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ORIGINAL

X-Ray Induces Pyroptosis in Hela Cells Through Caspase-3/Gsdme Signaling Pathway: Implications for Anti-Tumor Immunity and its Impact on Mental and Physical Health in Athletes

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ABSTRACT

Pyroptosis, a form of programmed cell death characterized by inflammation and the recruitment of inflammatory cells, plays a pivotal role in mitigating immunosuppression and sparking systemic immune responses within the tumor microenvironment. The potential of cancer cell pyroptosis to be triggered during radiotherapy, however, remains underexplored. This study delves into the induction of pyroptosis in cervical cancer HeLa cells following irradiation, seeking to pinpoint optimal radiation conditions to incite this process, alongside evaluating the subsequent anti-tumor immune effects. Our findings indicate that exposing HeLa cells to an 8 Gy radiation dose for 72 hours optimally initiates pyroptosis via the caspase-3/GSDME signaling pathway, enhancing phagocytosis and bolstering anti-tumor immunity. This research not only sheds light on a novel approach to cancer treatment through the manipulation of pyroptosis but also opens avenues for investigating the broader implications of this therapy on the mental and physical health of athletes. Enhanced understanding of these dynamics could revolutionize therapeutic strategies, ensuring they support the holistic well-being of individuals undergoing cancer treatment, particularly those in physically demanding professions or activities.

KEYWORDS: Pyroptosis; Irradiation; caspase3/GSDME; Phagocytosis; Anti-tumor immunity

1. INTRODUCTION

The interplay between cancer treatment modalities and the immune system has emerged as a pivotal area of research, particularly in the context of enhancing therapeutic efficacy while minimizing adverse effects. Pyroptosis, a form of programmed cell death distinguished by its pro-inflammatory nature, has garnered attention for its potential to alter the tumor microenvironment and stimulate anti-tumor immune responses(C.-c. Zhang et al., 2019). Unlike apoptosis(Y. Wang et al., 2017), which is generally immunologically silent, pyroptosis results in cell lysis, releasing inflammatory mediators that attract immune cells to the site of the tumor(Friedlander, 1986) (Zhao et al., 2020), thereby facilitating the clearance of cancer cells. This study focuses on the induction of pyroptosis in cervical cancer HeLa cells through radiotherapy, specifically via the caspase-3/GSDME signaling pathway, and explores its implications for anti-tumor immunity(Siegel, Miller, Fuchs, & Jemal, 2021).

Radiotherapy remains a cornerstone in cancer treatment, leveraging ionizing radiation to induce DNA damage in cancer cells, leading to cell death or growth arrest(Rogers et al., 2017). Recent advancements have revealed that beyond its direct cytotoxic effects, radiotherapy can modulate the tumor microenvironment(B. Zhou et al., 2018), influencing the immune system's ability to recognize and eliminate tumor cells. The caspase-3/GSDME signaling pathway has been identified as a critical mediator in this process, facilitating the transition from apoptosis to pyroptosis under certain conditions and thereby enhancing the immunogenicity of the cancer cells(Rébé, Derangère, & Ghiringhelli, 2015).

The potential of radiotherapy-induced pyroptosis to serve as a dual-function treatment modality—directly targeting cancer cells(Jorgensen & Miao, 2015) and simultaneously(Lu et al., 2018) promoting anti-tumor immunity—presents a promising therapeutic strategy(Arbyn et al., 2020). However, the broader implications of this approach, particularly concerning the mental and physical health of patients undergoing treatment(Fang et al., 2020), warrant thorough investigation(Hausman, 2019). This is especially relevant for individuals with high physical activity levels(Allen, Her, & Jaffray, 2017), such as athletes, whose physical and mental well-being are crucial for their professional performance and overall quality of life(Rébé et al., 2015).

This study aims to delineate the optimal radiation conditions to induce pyroptosis in cervical cancer HeLa cells and to assess the subsequent effects on anti-tumor immunity(Islami, Fedewa, & Jemal, 2019). By integrating

oncological research with considerations for patient well-being, we endeavor to contribute to the development of cancer treatments that not only effectively target the disease but also support the holistic health of the patient, offering new insights into the complex relationship between cancer therapy, immune response, and patient quality of life(Forastiere et al., 2003; Lefebvre et al., 1996).

2. Materials and methods

2.1. Materials and reagents

GSDME (ab225893) antibodies were bought from Abcam. GAPDH (EM1101) antibodies were purchased from Huaan Biological Company. Caspase-3 (9662S) and horseradish peroxidase labeled mouse second antibody (7076S) and rabbit second antibody (7074S) were all purchased from Cell Signaling Technology (CST). Cell membrane red fluorescent staining kit (Dil) (C1991S-1), Hoechst33342 living cell staining solution (100*) (C1028), and BCA protein concentration determination kit (P0011) were all purchased from Beyotime Biotechnology Research Institute. PMA（P1585）and LPS were purchased from Sigma-Aldrich (China). RNA extraction Reagent (AG21101) was purchased from Accurate Biology. FITC-ANNEXIN V Apoptosis Detection Kit (556547) was bought from Becton, Dickinson and Company. Cytotoxicity Test Kit (G1780) purchased from Promega Biotechnology Co., Ltd. IFN-γ was purchased from Ericsson (Shanghai) Biotechnology Co., Ltd. CD86 fluorescent flow Antibody (B7-2) was purchased from Thermofisher Scientific.

2.2. Cell culture

The Hela cells used in this study are used in our previous study (Hu et al., 2020). It can ensure that Hela cells are not contaminated by other cell lines. Hela cells and M1 phagocytes are adherent cells. Cultured in a 10 cm petri dish (430167), the medium used by Hela cells is DMEM, and the medium used by M1 macrophages is 1640. THP-1 cells are suspended cells and cultured in 50mL culture flask (TCF001050, JET). Both Hela cells and THP-1 cells were cultured; M1 macrophages were maintained in a pure 1640 medium without FBS and PS, and cultured to the appropriate density in a humidified incubator with 37 ℃, 5% carbon dioxide, and 95% air.

2.3. Cell irradiation

Inoculated Hela cells into a 6-well plate with radiation dose gradients of 2Gy, 8Gy, and 15Gy. After irradiation, the culture time gradients were 0h, 48h, 72h, and 96h, and took the cell microscopic pictures at the corresponding point in time and collected the cells for follow-up experiments.

2.4. Macrophages obtained by THP-1 transformation

The method of transforming macrophages obtained by THP-1 has been modified according to previous studies (Xu et al., 2018). THP-1 cells were cultured with 200 ng/mL PMA solution (PMA powder dissolved in DMSO to obtain PMA solution) for 18 hours, and M0 type macrophages were obtained. THP-1 cells were cultured with 200 ng/mL PMA solution for 6 h, then mixed with 100 ng/mL LPS solution (LPS dissolved in PBS buffer to obtain LPS solution) and 20 ng/mL IFN- γ (IFN- γ diluted with water) for 12 h, and then cultured for 24 h, M1 macrophages were obtained.

2.5. Flow cytometry analysis

Collected the cells, rinsed with precooled 10% PBS, and stained. Obtained and analyzed the data.

2.6. LDH release experiment

Inoculated the cells into a 6-well plate and treated according to the operation instructions. Collected 250 g the supernatant for 4 min. Added the supernatant to a 96-well plate, and detected with a Cytotox96 kit. The absorbance was measured at the 492nm wavelength. Using the formula (LDH sample-LDH negative control)/ (LDH maximum release-LDH negative control) × 100%to calculate the percentage of lactate dehydrogenase release. Obtain the maximum release of LDH follow the reagent instructions.

2.7. Western blot analysis

Cells were extracted with RIPA lysate and incubated at 4 ° c for 30min.Supernatant was obtained by rapid freezing after centrifugation at 12,000 rpm for 20 min, the protein concentration was determined by BCA protein analysis kit, the extracted protein samples were added to SDS-PAGE gel for electrophoresis separation, and then transferred to polyvinylidene fluoride (PVDF) membrane. After the film was transferred, the PVDF film was sealed with 5% milk at room temperature for 2 h, and the corresponding primary antibody was incubated overnight at 4℃. Then, the membrane was incubated with the corresponding horseradish peroxidase second antibody at room temperature, and the chemiluminescence signal of the protein band was detected by ultra-sensitive ECL reagent in chemiluminescence imager. Obtaining the gray value of protein by Image J.

