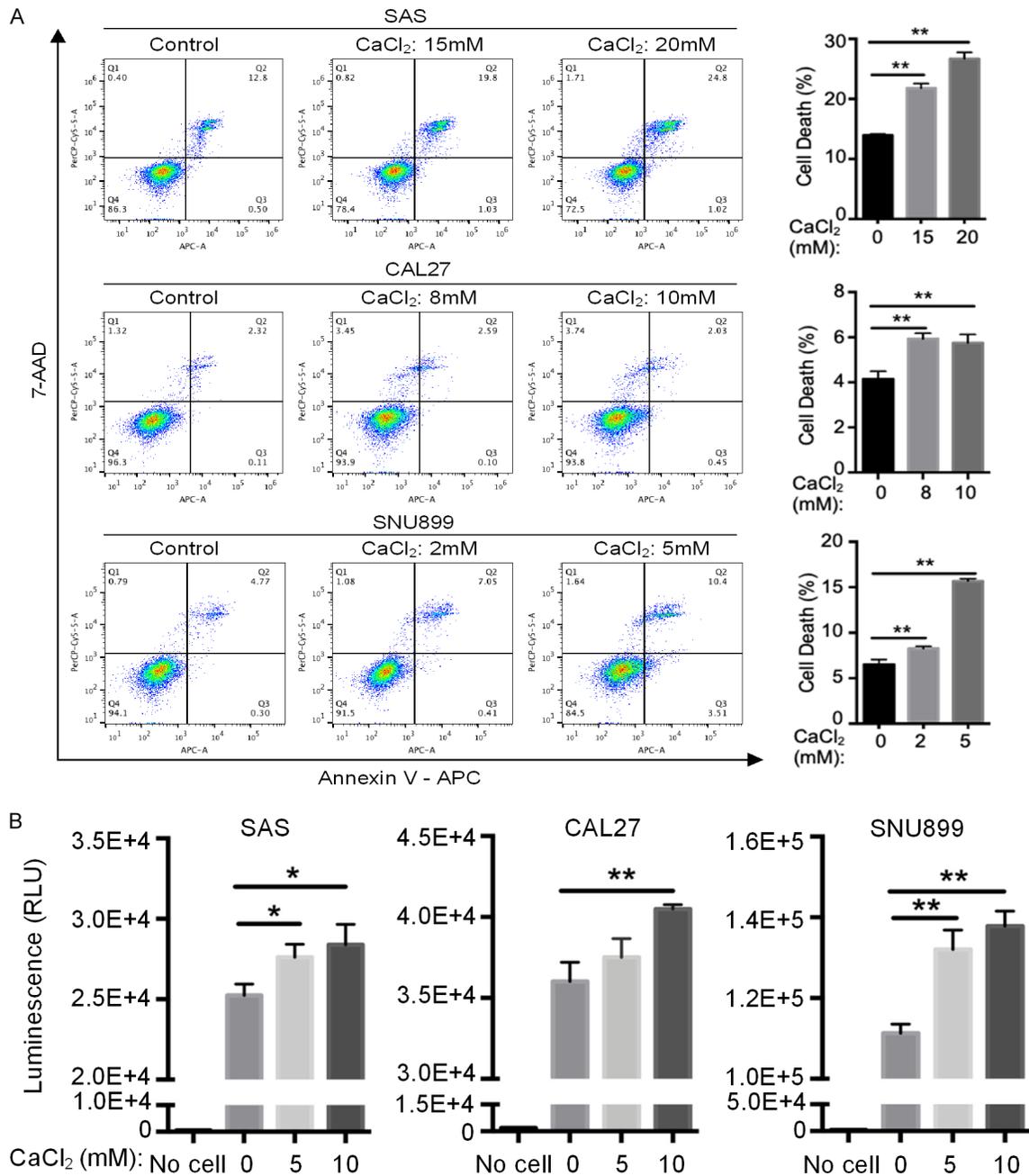
Figure 2. Calcium promoted cell death and caspase-1 activity in HNSCC cells. A. Cell death was measured by flow cytometry following Annexin V and 7-ADD staining in HNSCC cells upon $CaCl_2$ treatment (left panel). The percentage of death cells (Q1+Q2+Q3) was shown in the right panel. B. Caspase-1 activity was measured by Caspase-Glo® 1 inflammasome assay in HNSCC cells treated by $CaCl_2$. Data are expressed as mean ± SD (n=3). *P<0.05; **P<0.01.

