ISSN: 1813-4890

Cytotoxic CTLL-2 cells kill human breast cancer MCF-7 cells through the GSDMD-NT-mediated cellular pyroptosis

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Abstract

Cytotoxic T lymphocytes (CTLs) are crucial in defending against intracellular pathogens and tumors. It is well known that they mainly exert the cytotoxic effect of killing target cells through Fas/FasL and perforin-granzyme. However, Gasdermin D (GSDMD) is recently found a key protein that induces cellular pyroptosis through its active N-terminal (GSDMD-NT) that combines with phosphatidylserine to form holes on the cell membrane. Since CTLs contain rich GSDMD, we wonder whether or not GSDMD-NT also functions in the CTLL-2 cells-killed tumor cells. The current results showed that CTLL-2 cells could significantly attack human breast cancer MCF-7 cells with the release of lactate dehydrogenase, the formation of obvious pyroptotic vesicles and the enhancement of propidium iodide-stained cells, which is associated with the release of GSDMD-NT in the cells. These suggest that GSDMD-NT might participate in the process of cytotoxic T -cell killing of tumor cells.

Keywords

GSDMD, T cell, cytotoxicity, pyroptosis, breast cancer.

1. Introduction

Cytotoxic T lymphocytes (CTLs) play a vital role in combating viral infections and tumors[1]. It is well known that the process of CTL killing target cells is involved in several steps: (1) migration of CTLs to the location of target cells, (2) recognition of the target through the T cell receptor (TCR) binding to the specific antigen on the target cell surface, (3) formation of a cytotoxic synapse between CTLs and target cells, and (4) induction of target cell death by CTLs through the release of effector molecules like perforin and granzyme, or via Fas/FasL interaction[2-6]. Perforin makes pores in cell membrane, allowing granzymes and granulysin to get into the cytoplasm directly, or on endosomes to deliver granzymes indirectly[7]. Research suggests that the pores formed by perforin on the cell membrane are temporary due to a wound healing initiated by the cells[8]. Furthermore, research indicates a relationship between the expression of Gasdermin D (GSDMD) and $CD8^+$ T cell markers in The Cancer Genome Atlas (TCGA) cohort, with increased GSDMD cleavage observed in OT-1 CTLs and human activated $CD8^+$ T cells[9].

GSDMD is a member of the Gasdermin family, which contains 6 paralogs in humans, including GSDMA, GSDMB, GSDMC, GSDMD, DFNA5 and DFNB59. In mice, there are 3 GSDMA homologues, 4 GSDMC homologs, GSDMD, DFNA5, and DFNB59. GSDMD is the most extensively studied member of this family and plays a crucial role in the inflammasome-dependent pyroptotic cell death[10, 11]. It is made up of two parts, the N-terminal and the C-terminal, and is activated by inflammatory caspases. Once activated GSDMD-NT binds to specific membrane lipids[12, 13] to form a hollow oligomeric complex (hole) with an inner ring diameter of 10~20 nm that allows the passage of molecules like IL-1β[14-16]. Excessive pore formation leads to lytic cell

ISSN: 1813-4890

death or pyroptosis[1]. In normal human or mouse cells, GSDMD remains inactive in the cytoplasm, GSDMD-CT inhibits the activity of GSDMD-NT. The proteolytic cleavage of GSDMD at specific sites is necessary for its pore-forming activity[17]. This cleavage is primarily carried out by caspases, like human caspase-1, caspase-4 and caspase-5, and mouse caspase-11. Inflammasomes are activated in response to infections or metabolic disorders, recruiting inflammatory caspases for GSDMD activation[18]. Apart from inflammatory caspases, other enzymes such as cathepsin G and elastase can also cleave GSDMD and trigger its pore-forming function, producing pore-forming GSDMD-NT fragments in neutrophils[19, 20].

The perforin pores created by CTL cells on the plasma membrane of target cells can trigger a wound repair response in the target cells, which ultimately leads to restricted cell killing. It is hypothesized that there might be additional mechanisms contributing to the cytotoxic activity of CTL cells against target cells. Furthermore, the expression and cleavage level of GSDMD in $CD8^+$ T cells show a positive correlation with cell markers and activation status, it is reasonable to speculate that GSDMD might play a role in killing effect of CTL cells on target cells with its enhanced effectiveness of CTL cell-to-target cell killing. This research seeks to explore the potential involvement of GSDMD in the killing of tumor cells by CTL cells.