2.8. Quantitative real-time PCR analysis

Total RNA was extracted from cells with an AGRNAexProRNA extraction reagent. Then, using the Prime Scrip RT kit, the first strand of cDNA was synthesized according to the instructions and stored in a refrigerator at

20℃. qRT-PCR amplification is carried out on CFXConnectTM real-time system with SYBRGreenPreMixProTaqHS. The result of the thermal cycle is expressed as mean ± standard deviation (SD). GAPDH was used as the internal standard for quantitative gene expression, and the $2^{-\Delta\Delta CT}$ method was used to deal with it. The primer sequence is shown in Table 1.

Table 1: Real-time quantitative polymerase chain reaction

Protein	Gene	Forward (5′ – 3′)	Reverse (5′ – 3′)
IL-1β	IL-1β	GCCAGTGAAATGATGGCTTATT	AGGAGCACTTCATCTGTTTAGG
IL-8	IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTCGGACTTTTGTACTCATCTGCAC
IL-6	IL-6	CACTGGTCTTTTGGAGTTTGAG	
TNF-α	TNF	AAGGACACCATGAGCACTGAAAGC	AGGAAGGAGAAGAGGCTGAGGAAC
GAPDH	GAPDH	GCTATCCAGGCTGTGCTATC	TGTCACGCACGATTTCC

2.9. Fluorescence microscopic imaging

After inducing THP-1 cells into M1 macrophages, a phagocytosis test was carried out. Hela cells were stained with Hoechst33342 (100*) at 37 ℃ for 10 min, and M1 macrophages were stained with DiI for a membrane 20min at 37 ℃. Then use the Saimfei EVOSM5000 take photos. Randomly selected 5 visual fields for each hole, and calculated the phagocytic ratio. Hoechst33342 (100*) emits blue fluorescence and DiI emits red fluorescence.

2.10. Statistical analysis

Repeated all the experiments at least 3 times, and expressed the experimental data as mean ± standard deviation (SD). GraphPadPrism8.0 software is used to perform one-way ANOVA. When $P<0.05$, the difference was considered to be statistically Statistical significance.

3. Results

3.1. Effects of 2GY irradiation on Hela cell pyroptosis

In order to study the effect of 2GY irradiation on pyroptosis of Hela cells. We explored the pyroptosis of Hela cells by culturing Hela cells for different times under 2GY dose irradiation. The results showed that when Hela cells were irradiated with 2GY dose, the morphology of Hela cells hardly changed with the increase of culture time (Fig. 1A), the release of LDH was not obvious, and there was almost no rupture and leakage of plasma

membrane (Fig. 1B). Flow cytometry results showed that compared with 8GY and 15GY irradiation, the cell death rate was very low and had little effect on cells (Fig. 1C, D). The results of Western blot suggested that the activation of the pyroptosis protein GSDME protein was not obvious with time. There was no significant difference in the expression of the total band, no obvious cleavage band GSDME-N and GSDME-C protein expression, and no obvious pyroptosis (Fig. 1E)

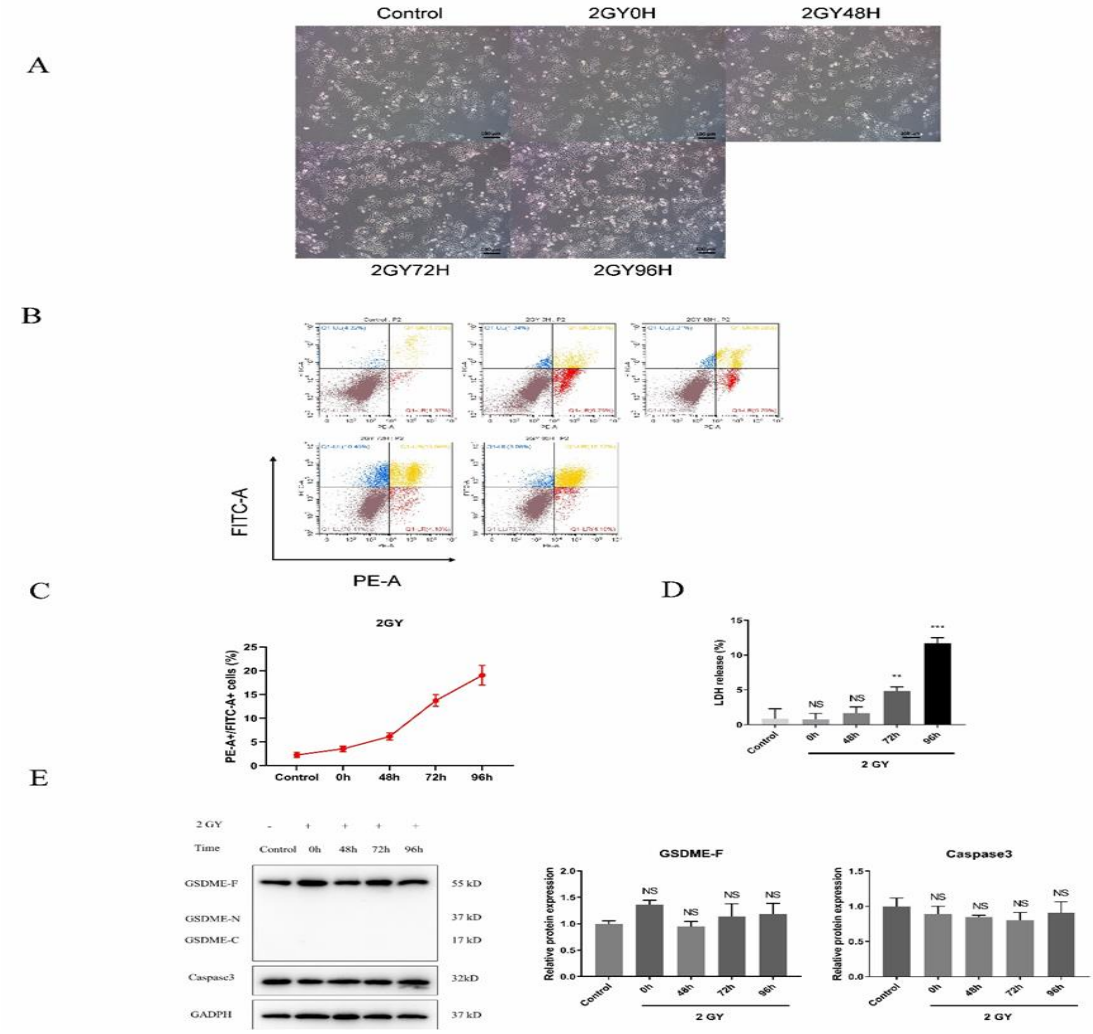


Figure 1: Effects of 2 GY irradiation on Hela cell pyroptosis. (A) Cell map observed under the microscope, the scale bar is 100 μm. (B) The release rate of inflammatory mediators was measured by LDH. (C-D) Flow cytometry for Hela survival rate and quantitative results. (E) By Western blot, the expression of GSDME and Caspase3 in irradiated Hela cells was detected. All data were analyzed by one-way ANOVA. All data were expressed as mean ±SD (n = 3). Compared with the control group, NS > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001.