A

SAS-Mock

SAS-CD38

Cal27-Mock

Cal27-CD38

SNU899-Mock

SNU899-CD38

7-AAD

Annexin V - APC

B
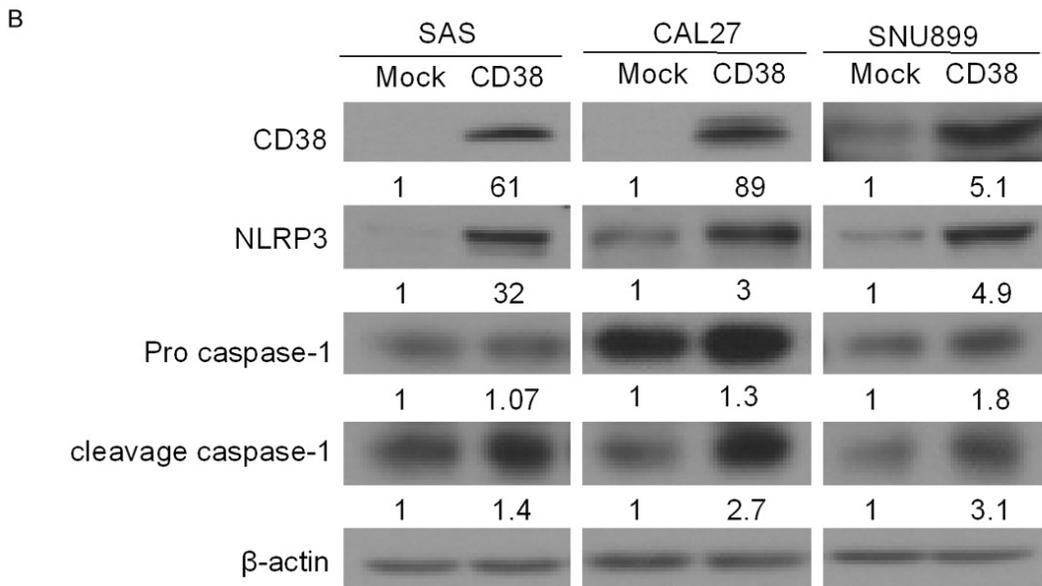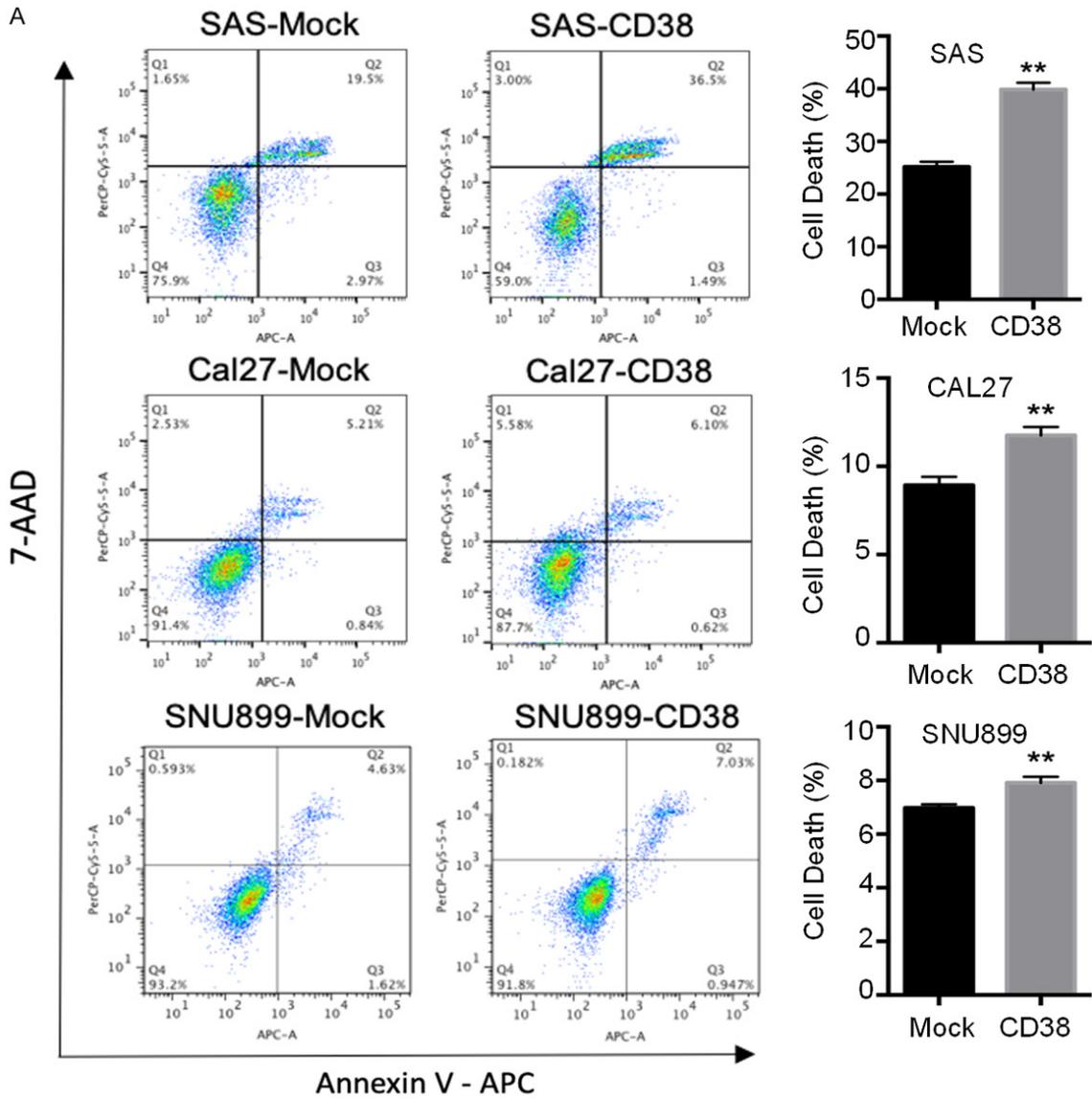
Figure 3. CD38 triggered cell death via NLRP3 and caspase-1. A. Cell death was measured by flow cytometry following Annexin V and 7-ADD staining in CD38-overexpressing HNSCC cells and mock cells (left panel). The percentage of death cells (Q1+Q2+Q3) was shown in the right panel. B. Western blot analysis of NLRP3, pro caspase-1 and cleavage caspase-1 expression in CD38-overexpressing HNSCC cells and mock cells. Densitometry values of bands were determined using Image Studio™ Lite software and were normalized