2. Materials and methods

2.1. Materials and reagents

The Live/Dead Staining Kit was procured from Jiangsu Kaiji Biotechnology Co., Ltd. A lactate dehydrogenase (LDH) cytotoxicity detection kit was obtained from Regan Biotech Company. Antibodies for total and cleaved N-terminal GSDMD, β-tubulin, and goat anti-rabbit mouse IgG-HRP were all offered from Abmart Pharmaceutical Technology Co., Ltd.

2.2. Experimental methods

2.2.1. Cell culture

MCF-7 cells and CTLL-2 cells are both maintained in our laboratory. MCF-7 cells were seeded in DMEM medium with 10% FBS. CTLL-2 cells were cultured in RMPI-1640 medium supplemented with 10% FBS and 0.5 ng/ml IL-2. Both cell lines were maintained in a 37 °C-incubator with 5% CO_2.

2.2.2. Western blotting

The cells were lysed using RIPA lysis buffer to extract proteins, which were then measured utilizing the BCA method. Subsequently, the samples were electrophoresed on a 12% SDS-PAGE gel, followed by transfer onto a nitrocellulose membrane for 50 minutes. This membrane was subsequently blocked using skim milk powder for 2 hours and rinsed thrice with TBST for 5 minutes each time. The membrane was then exposed overnight to antibodies against Total and cleaved N-terminal GSDMD (1:1500) as well as β-Tubulin (1:5000), followed by incubation with the secondary antibody, goat anti-rabbit mouse IgG-HRP (1:5000), for 1 hour. The visualization of bands was achieved by incubation with enhanced chemiluminescence (ECL) and documented using the FluorChem 8000 image system.

2.2.3. Live/Dead staining

MCF-7 cells were seeded at a density of 1×10^4 cells per well in a 96-well plate, with 3 wells serving as replicates, and were left to incubate overnight at 37 °C with 5% CO2. The next day, after the MCF-7 cells had attached to the surface, the original culture medium was removed and CTLL-2 cell suspension was introduced for co-culture. The experiment included a control group with no cells, a positive control group (1% Triton X-100), and various Effector-to-Target (E:T) ratios (10:1, 20:1, 40:1). Each well was supplemented with 100 μl of RPMI1640 and the respective quantities of CTLL-2 cell suspension (1×10^5, 2×10^5, 4×10^5 cells per well), followed

ISSN: 1813-4890

by co-culture at 37 °C with 5% CO_2 for 6 hours. The positive control group was exposed to 1% Triton X-100 for 5 minutes. Effector cells (E) and target cells (T) were clearly distinguished. After co-culturing, a gentle wash with PBS was carried out to remove the supernatant and deactivate active esterases in the culture medium. Subsequently, 100 μl of staining solution containing 2 μM calcein AM and 8 μM PI were added and left to incubate at room temperature for 30 minutes. The staining solution was then removed, and 100 μl of PBS was added to halt the incubation. Lastly, the observation and image capture were conducted using an inverted fluorescence microscope.

2.2.4. LDH release test

MCF-7 cells were grown in a 96-well plate and attached overnight before CTLL-2 cells were added at different effector to target ratios. The cells were then incubated for 6 hours at 37 °C with 5% CO_2 to assess tumor cell death using a LDH cytotoxicity assay kit (LEAGENE).

2.2.5. Statistical analysis

Experimental results are displayed as mean ± standard deviation and statistically analyzed using GraphPad Prism 9. One-way ANOVA and t-tests were employed to compare data between multiple groups. P-values less than 0.05 are considered to be statistically significant.

3. Results

3.1. Expressions of GSDMD in MCF-7 and CTLL-2 cells

To explore roles of GSDMD in pyroptosis of CTLL-2 or MCF-7 cells, we first checked expressions of GSDMD in MCF-7 and CTLL-2 cells on the DepMap database (https://depmap.org/portal/), but no expression in CTLL-2 cells was found. The GSDMD expression level in MCF-7 cells was only 0.163, which is extremely low. To further confirm the normal GSDMD expression of these two cells, cytoplasmic proteins from them were extracted when they were in a good condition. Its expression was determined by Western Blot as shown in Figure 1A. Quantitative analysis of band grayscale calculated the expression levels of GSDMD, as illustrated in Figure 1B. The results showed that CTLL-2 cells had a certain amount of GSDMD expression, but compared with CTLL-2 cells the level of GSDMD in MCF-7 cells was almost negligible. Moreover, GSDMD-NT with the activity of forming pores could not be detected out in the effector cells and the target ones, respectively.