3.2. Effects of 8GY irradiation on Hela cell pyroptosis

In order to study the effect of 8GY irradiation on pyroptosis of Hela cells. We explored the pyroptosis of Hela cells by culturing Hela cells for different times under 8 GY dose irradiation. The results showed that Hela cells

were irradiated at the dose of 8GY, and with the increase of treatment time, large bubbles and cell swelling were observed under the microscope, resulting in cell death (Fig. 2A). LDH release increased significantly with the extension of culture time, and rupture and leakage of plasma membrane occurred (Fig. 2B), In addition, flow cytometry results showed that compared with 2GY irradiation, the cell death rate gradually increased with the increase of culture time, reaching more than 50% at 72h (Fig. 2C, D). Western blot results showed that the pyroptosis protein GSDME protein increased with the prolongation of time, the activation was significantly enhanced, the expression of the total band decreased, and the expression of GSDME-N and GSDME-C proteins in the cleavage band increased, which was significantly enhanced at 72h, and the cells underwent pyroptosis (Fig. 2E)

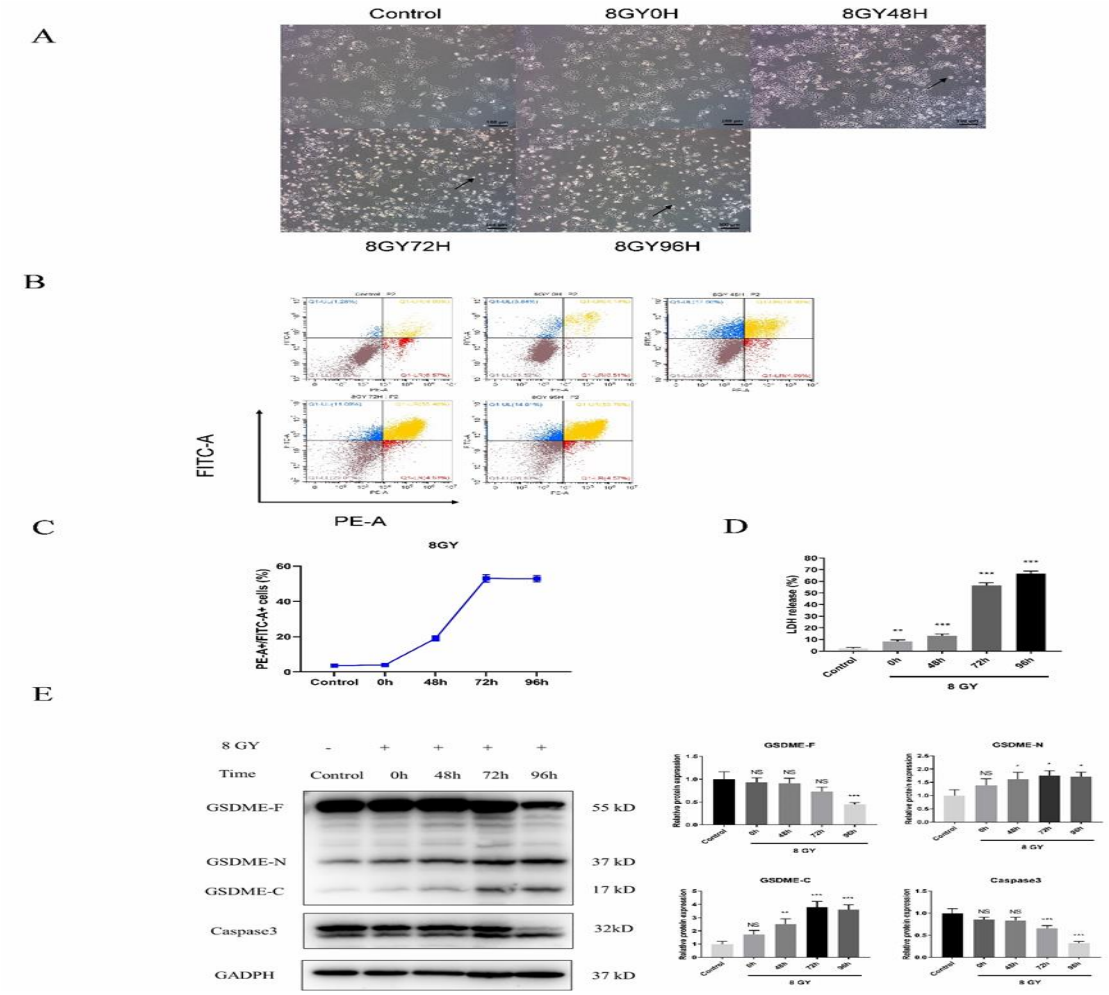
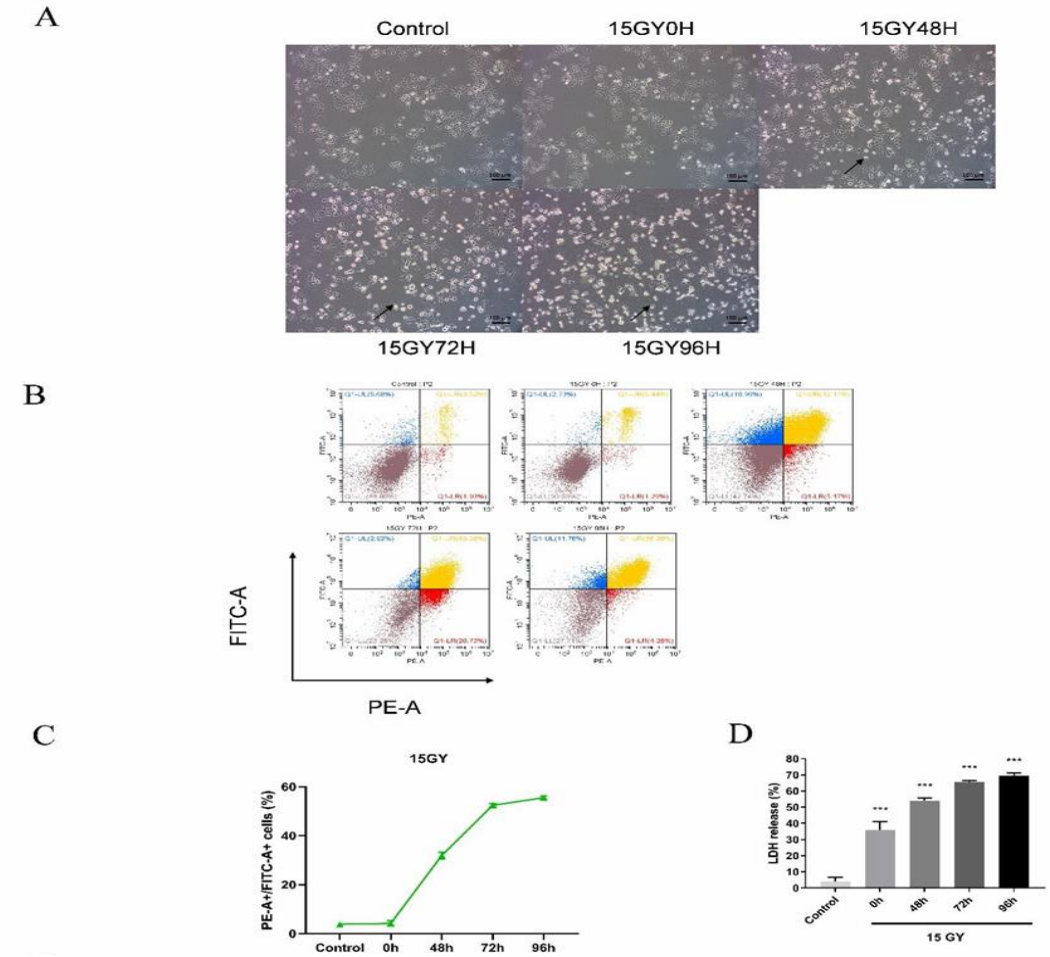


Figure 2: Effects of 8 GY irradiation on Hela cell pyroptosis. (A) Cell map observed under the microscope, the scale bar is 100 μm, the black arrow is where the large bubbles appear. (B) The release rate of inflammatory mediators was measured by LDH. (C-D) Flow cytometry for Hela survival rate and quantitative analysis results. (E) The expression of GSDME and Caspase3 in irradiated Hela cells was detected by Western blot. ImageJ software was used for quantitative detection of related protein levels, with GADPH as internal reference. Please refer to the previous for the format required for data analysis and presentation.

3.3. Effects of 15GY irradiation on Hela cell pyroptosis

In order to study the effect of 15GY irradiation on pyroptosis of Hela cells. We explored the pyroptosis of Hela cells by culturing Hela cells for different times under 15 GY dose irradiation. The results showed that Hela cells were irradiated with a dose of 15GY, and with the extension of treatment time, it was observed under the microscope that the plasma membrane cells of Hela cells appeared bubble earlier, and a large number of cells died in a short time (Fig. 3A), and the release of LDH also increased significantly (Fig. 3B).