to β-actin. Relative band intensities were displayed under each blot. Data are expressed as mean ± SD (n=3). **P<0.01.
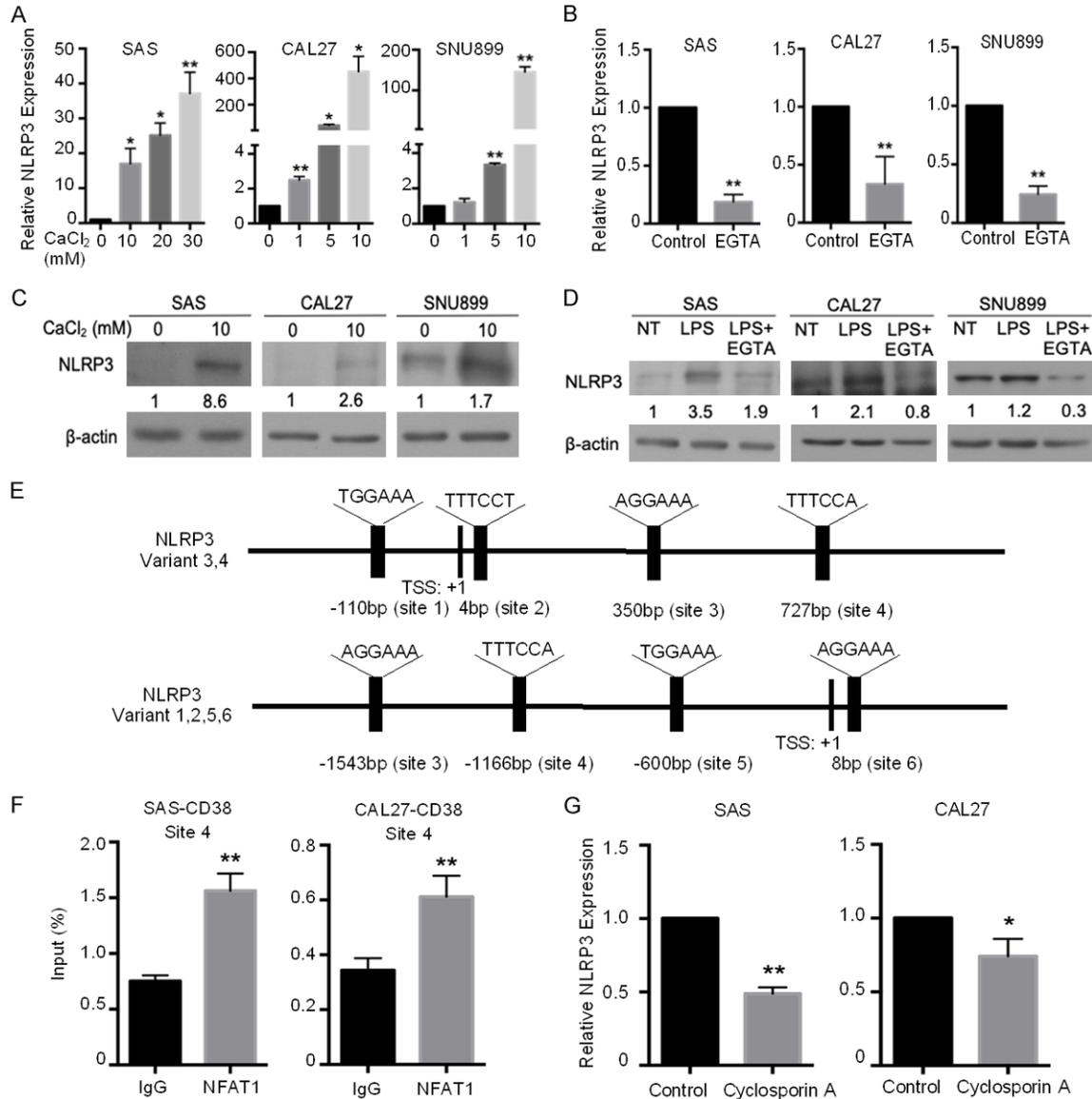
Figure 4. CD38 enhanced NLRP3 expression through calcium-NFAT signalling. A. qPCR analysis of NLRP3 expression in HNSCC cells treated by CaCl$_2$. B. Western blot analysis of NLRP3 expression in HNSCC cells treated by CaCl$_2$. Densitometry values of bands were determined using Image Studio™ Lite software and were normalized to β-actin. Relative band intensities were displayed under each blot. C. qPCR analysis of NLRP3 expression in HNSCC cells treated by extracellular Ca^{2+} chelator, EGTA (2 mM for SAS; 1 mM for CAL27 and SNU899). D. Western blot analysis of NLRP3 expression in HNSCC cells treated by LPS (500 ng/ml) in the presence or absence of EGTA (2 mM for SAS, 1 mM for CAL27 and SNU899). E. Schematic representation of the position and sequences of potential NFAT1 binding motifs in NLRP3 promoter. Each motif was indicated as black block and sequences were showed on the top of black block. TSS: transcription start site. F. ChIP analysis of NFAT1 binding to potential NFAT1 binding motifs in NLRP3 promoter in CD38-overexpressing HNSCC cells. G. qPCR analysis of NLRP3 expression in HNSCC cells upon cyclosporin A (NFAT inhibitor, 10 µM) treatment. Data are expressed as mean ± SD (n=3). *P<0.05; **P<0.01.