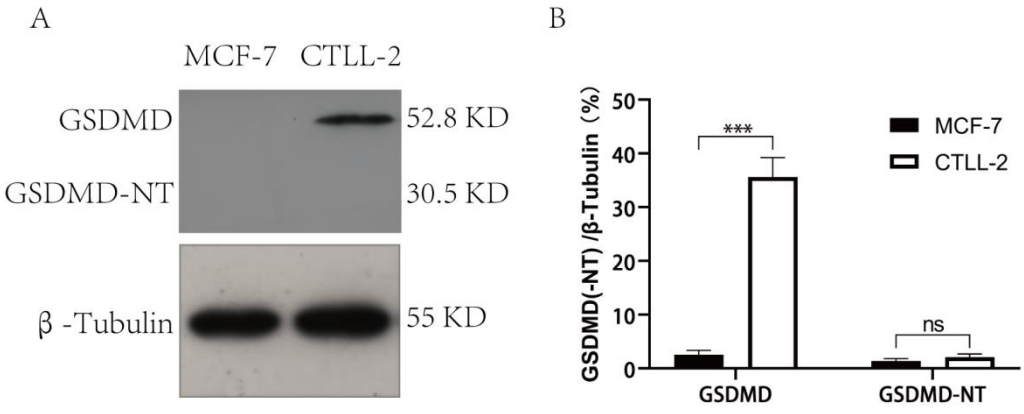

Figure 1. Expressions of GSDMD in MCF-7 and CTLL-2 cells. A: Proteins were extracted from MCF-7 and CTLL-2 cells, followed by Western blot to detect GSDMD protein expression. The panel shows the protein expression levels of GSDMD in MCF-7 and CTLL-2 cells; B: Quantitative analysis of band grayscale in Figure A determined the expression levels of GSDMD, with the results presented as mean ± SD. n=3. *** $p < 0.001$.

ISSN: 1813-4890

3.2. CTLL-2 cells effectively kill MCF-7 cells

As shown in Figure 2, the dead MCF-7 cells in the co-culture group were surrounded by CTLL-2 cells closely adjacent to them, and pyroptotic vesicles were produced on the surface of MCF-7 cells (white arrow in the magnified image in Figure 2A). The cells that emitted red and green fluorescence were counted under different visual fields, respectively. As the E:T ratio was increased the proportion of MCF-7 cells undergoing pyroptosis was also increased. However, no cell pyroptosis was found in the control group. This showed that MCF-7 underwent pyroptosis under the attack of CTLL-2 cells, while CTLL-2 cells had no obvious damage. As the proportion of CTLL-2 cells was increased, the pyroptosis phenomenon became more obvious (Figure 2B). These indicate that CTLL-2 cells appear cytotoxic and can kill human breast cancer cells.

In order to further quantify cytotoxicity of CTLL-2 to MCF-7, LDH assay on the co-cultured cells was performed. In view of the above experimental results, we only made comparisons between the control, E:T(10:1), and E:T(25:1) groups. The results showed that in compared with the untreated group, the E:T(10:1) and E:T(25:1) groups all had extremely significant cytotoxic reactions, indicating that CTLL-2 cells have a significant killing effect on MCF-7 cells under a certain cell ratio (Figure 2C).