Flow cytometry results showed that the cell death rate gradually increased with the increase of culture time compared with 2GY irradiation (Fig. 3C, D), Western blot results showed that the activation of pyroptosis protein GSDME protein was significantly enhanced in a short time, and the expression of total band decreased, the expression of GSDME-N and GSDME-C proteins in the cleavage zone increased, but the expression decreased to insignificant after 48h. Under long-term culture, a large number of cells died and proteins were degraded (Fig. 3E), which was not conducive to the experiment



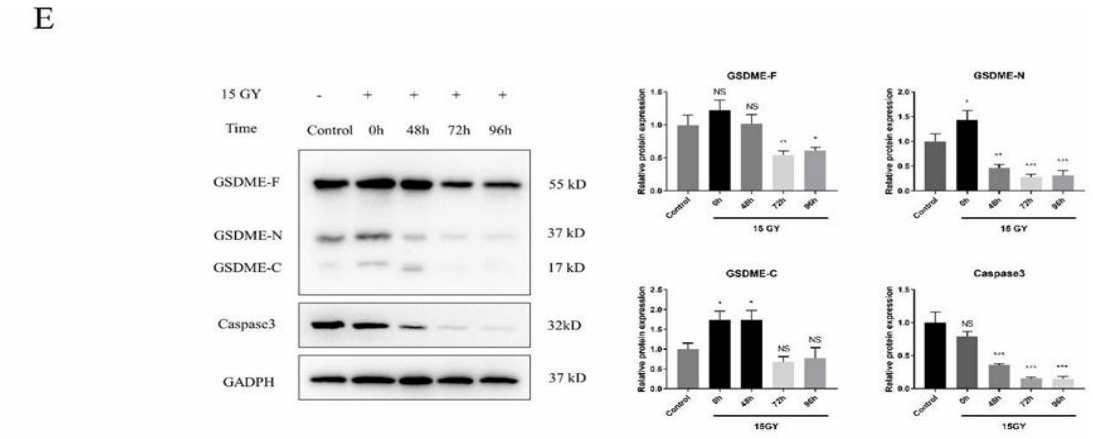
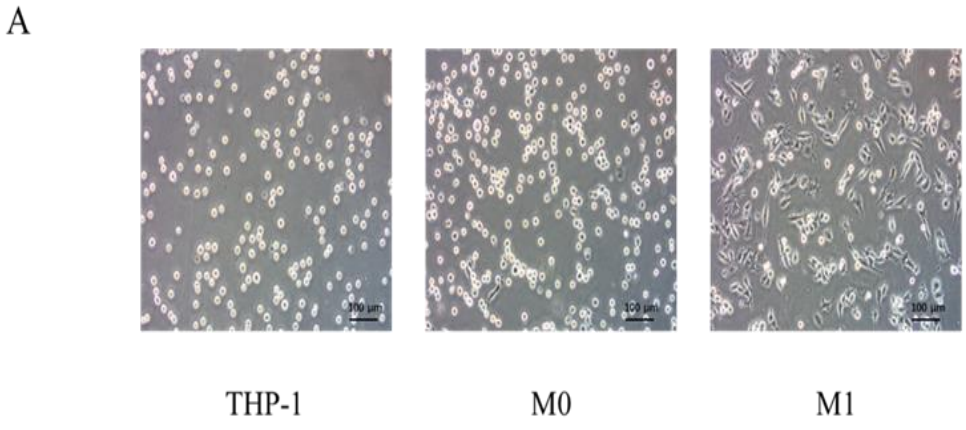


Figure 3: Effects of 15GY irradiation on Hela cell pyroptosis. (A) Cell map observed under the microscope, the scale bar is 100 μm, the black arrow is where the large bubbles appear. (B) The release rate of inflammatory mediators was measured by LDH. (C, D) Flow cytometry for Hela survival rate and quantitative analysis results. (E) The expression of GSDME and Caspase3 and their cutting band in irradiated Hela cells was detected by Western blot. ImageJ software was used for quantitative detection of related protein levels, with GADPH as the internal reference. Please refer to the previous for the format required for data analysis and presentation.

3.4. Differentiation of THP-1 cells into M1-macrophage-like cells

So as to investigate the relationship between pyroptosis and anti-tumor immunity, THP-1 cells were first selected to induce the generation of M1 macrophages. Under cytoscopic observation, THP-1 cells were in round suspension, M0 macrophages were in round adherent state, and M1 macrophages with relatively long pseudopodia adherent state (Fig. 4A). In addition, flow cytometry with M1-specific fluorescent antibody CD86 showed that CD86 was highly expressed in M1 macrophages. However, it was almost not expressed in THP-1 cells and M0 cells, indicating successful induction of M1 (Fig. 4B, C). qRT-PCR results suggested that the expressions of inflammatory factors IL-6, TNF-α, IL-1β and IL-8 in M1 macrophages were significantly increased (Fig. 4D).