(NFAT inhibitor) and NLRP3 expression was determined. Results from qPCR indicated that cyclosporin A treatment resulted in a significant downregulation of NLRP3 expression in HNSCC cells (**Figure 4G**), suggesting that NFAT1 modulated NLRP3 expression. Taken together, CD38 promoted NLRP3 expression via Ca^{2+}-NFAT signaling in HNSCC.

CD38 activated GSDMD-N terminal mediated pyroptotic cell death via promoting caspase-1 activity

Given that $CaCl_2$ treatment induced caspase-1 activity in HNSCC cells, we further test whether CD38 promoted caspase-1 activity. The activity of caspase-1 was significantly increased in CD38-overexpressing cells compared to mock cells (**Figure 5A**). In light of that caspase-1 could cleave GSDMD to generate GSDMD-N terminal which was an executioner of pyroptosis, we evaluated whether CD38 promoted GSDMD-N terminal production. Results from western blot showed that GSDMD-N terminals and GSDMD-C terminals were increased in CD38-overexpressing cells compared to mock cells (**Figure 5B**). These data proved that CD38 induced GSDMD cleavage by activating caspase-1 activity, eventually leading to pyroptosis.

In order to further confirm the functional role of CD38 *in vivo*, we performed animal experiments using mouse xenograft model. CD38-overexpressing SAS cells and mock cells were injected subcutaneously into the left and right flank of nude mice (n=6). The rate of tumour growth was significantly lower in CD38-overexpressing group in comparison with mock group (**Figure 5C**, **5D**). Furthermore, the final tumor weight was also lower in CD38-overexpressing group compared to mock group (**Figure 5E**).

Discussion

In cancers, the role of CD38 in cancer remains controversial. CD38 enhanced tumorigenesis is also reported in lung, nasopharyngeal and cervical cancer [28-30]. Daratumumab, the first CD38-targeting monoclonal antibody, is approved for use in the treatment of multiple myeloma (MM) cells with high CD38 expression. Increased CD38 expression is also found in pancreatic cancer [31]. In contrast, expressing CD38 in prostate cancer can induce the formation of a non-proliferative phenotype [32]. It is believed that CD38 inhibits proliferation and metabolism through modulating NAD^+ levels [32]. To the best of our knowledge, the role of CD38 in HNSCC remains undefined.

We first examined the clinical relevance of CD38 in TCGA head and neck cancer datasets and found that patients with low expression of CD38 had a significant reduction in overall and disease-free survival. In EBV-expressing nasopharyngeal carcinoma cell line C666, stable CD38 expression increased ATP concentration, enhanced proliferation, and colony-forming ability [29]. As the presence of viral transcriptome in the NPC cell line would affect our interpretation of the role of CD38 in HNSCC, we repeated the functional studies using tongue and laryngeal cancer cell lines. Our results indicated that CD38 is low or beyond the detectable level in the HNSCC models. Reduced proliferation is observed in HNSCC cell lines with stable CD38 expression. Also, the colony formed by CD38 expressing HNSCC had a relatively smaller size and number. The data suggested that CD38 might exhibit a tumor-suppressing function in the non-viral associated carcinoma in the head and neck regions.