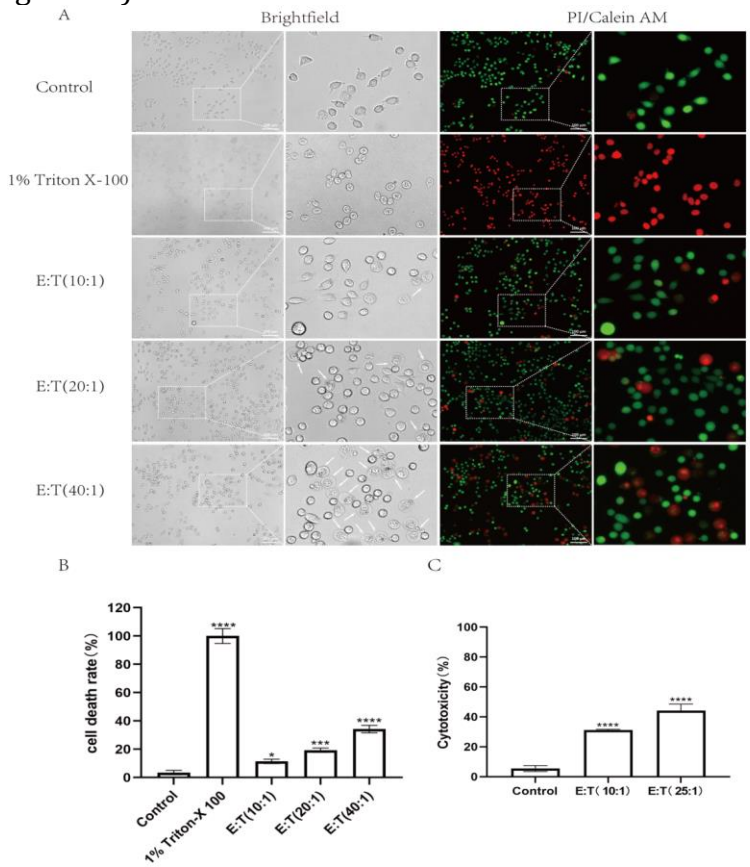

Figure 2. Killing of MCF-7 cells by CTLL-2 cells. A: Live/dead staining is performed on CTLL-2 and MCF-7 cells after co-culture. The cells were treated with calcein AM and PI staining solution and incubated at room temperature for 30 minutes. They were imaged under an inverted fluorescence microscope. The white arrows point to the pyroptotic vesicles (100-fold magnification); B: Quantitative analysis was conducted on the numbers of red/green fluorescence-emitting cells across various visual fields; C: After the cells were co-cultured in 96-well plates for 6 hours their culture supernatant was collected to measure the LDH content for evaluating the cell mortality rate. Data were displayed as mean ± SD. n=3. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ vs the control. E:T, effector cells to target cells.

ISSN: 1813-4890

3.3. GSDMD-NT participates in the killing of MCF-7 cells by CTLL-2 cells

To investigate a potential role of GSDMD in the cytotoxicity of CTLL-2 cells towards MCF-7 cells, co-cultured cells were stained for live and dead cells. Protein extraction was performed for Western Blot analysis. The experiment included the control group, i.e., MCF-7 cells without nay treatment, and the E:T (25:1) group, that is, the ratio of effector cells to target cells is at 25:1. After 6 hours of co-culture, fluorescence imaging revealed pyroptotic bubbles in the E:T (25:1) group with higher red fluorescence intensity compared to the control group. Western Blot analysis showed the presence of GSDMD-NT in the E:T (25:1) group, in which the effector cells were washed out before the extraction of cytoplasmic proteins, while its expression in the control group was minimal. These findings support the involvement of GSDMD in the CTLL-2 cell-mediated cytotoxicity towards MCF-7 cells to eventually lead to cellular pyroptosis.