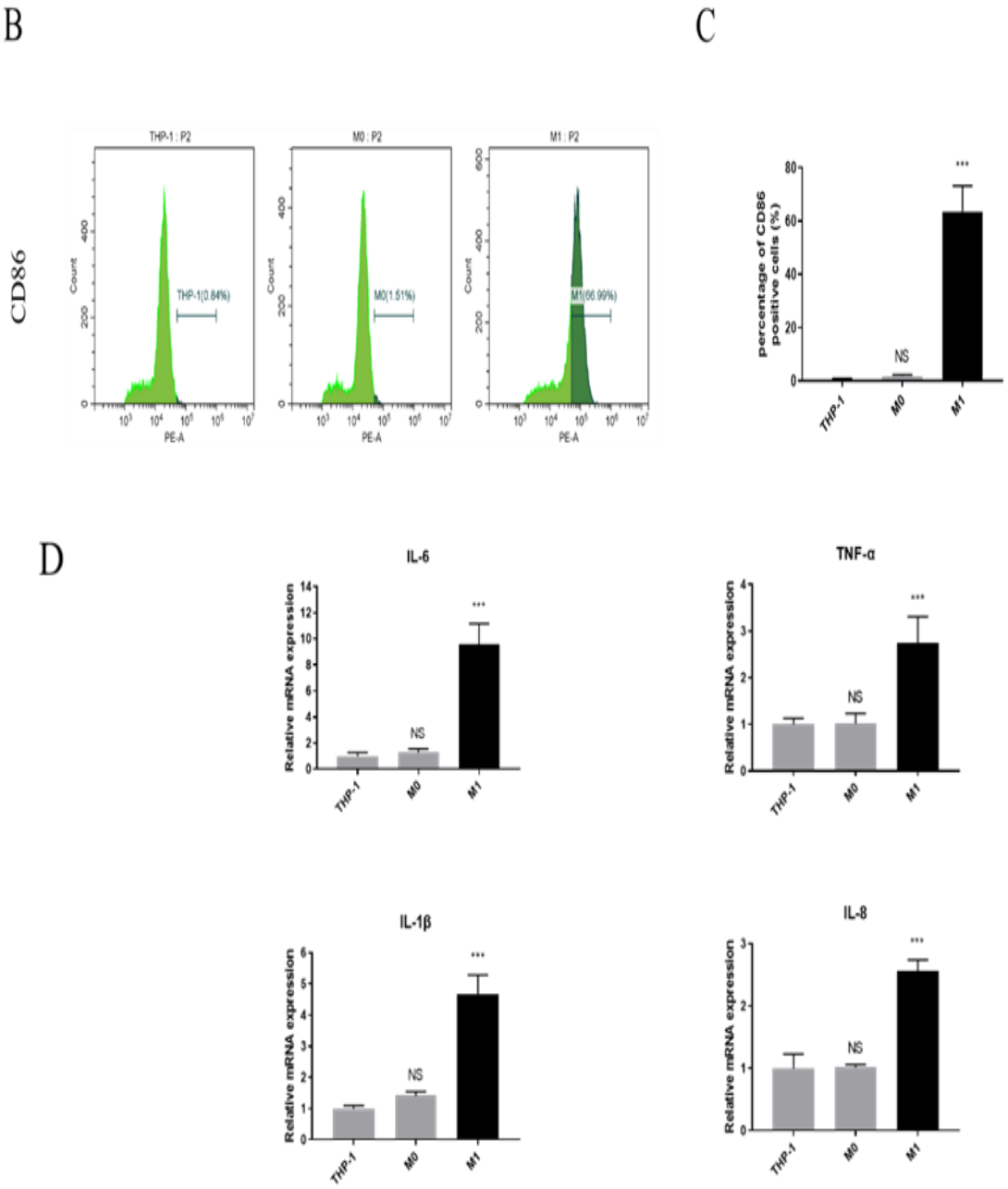


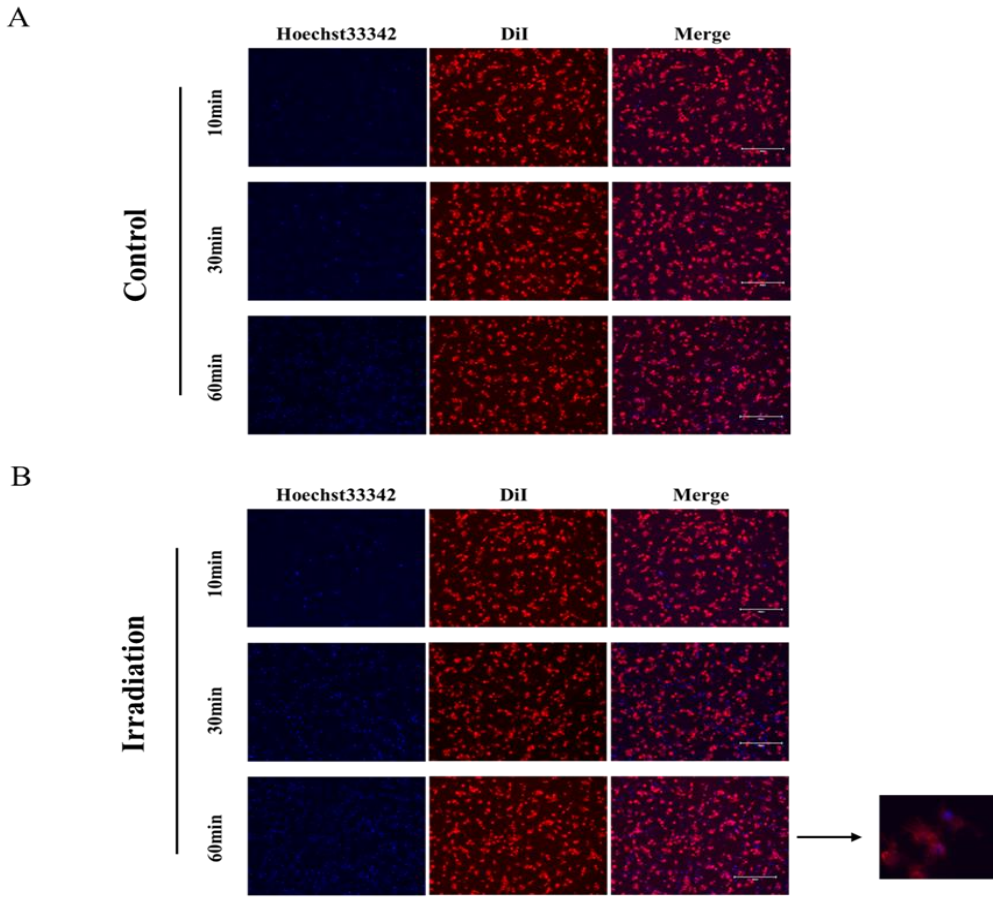
Figure 4: THP-1 induces the production of M1 macrophages. (A) Cell map observed under the microscope, the scale bar is 100 μm. The flow cytometry detected the (B-C) THP-1-induced M1 macrophage production. (D) The mRNA expressions of inflammatory factors TNF-α, IL-1β, IL-6, and IL-8 were detected by qRT-PCR with GADPH as the internal reference. Please refer to the previous for the format required for data analysis and presentation.

3.5 Effect of M1 macrophages on pyroptosis Hela cells

To explore the relationship between pyroptosis and anti-tumor immunity, we stained the cell membrane of the induced M1 macrophages with DiI (red fluorescence), and then used Hoechst333342 to stain the nuclei (blue fluorescence) of Hela cells after trypsinization and centrifugation. M1

macrophages and Hela cells were 1:2 Proportional co-culture. When M1 macrophages phagocytosed Hela cells, the two fluorophores of the phagocytosed cells overlapped. For cells in each treatment group, 5 fields of view were randomly selected under a fluorescence microscope, the percentage of phagocytic cells in the total number of cells was counted, and then the average was calculated. The control group was re-suspended with 1640 medium without serum and penicillin-streptomycin, and the irradiation treatment group was res-uspended with the supernatant of Hela cell medium treated with 8Gy irradiation for 72h.

Fluorescence imaging was performed at 10 min, 30 min, and 60 min, respectively, and the results showed that compared with the unirradiated control group, M1 macrophages in the irradiated group had a better phagocytosis effect on the pyroptotic HeLa cells (Fig. 5A, B). The fluorescence results were counted, and the phagocytosis rate was calculated. The results showed that compared with the control group, the proportion of Hela cells phagocytosed by M1 macrophages in the treatment group was significantly higher, indicating that the irradiation-induced pyroptotic hela cells released certain substances into the culture. In the basal supernatant, the phagocytosis of phagocytes was promoted to a certain extent, thereby promoting anti-tumor immunity (Fig. 5C).



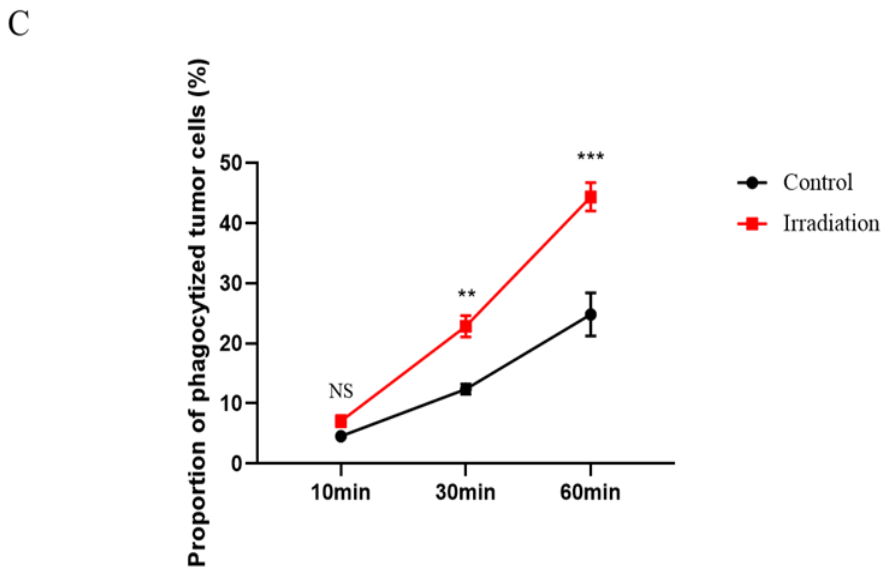


Figure 5: The phagocytosis of pyroptosis Hela cells by M1 macrophages. (A, B) The phagocytosis of tumors by M1 macrophages was observed by fluorescence imaging. (C) The phagocytic ratio of tumour cells was analyzed by cell count. Hela cells (blue fluorescence, Hoechst33342 staining) and M1 macrophages (red fluorescence, DiI staining) were 300 μm in scale. Please refer to the previous for the format required for data analysis and presentation.

4. Discussion

Cancer is an important public health problem around the world, and it's also one of the most concerning diseases in developed countries, and a great deal of energy has been invested in the discovery and development of treatments for cancer. Although anti-tumor treatment has improved in the past decades, cancer is still one of the most harmful diseases in the world (Viegas, Ladeira, Costa-Veiga, Perelman, & Gajski, 2017). As the World Health Organization predicts that the incidence rate of cancer in the general population will increase significantly, the current epidemiological data is still worrying. By 2025, 2030 and 2035, the annual number of new cases will increase from 14 million to 19 million, 21.7 million and 24 million respectively. Cervical cancer is the second leading cause of death of gynecological cancer (Parkin, Bray, Ferlay, & Pisani, 2005). Treatments for cancer include radiotherapy, surgical operation, chemotherapy, immunotherapy, and hormone therapy. Of these, Radiotherapy is very important, and about half of all cancer patients receiving it. Great advances in radiotherapy (RT) were attributed to the research findings of X-rays (1895) and the first application in clinical radiology (1896) (Bernier, Hall, & Giaccia, 2004). Advances in precision radiotherapy have enhanced local control and improved outcomes in clinical applications, effectively enhancing the clinical efficacy of radiotherapy. Meanwhile the recent research into radiobiology has transformed the paradigm to combine biological accuracy with physical targeting to realize the

personalization of cancer treatment. In the current era, radiotherapy serves as the primary treatment for more than half of all cancer patients (Delaney, Jacob, Featherstone, & Barton, 2005).