Intracellular calcium mobilization is an important physiological process for tissue homeostasis, immunity, proliferation, and development. Calcium influx could trigger assembly and activation of the NLRP3 inflammasome complex. Disruption of calcium mobilization inhibits NLRP3 inflammasome activation [17, 33, 34]. Thus, exiting data supported the idea that calcium ion is a regulator for triggering inflammasome activation. Another well-documented function of calcium is in cell death regulation. Calcium perturbation has been implicated in different forms of cell death pathways, including anoikis, apoptosis, autophagy, and necrosis [35]. Whether calcium is involved in pyroptotic cell death remains elusive. In HNSCC cell lines, the proportion of dead cells was increased in parallel to the increasing Ca^{2+} concentration. Also, it is noted that caspase-1 activity is also increased in response to exogenous calcium. The results implied that calcium mobilizing signals might be involved in inflammasome-mediated cell death in HNSCC.
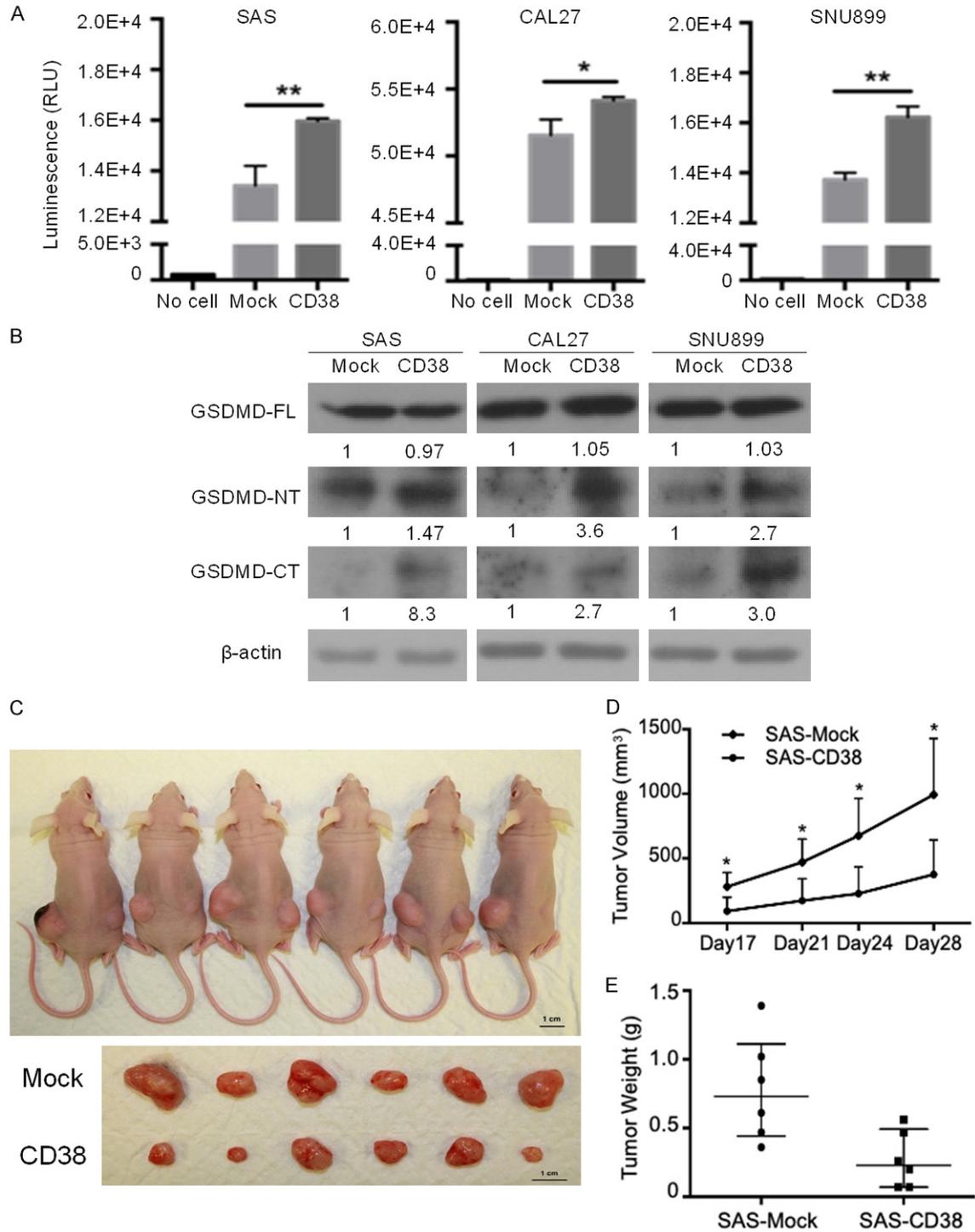
Figure 5. CD38 induced GSDMD cleavage via activating caspase-1 activity. A. Caspase-1 activity was measured by Caspase-Glo® 1 inflammasome assay in CD38-overexpressing HNSCC cells and mock cells. B. Western blot analysis of full-length gasdermin D (GSDMD-FL), GSDMD-N terminal (GSDMD-NT) and GSDMD-C terminal (GSDMD-CT) levels in CD38-overexpressing HNSCC cells and mock cells. Densitometry values of bands were determined using Image Studio™ Lite software and were normalized to β-actin. Relative band intensities were displayed under each blot. C. The images showed the tumors derived from CD38-overexpressing SAS cells and mock cells. D and E. Tumor volume over time and final tumor weight of xenografts derived from CD38-overexpressing SAS cells and mock cells. Data are expressed as mean ± SD (n=3 for a; n=6 for c). *P<0.05, **P<0.01.