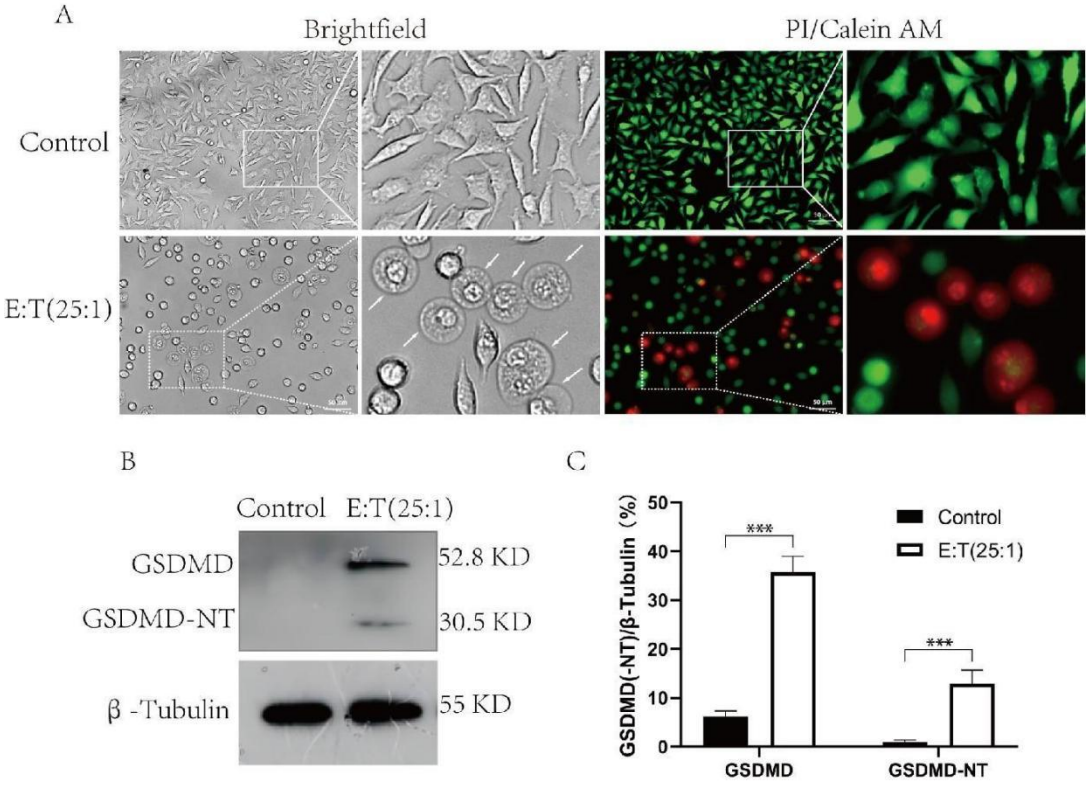

Figure 3. GSDMD participates in the killing of MCF-7 cells by CTLL-2 cells. A: Live/dead staining was conducted on CTLL-2 and MCF-7 cells following co-culture. The cells were treated by the addition of calcein AM and PI staining solution, and then incubated at room temperature for 30 minutes. Observation and image capture were carried out under an inverted fluorescence microscope. The white arrows point to the pyroptotic vesicles (200-fold magnification); B: GSDMD expression and activation are observed in MCF-7 cells through Western Blotting, both in the presence and absence of CTLL-2 cell co-culture; C: Quantitative analysis of band grayscale in Figure B determined the expression levels of GSDMD, with the results presented as mean ± SD. n=3. *** $p < 0.001$. E:T, effector cells to target cells.

4. Discussion

One of the key issues in cancer treatment is the effective killing of tumor cells while sparing healthy cells. Promoting apoptosis is one important therapeutic method to kill tumor cells. However, the efficiency of apoptosis in tumor cells is often limited due to either intrinsic or acquired resistance[21]. Therefore, the introduction of pyroptosis may be another effective method to treat apoptosis-resistant tumor diseases. Currently, extensive research has been

conducted on pyroptosis in many inflammatory diseases, but the exploration of its profound molecular mechanism and its application in tumor treatment is still in its early stages. Moreover, it is important to note that pyroptosis may have dual mechanisms in tumor occurrence, both inhibiting and promoting it, as it can cause damage to normal cells and create a microenvironment favorable for tumor growth and metastasis[22, 23]. Research indicates that cucurbitacin B, a natural bioactive compound derived from melon pedicles, can trigger pyroptosis through the TLR4/NLRP3/GSDMD pathway in non-small cell lung cancer (NSCLC) cells and mouse models, thereby exhibiting anti-lung cancer properties[24]. However, conflicting findings suggest that inhibiting GSDMD expression can impede tumor cell proliferation in NSCLC[25], while GSDMD overexpression may enhance tumor growth in bladder cancer[26]. Therefore, caution is advised in the context of GSDMD-mediated pyroptosis in cancer therapy, and further investigation is warranted to elucidate its impact on tumor survival across various tissues and genetic backgrounds.

CTL cells have been shown to eliminate tumor cells through various mechanisms such as granzyme, perforin, granulysin, and killer cytokines. Our findings suggest that CTL cells could facilitate the entry of GSDMD into tumor cells via perforin-induced pores, leading to increased pyroptosis within the tumor cells. This, in turn, enhances the cytotoxic function of CTL cells. However, mechanism by which GSDMD is cleaved and potential negative impact of cytokines released by pyroptotic tumor cells on tumor microenvironment remain to be investigated.

Acknowledgements

We thank our colleagues in the Department of Immunology of Jinan University for their support.

References

[1] S. Halle, O. Halle, and R. Förster, Mechanisms and Dynamics of T Cell-Mediated Cytotoxicity In Vivo, Trends in Immunology, vol. 38(2017), no. 6, p. 432–443.

[2] G. Bossi, C. Trambas, S. Booth, R. Clark, J. Stinchcombe, and G. M. Griffiths, The secretory synapse: the secrets of a serial killer, Immunological Reviews, vol. 189(2002), no. 1, p. 152–160.

[3] A. Kabanova, V. Zurli, and C. T. Baldari, Signals Controlling Lytic Granule Polarization at the Cytotoxic Immune Synapse, Front Immunol, vol. 9(2018), p. 307.

[4] A. T. Ritter et al., Actin Depletion Initiates Events Leading to Granule Secretion at the Immunological Synapse, Immunity, vol. 42(2015), no. 5, p. 864–876.