A radiotherapy treatment induces various types of cellular death in order to treat cancer, as do most anticancer treatments (Verheij, 2008). There is evidence that pyroptosis may serve as a mechanism to inhibit tumor growth (F. Wang et al., 2018). A recent study provided an alternative way to kill cancer cells induced by pyroptosis (Li et al., 2021). In pyroptosis, gasdermin family proteins form pores, which are essential for the execution of the process. Recent researchs have shown that the gasderminE (GSDME) can transform caspase3-mediated apoptosis induced by chemotherapeutic drugs into pyroptosis (Y. Wang et al., 2017). GSDME includes N-terminal and C-terminal domains, and N-terminal monomers oligomerize on the plasma membrane to form pores. Activated caspase-3 cleaves GSDME on interdomain connectors, releases N-terminal domains, forms membrane pores, and causes pyroptosis. Structure-guided mutagenesis shows that the implementation of pyroptosis depends on the pore-forming activity of the gasdermin-N domain (Ding et al., 2016). These findings change the way programmed cell death is understood because caspase-3 has long term been taken for a marker of apoptosis. In fact, it is the expression or level of GSDME that decides the form of death of caspase-3-activated cells. GSDME-High cells were scorched to death under the action of "apoptosis stimulation" of similar chemotherapeutic drugs, and cells lacking enough GSDME occurred to secondary necrosis after apoptosis. In a study led by Hu et al. (Hu et al., 2020), GSDME-C palmitoylation and GSDME-N membrane pore effect were shown to have an effect on drug-induced pyroptosis. The absence of GSDME invalidates some chemotherapeutic drugs. There is evidence from studies that DNA methyltransferase inhibitor dexitabine inhibits hypermethylation of the GSDME promoter in cancerous tissues with low expression of GSDME, induce pyroptosis by enhancing the expression of GSDME (Fan et al., 2019; Ruan, Wang, & Wang, 2020).

In colon cancer cells, Yu et al. (Yu et al., 2019) discovered that GSDME activated caspase-3/-9 ,and induced pyroptosis downstream of the ROS/JNK/bax-mitochondrial apoptosis pathway. Research has shown that GSDME-dependent pyroptosis is a pathway where carboplatin scavenges tumor cells, which may be of great importance for anticancer therapy. However, some data showed that both cisplatin and paclitaxel-induced apoptosis and secondary necrosis or pyroptosis of A549 cells, but the secondary necrosis or pyroptosis induced by cisplatin were more obvious, and the standards of caspase-3 activation and GSDME-NT production were higher than those of paclitaxel. These results suggest that the different modes of death induced by paclitaxel and cisplatin in A549 lung cancer cells may be due to the obvious activation of caspase-3 and the cleavage of GSDME.

Many researchs have shown that pyroptosis is of great benefit to cancer treatment during cancer treatment, mainly through the activation of caspase-3 and the cutting of GSDME (An et al., 2021; J. Chen et al., 2022; Jiang, Qi, Li, & Li, 2020). However, its not clear whether the cervical cancer Hela cells will also kill cancer cells through pyroptosis during radiotherapy. Therefore, this paper uses cervical cancer Hela cells as the basis to perform radiotherapy on them to investigate the changes in cancer cells. Our study found that under different irradiation doses and times, Hela cells died less under low-dose 2 GY irradiation. Observe the cells under the microscope and find that the morphology of Hela cells hardly changes with the increase of culture time (Fig. 1A). In addition, LDH release is also not obvious, indicating that the plasma membrane hardly ruptures and leaks (Fig. 1B). The results of flow cytometry show that the cell death rate is very low with the increase of culture time under the irradiation dose of 2 GY, which has little effect on cells (Fig. 2C, D). Western blot results also found that GSDME only have the main band, and their cleavage bands GSDME-N, GSDME-C, (Fig. 1D), which shows that the low-dose 2 GY irradiation has little effect on cells. However, under the irradiation conditions of medium dose 8 GY and high dose 15 GY, compared with the non-irradiated group, the expression of GSDME-N and GSDME-C in the irradiated group was significantly increased (Fig. 2D and 3D). Microscopic observation of cells found that with the increase of culture time, large bubbles and cell swelling appeared in the cytoplasmic membrane (Fig. 2A and 3A), and the release of LDH increased significantly, indicating that the plasma membrane ruptured and leaked (Fig. 2B and 3B). The outcomes of flow-cytometry also suggested that the cell death rate accumulated with the increase of culture time under the irradiation dose of 8GY and 15GY, and the cell death rate increased significantly when it reached 72h. (Fig. 2C, D and 3C, D). However, after culturing for 72h under 15GY high-dose irradiation, a large number of cells died, and the protein expression was significantly reduced, which was not suitable for subsequent experiments. The above results suggest that cells cultured under 8GY dose irradiation for 72h may be suitable conditions for cell pyroptosis.

Therefore, we chose 8GY dose culture conditions for 72h for subsequent experiments. In conclusion, we can infer that the death of cancer cells caused by irradiation may be partially completed by GSDME/Caspase-3-induced pyroptosis, that is, radiotherapy for cancer may be partially achieved by pyroptosis. Pyroptosis has important implications for the treatment of cancer. Some researchers have found that inflammation caused by pyroptosis triggers strong anti-tumor immunity through a bioorthogonal system (Q. Wang et al., 2020). In addition, inflammatory mediators released by pyroptosis can adjust the number and function of immunocyte, and metabolic status, in short, cell pyroptosis is closely associated with the tumor immune microenvironment. Therefore, based on the research finding that cervical cancer Hela cells can be induced to undergo pyroptosis by radiation therapy,

we investigated the relationship between pyroptosis and anti-tumor immunity by co-culture with M1 macrophages induced by the differentiation of human monocyte line THP-1 relationship between. THP-1 cells are frequently-used as models of human monocytes and are widely used to research the function, mechanism, and signal pathways of monocytes (Chanput, Mes, & Wichers, 2014). THP-1 macrophages can differentiate into M1 and M2 macrophages according to the stimuli used (Tjiu et al., 2009). Previous studies have found that THP-1 stimulated by PMA, IFN-γ, and LPS can be induced to differentiate into M1 macrophages, while THP-1 stimulated by PMA and IL-4 can be induced to differentiate into M2 macrophages (Zong et al., 2019). This classification is based on the involvement of T1 and T2 helper molecules in an inflammatory response that causes macrophages to secrete certain cytokines. The type of cytokines released determines whether macrophages belong to type M1 or type M2. According to the existing studies, it is found that M1 macrophages synthesize proinflammatory cell factor such as TNF- α, IL-1β, IL-6 and IL-8. In addition, specific markers show that M1 macrophages show polarization characteristics of increased CD86 expression at 24 hours (Q. W. Chen, Wu, & Yan, 2020). Some studies have shown that M2 macrophages are generally considered to promote the occurrence and progression of tumors, while M1 macrophages can kill and clear tumor cells, which is consistent with the main physiological function of M1 macrophages in scavenging foreign bodies (Wanderley et al., 2018). In this study, we used THP-1 to induce M1 macrophages to differentiate into M1 macrophages by PMA, IFN-γ, and LPS stimulation.