Given the CD38 is a Ca^{2+} modulator, we speculated the CD38 could play a part in calcium-associated cell death. CD38 expression was very low in the HNSCC cells. In CD38-over-expressing HNSCC, the proportion of dead cells was significantly increased as compared with the mock control. Further, we noted that the cleavage form of caspase-1 was increased significantly in the CD38-overexpressing HNSCC. The high caspase-1 activity is an indicator of inflammasome activation in the CD38-overexpressing HNSCC. NLRP3 protein level remains low in the control cells. The canonical activation model of NLRP3 inflammasome suggested that the inflammasome is activated by self-assembly into functional units with proteolytic ability. Our new data reveals that CD38 overexpression could stimulate NLRP3 production in HNSCC. Expression activation of NLRP3 was also noted in LPS/calcium-treated HNSCC cell lines. Our data showed for the first time that CD38 could activate inflammasome-mediated caspase-1 activation by activating NLRP3 expression in HNSCC.

Next, we asked whether NLRP3 upregulation in HNSCC is attributed to the Ca^{2+} signaling effects. In HNSCC cell lines, $CaCl_2$ treatment can stimulate NLRP3 upregulation at the transcription level. In contrast, NLRP3 transcription in HNSCC is profoundly inhibited in the presence of calcium chelator. The data reinforce our speculation that CD38 is involved in stimulating NLRP3 expression transcriptionally. Ca^{2+} can activate a group of transcription factor known as NFAT. In the transcription regulating region of the NLRP3 gene, we mapped six putative NFAT1 binding sites. In the presence of the NFAT inhibitor, NLRP3 mRNA expression was significantly suppressed. Additionally, the physical association of NFAT1 on the NLRP3 promoter is confirmed by the ChIP assays. Together, the data implied that Ca^{2+} is participating in regulating NLRP3 transcription via calcium-sensitive transcription factor NFAT.

Finally, we questioned whether CD38 is playing a crucial part in governing pyroptosis in HNSCC. In CD38-overexpressing HNSCC, the inflammasome activity was significantly increased. Further, the pore-forming N-terminal of GSDMD was also increased. Given that pyroptosis has an inhibitory effect on tumor growth and CD38 protein levels are low/absent in the HNSCC cell lines, we speculated that CD38 has a tumor-suppressing property in HNSCC. As showed in the pre-clinical model, HNSCC xenograft with CD38 overexpression was significantly reduced in size and volume. Thus, by inhibiting CD38 expression, HNSCC might be able to escape from the NLRP3 inflammasome-mediated programmed cell death.

Conclusions

Our data provide new insights into the putative tumor-suppressing functions of CD38 in HNSCC. CD38 is participating in NLRP3 expression by stimulating transcription via calcium-sensitive transcription factor. Given that CD38 has a profound impact on pyroptosis, further study is warranted to evaluate the use of CD38-mediated signaling cascade for HNSCC treatment.

Disclosure of conflict of interest

None.

Abbreviations

ATP, Adenosine triphosphate; ASC, Apoptotic speck-like protein containing caspase-recruitment domain; cADPR, Cyclic adenosine diphosphate-ribose; CD38, Cluster of differentiation 38; ChIP, Chromatin immunoprecipitation; CI, Cell index; EGFR, Epidermal growth factor receptor; ER, Endoplasmic reticulum; GSDMD, Gasdermin D; GSDMD-NT, Gasdermin D-N terminal; HNSCC, Head and neck squamous cell carcinoma; IP3R, Inositol triphosphate receptor; LPS, Lipopolysaccharides; MM, Multiple myeloma; $NADP^+$, Nicotinamide adenine dinucleotide phosphate; NAADP, Nicotinic acid adenine dinucleotide phosphate; NFAT, Nuclear Factor of Activated T cell; NLRP3, Nucleotide-binding domain, leucine-rich repeat-containing family, pyrin domain-containing 3; PRRs, Pattern-recognition receptors; PVDF, Polyvinylidine difluoride; SOCE, Store-operated Ca^{2+}

entry; SD, Standard deviation; β-NAD⁺, β-nicotinamide adenine dinucleotide.

Address correspondence to: Dr. Thian-Sze Wong, Department of Surgery, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong, China. Tel: +86-852-3917-9604; Fax: +86-852-3917-9634; E-mail: thiansze@gmail.com

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