[5] M. R. Jenkins, A. Tsun, J. C. Stinchcombe, and G. M. Griffiths, The Strength of T Cell Receptor Signal Controls the Polarization of Cytotoxic Machinery to the Immunological Synapse, Immunity, vol. 31(2009), no. 4, p. 621–631.

[6] M.L. Dustin and E.O. Long, Cytotoxic immunological synapses, Immunological Reviews, vol. 235(2010), no. 1, p. 24–34.

[7] J. Thiery et al., Perforin pores in the endosomal membrane trigger release of endocytosed granzyme B to the cytosol of target cells, Nat Immunol, vol. 12(2011), no. 8, p. 770–777.

[8] J. A. Lopez et al., Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack, Blood, vol. 121(2013), no. 14, p. 2659–2668.

[9] G. Xi et al., GSDMD is required for effector CD8+ T cell responses to lung cancer cells, International Immunopharmacology, vol. 74(2019), p. 105713.

[10] J. Shi et al., Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death, Nature, vol. 526(2015), no. 7575, p. 660–665.

[11] N. Kayagaki et al., Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling, Nature, vol. 526(2015), no. 7575, p. 666–671.

[12] S. Kuang et al., Structure insight of GSDMD reveals the basis of GSDMD autoinhibition in cell pyroptosis, Proc Natl Acad Sci U S A, vol. 114(2017), no. 40, p. 10642–10647.

[13] Y. Zhang, X. Chen, C. Gueydan, and J. Han, Plasma membrane changes during programmed cell deaths, Cell Res, vol. 28(2018), no. 1, p. 9–21.

[14] X. Liu et al., Inflammasome - activated gasdermin D causes pyroptosis by forming membrane pores, Nature, vol. 535(2016), no. 7610, p. 153–158.

[15] L. Sborgi et al., GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death, EMBO J, vol. 35(2016), no. 16, p. 1766–1778.

[16] M. Karmakar et al., N-GSDMD trafficking to neutrophil organelles facilitates IL-1β release independently of plasma membrane pores and pyroptosis, Nat Commun, vol. 11(2020), p. 2212.

[17] P. Devant and J. C. Kagan, Molecular mechanisms of gasdermin D pore-forming activity, Nat Immunol, vol. 24(2023), no. 7, p. 1064–1075.

[18] C.L. Evavold and J.C. Kagan, Inflammasomes: Threat Assessment Organelles of the Innate Immune System, Immunity, vol. 51(2019), no. 4, p. 609–624.

[19] H. Kambara et al., Gasdermin D Exerts Anti-inflammatory Effects by Promoting Neutrophil Death, Cell Rep, vol. 22, no. 11, p. 2924–2936, Mar. 2018.

[20] S. S. Burgener, N. G. F. Leborgne, S. J. Snipas, G. S. Salvesen, P. I. Bird, and C. Benarafa, Cathepsin G inhibition by Serpinb1 and Serpinb6 prevent programmed necrosis in neutrophils and monocytes and reduce GSDMD-driven inflammation, Cell Rep, vol. 27(2019), no. 12, p. 3646-3656.e5.

[21] H. Okada and T. W. Mak, Pathways of apoptotic and non-apoptotic death in tumour cells, Nat Rev Cancer, vol. 4(2004), no. 8, p. 592–603.

[22] D. Wu, S. Wang, G. Yu, and X. Chen, Cell Death Mediated by the Pyroptosis Pathway with the Aid of Nanotechnology: Prospects for Cancer Therapy, Angew Chem Int Ed, vol. 60(2021), no. 15, p. 8018–8034.

[23] Z. Dai, W.C. Liu, X.Y. Chen, X. Wang, J.L. Li, and X. Zhang, Gasdermin D-mediated pyroptosis: mechanisms, diseases, and inhibitors, Front Immunol, vol. 14(2023), p. 1178662.

[24] R. Yuan et al., Cucurbitacin B inhibits non-small cell lung cancer in vivo and in vitro by triggering TLR4/NLRP3/GSDMD-dependent pyroptosis, Pharmacological Research, vol. 170(2021), p. 105748.

[25] S. Chen et al., Macrophages in immunoregulation and therapeutics, Signal Transduct Target Ther, vol. 8(2023), p. 207.

[26] J. Peng et al., CD147 Expression Is Associated with Tumor Proliferation in Bladder Cancer via GSDMD, Biomed Res Int, vol. 2020(2020), p. 7638975.