By observing the morphology of M1 macrophages under the microscope, we can see that M1 macrophages showed fusiform with a longer pseudopod attachment state (Fig. 4A) compared with round suspended THP-1 cells. We analyzed the M1 marker CD86 by flow cytometry and found that the expression of CD86 in M1 macrophages was significantly higher than that in M0 macrophages and THP-1 cells (Fig. 4B, C), indicating that M1 macrophages successfully induced differentiation. Moreover, we also analysed the expression of M1 related inflammatory factors by qRT-PCR experiment. The results suggested that the expression of inflammatory cytokines IL-6, TNF-α, IL-1β, and IL-8 increased significantly compared with M0 macrophages and THP-1 (Fig. 4D), which was consistent with the characteristic that M1 phagocytes could cause the release of pro-inflammatory cytokines. After confirming that the induction of M1 macrophages was successful, we used M1 macrophages to co-culture with Hela cells. By staining the cells, Hela cells were blue, and M1 macrophages were red. By fluorescence imaging at different times, we could clearly see that compared with un-irradiated Hela cells, the irradiated Hela cells were more phagocytosed by M1 macrophages (Fig. 5A, B). It was proved that the ratio of phagocytosis by macrophages of Hela cells undergoing pyroptosis was significantly higher than that of Hela cells that were not irradiated (Fig. 5C).

Therefore, it can be explained that the irradiated Hela cells that have undergone pyroptosis are better phagocytosed by M1 macrophages, the phagocytosis rate is higher, and the anti-tumor immune effect is better.

Macrophages are important members of the mononuclear macrophage immune system and widely exist in almost all tissues of multicellular organisms. These macrophages are indispensable for regulating innate immunity and helping to initiate adaptive immunity (Ley, 2017). Macrophages are white blood cells that play an important role in maintaining tissue stability, killing microorganisms, regulating inflammation, immunosuppression, antigen processing, and tissue remodelling (Haniffa, Bigley, & Collin, 2015; Seyedizade et al., 2020; Yona & Gordon, 2015). Furthermore, macrophages can activate the complement system by secreting various cytokines and participating in the process of immunoregulation (J. Zhou et al., 2020). Therefore, macrophages have become the central drug targets in various disease states, including the tumor microenvironment (TME). Researches have shown that M1 macrophages can lead to the production of cytokines in TME and promote the destruction of tumor cells by recruiting immunostimulatory white blood cells and phagocytosis of tumor cells. However, M2 macrophages play an important role in tumor proliferation (H.-W. Wang & Joyce, 2010). It drives the development of primary and metastatic tumors by its contribution to basement membrane rupture and deposition, angiogenesis, leukocyte recruitment, and global immunosuppression (Caux, Ramos, Prendergast, Bendriss-Vermare, & Ménétrier-Caux, 2016; Quail & Joyce, 2013). Furthermore, the combination of radiotherapy and immunotherapy is a reasonable way to enhance anti-tumor immune response, which has actually been used in clinical trials of many kinds of human cancers (Jagodinsky, Harari, & Morris, 2020; Johnson & Jagsi, 2016; Kosinsky et al., 2018). Therefore, in our experiment, we used differentiation-induced M1 macrophages for anti-tumor immunity experiments. In the results of co-culture with Hela cells, Hela cells were phagocytosed by M1 macrophages. In the irradiated Hela cells, the phagocytosis effect was more pronounced, which was significantly different from that of unirradiated Hela cells. It may be due to the pyroptosis caused by its irradiation, and the pyroptotic cells release a certain cytokine, which promotes the phagocytosis of macrophages. Pyroptosis is a new mode of immunogenic cell death (ICD), which has gradually become an excellent opportunity to improve the effectiveness of cancer immunotherapy. In general, pyroptosis appears in macrophages after pathogen infection. It's very important to eliminate pathogens (M. Wang, Jiang, Zhang, Li, & Wang, 2019). Pyroptosis involves swelling of the cells and rupture of the plasma membrane, resulting in the release of pro-inflammatory cytokines such as IL-1β, as well as cell contents released into the extracellular space and activation of the immune response. As a form of cell death highly immunogenic, pyroptosis causes local inflammation and attracts inflammatory cells to the site. It presents a good opportunity to alleviate the

immunosuppression of TME while inducing a systemic immune response to treat solid tumors (Z. Zhang et al., 2020). M1 macrophages can also lead to the production of cytokines in TME, resulting in the accumulation of a large number of inflammatory mediators in TME, and better promoting the occurrence of anti-tumor immunity. It can be explained that when irradiated Hela cells were co-cultured with M1 macrophages, the phagocytosis and destruction of cervical cancer Hela cells were more obvious. Although our study has shown that pyroptosis can promote anti-tumor immunity, its specific influencing factors are still uncertain. In our follow-up study, we will perform transcriptome sequencing of co-cultured phagocytes. After detecting the altered transcriptome sequence, corresponding explorations are made to identify specific factors that affect anti-tumor immunity and further improve our research.

5. Conclusion

In conclusion, our study has highlighted the significant potential of radiotherapy to induce pyroptosis in cervical cancer HeLa cells via the caspase-3/GSDME signaling pathway, presenting a novel mechanism to enhance anti-tumor immunity. By identifying optimal radiation conditions that promote this form of programmed cell death, we have uncovered a promising avenue for improving the efficacy of cancer treatments, emphasizing the role of pyroptosis in activating systemic immune responses within the tumor microenvironment. Moreover, the implications of this therapy extend beyond the immediate realm of tumor suppression, suggesting potential effects on the mental and physical health of patients, including athletes who may face unique challenges during cancer treatment.

Understanding how therapeutic strategies like radiotherapy-induced pyroptosis impact the overall well-being of individuals, particularly those engaged in high levels of physical activity, is crucial for developing comprehensive care plans that address both the oncological and holistic health needs of cancer patients. Future research should focus on further elucidating the mechanisms underlying pyroptosis induction by radiotherapy and its effects on the immune system, as well as exploring the broader implications of such treatments on patient health and quality of life. By integrating insights from oncology with considerations for mental and physical health, especially in populations with specific health considerations like athletes, we can move toward more personalized and effective cancer care strategies that support not only the eradication of cancer cells but also the overall well-being of patients.

Declaration of competing interests

The authors declare that there is no conflict of interest regarding the

publication of this paper.

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REFERENCES

Allen, C., Her, S., & Jaffray, D. A. (2017). Radiotherapy for cancer: present and future. In (Vol. 109, pp. 1-2).

An, H., Heo, J. S., Kim, P., Lian, Z., Lee, S., Park, J., . . . Ooshima, A. (2021). Tetraarsenic hexoxide enhances generation of mitochondrial ROS to promote pyroptosis by inducing the activation of caspase-3/GSDME in triple-negative breast cancer cells. *Cell Death & Disease, 12*(2), 159.

Arbyn, M., Weiderpass, E., Bruni, L., de Sanjosé, S., Saraiya, M., Ferlay, J., & Bray, F. (2020). Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. *The Lancet Global Health, 8*(2), e191-e203.

Bernier, J., Hall, E. J., & Giaccia, A. (2004). Radiation oncology: a century of achievements. *Nature Reviews Cancer, 4*(9), 737-747.

Caux, C., Ramos, R. N., Prendergast, G. C., Bendriss-Vermare, N., & Ménétrier-Caux, C. (2016). A milestone review on how macrophages affect tumor growth. *Cancer research, 76*(22), 6439-6442.

Chanput, W., Mes, J. J., & Wichers, H. J. (2014). THP-1 cell line: an in vitro cell model for immune modulation approach. *International immunopharmacology, 23*(1), 37-45.

Chen, J., Ge, L., Shi, X., Liu, J., Ruan, H., Heng, D., & Ye, C. (2022). Lobaplatin induces pyroptosis in cervical cancer cells via the caspase-3/GSDME pathway. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 22*(11), 2091-2097.

Chen, Q. W., Wu, G. D., & Yan, Z. Y. (2020). Zhongguo ying yong sheng li xue za zhi = Zhongguo yingyong shenglixue zazhi. *Chinese journal of applied physiology, 36*(6), 534-538. doi:https://doi.org/10.12047/j.cjap.5865.2020.113

Delaney, G., Jacob, S., Featherstone, C., & Barton, M. (2005). The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. *Cancer: Interdisciplinary International Journal of the American Cancer Society, 104*(6), 1129-1137.

Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., . . . Shao, F. (2016). Pore-forming activity and structural autoinhibition of the gasdermin family. *Nature, 535*(7610), 111-116.

Fan, J.-X., Deng, R.-H., Wang, H., Liu, X.-H., Wang, X.-N., Qin, R., . . . Zhou,

P.-H. (2019). Epigenetics-based tumor cells pyroptosis for enhancing the immunological effect of chemotherapeutic nanocarriers. *Nano letters, 19*(11), 8049-8058.

Fang, Y., Tian, S., Pan, Y., Li, W., Wang, Q., Tang, Y., . . . Ma, P. (2020). Pyroptosis: a new frontier in cancer. *Biomedicine & Pharmacotherapy, 121*, 109595.

Forastiere, A. A., Goepfert, H., Maor, M., Pajak, T. F., Weber, R., Morrison, W., . . . Chao, C. (2003). Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. *New England Journal of Medicine, 349*(22), 2091-2098.

Friedlander, A. M. (1986). Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *Journal of Biological Chemistry, 261*(16), 7123-7126.

Haniffa, M., Bigley, V., & Collin, M. (2015). *Human mononuclear phagocyte system reunited.* Paper presented at the Seminars in cell & developmental biology.

Hausman, D. M. (2019). What is cancer? *Perspectives in biology and medicine, 62*(4), 778-784.

Hu, L., Chen, M., Chen, X., Zhao, C., Fang, Z., Wang, H., & Dai, H. (2020). Chemotherapy-induced pyroptosis is mediated by BAK/BAX-caspase-3-GSDME pathway and inhibited by 2-bromopalmitate. *Cell Death & Disease, 11*(4), 281.

Islami, F., Fedewa, S. A., & Jemal, A. (2019). Trends in cervical cancer incidence rates by age, race/ethnicity, histological subtype, and stage at diagnosis in the United States. *Preventive medicine, 123*, 316-323.

Jagodinsky, J. C., Harari, P. M., & Morris, Z. S. (2020). The promise of combining radiation therapy with immunotherapy. *International Journal of Radiation Oncology* Biology* Physics, 108*(1), 6-16.

Jiang, M., Qi, L., Li, L., & Li, Y. (2020). The caspase-3/GSDME signal pathway as a switch between apoptosis and pyroptosis in cancer. *Cell death discovery, 6*(1), 112.

Johnson, C. B., & Jagsi, R. (2016). The promise of the abscopal effect and the future of trials combining immunotherapy and radiation therapy. *International Journal of Radiation Oncology* Biology* Physics, 95*(4), 1254-1256.

Jorgensen, I., & Miao, E. A. (2015). Pyroptotic cell death defends against intracellular pathogens. *Immunological reviews, 265*(1), 130-142.

Kosinsky, Y., Dovedi, S. J., Peskov, K., Voronova, V., Chu, L., Tomkinson, H., . . . Helmlinger, G. (2018). Radiation and PD-(L) 1 treatment combinations: immune response and dose optimization via a predictive systems model. *Journal for immunotherapy of cancer, 6*, 1-15.

Lefebvre, J.-L., Chevalier, D., Luboinski, B., Kirkpatrick, A., Collette, L., & Sahmoud, T. (1996). Larynx preservation in pyriform sinus cancer: preliminary results of a European Organization for Research and

Treatment of Cancer phase III trial. *JNCI: Journal of the National Cancer Institute, 88*(13), 890-899.

Ley, K. (2017). M1 means kill; M2 means heal. *The Journal of Immunology, 199*(7), 2191-2193.

Li, L., Jiang, M., Qi, L., Wu, Y., Song, D., Gan, J., . . . Bai, Y. (2021). Pyroptosis, a new bridge to tumor immunity. *Cancer Science, 112*(10), 3979-3994.

Lu, H., Zhang, S., Wu, J., Chen, M., Cai, M.-C., Fu, Y., . . . Yu, Z. (2018). Molecular targeted therapies elicit concurrent apoptotic and GSDME-dependent pyroptotic tumor cell death. *Clinical Cancer Research, 24*(23), 6066-6077.

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global cancer statistics, 2002. *CA: a cancer journal for clinicians, 55*(2), 74-108.

Quail, D. F., & Joyce, J. A. (2013). Microenvironmental regulation of tumor progression and metastasis. *Nature medicine, 19*(11), 1423-1437.

Rébé, C., Derangère, V., & Ghiringhelli, F. (2015). Induction of pyroptosis in colon cancer cells by LXRβ. *Molecular & cellular oncology, 2*(1), e970094.

Rogers, C., Fernandes-Alnemri, T., Mayes, L., Alnemri, D., Cingolani, G., & Alnemri, E. S. (2017). Cleavage of DFNA5 by caspase-3 during apoptosis mediates progression to secondary necrotic/pyroptotic cell death. *Nature communications, 8*(1), 14128.

Ruan, J., Wang, S., & Wang, J. (2020). Mechanism and regulation of pyroptosis-mediated in cancer cell death. *Chemico-biological interactions, 323*, 109052.

Seyedizade, S. S., Afshari, K., Bayat, S., Rahmani, F., Momtaz, S., Rezaei, N., & Abdolghaffari, A. H. (2020). Current status of M1 and M2 macrophages pathway as drug targets for inflammatory bowel disease. *Archivum Immunologiae et Therapiae Experimentalis, 68*, 1-24.

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2021). Cancer statistics, 2021. *Ca Cancer J Clin, 71*(1), 7-33.

Tjiu, J.-W., Chen, J.-S., Shun, C.-T., Lin, S.-J., Liao, Y.-H., Chu, C.-Y., . . . Inoue, H. (2009). Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction. *Journal of Investigative Dermatology, 129*(4), 1016-1025.

Verheij, M. (2008). Clinical biomarkers and imaging for radiotherapy-induced cell death. *Cancer and Metastasis Reviews, 27*, 471-480.

Viegas, S., Ladeira, C., Costa-Veiga, A., Perelman, J., & Gajski, G. (2017). Zaboravljeni učinci raka na javno zdravstvo–pregled. *Arhiv za higijenu rada i toksikologiju, 68*(4), 287-297.

Wanderley, C. W., Colon, D. F., Luiz, J. P. M., Oliveira, F. F., Viacava, P. R., Leite, C. A., . . . Silva, R. L. (2018). Paclitaxel reduces tumor growth by reprogramming tumor-associated macrophages to an M1 profile in a TLR4-dependent manner. *Cancer research, 78*(20), 5891-5900.

Wang, F., Liu, W., Ning, J., Wang, J., Lang, Y., Jin, X., . . . Yang, F. (2018). Simvastatin suppresses proliferation and migration in non-small cell lung cancer via pyroptosis. *International journal of biological sciences, 14*(4), 406.

Wang, H.-W., & Joyce, J. A. (2010). Alternative activation of tumor-associated macrophages by IL-4: priming for protumoral functions. *Cell cycle, 9*(24), 4824-4835.

Wang, M., Jiang, S., Zhang, Y., Li, P., & Wang, K. (2019). The multifaceted roles of pyroptotic cell death pathways in cancer. *Cancers, 11*(9), 1313.

Wang, Q., Wang, Y., Ding, J., Wang, C., Zhou, X., Gao, W., . . . Liu, Z. (2020). A bioorthogonal system reveals antitumour immune function of pyroptosis. *Nature, 579*(7799), 421-426.

Wang, Y., Gao, W., Shi, X., Ding, J., Liu, W., He, H., . . . Shao, F. (2017). Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. *Nature, 547*(7661), 99-103.

Xu, F., Cui, W.-Q., Wei, Y., Cui, J., Qiu, J., Hu, L.-L., . . . Liu, B.-J. (2018). Astragaloside IV inhibits lung cancer progression and metastasis by modulating macrophage polarization through AMPK signaling. *Journal of Experimental & Clinical Cancer Research, 37*, 1-16.

Yona, S., & Gordon, S. (2015). From the reticuloendothelial to mononuclear phagocyte system–the unaccounted years. *Frontiers in immunology, 6*, 328.

Yu, J., Li, S., Qi, J., Chen, Z., Wu, Y., Guo, J., . . . Zheng, J. (2019). Cleavage of GSDME by caspase-3 determines lobaplatin-induced pyroptosis in colon cancer cells. *Cell Death & Disease, 10*(3), 193.

Zhang, C.-c., Li, C.-g., Wang, Y.-f., Xu, L.-h., He, X.-h., Zeng, Q.-z., . . . Ouyang, D.-y. (2019). Chemotherapeutic paclitaxel and cisplatin differentially induce pyroptosis in A549 lung cancer cells via caspase-3/GSDME activation. *Apoptosis, 24*, 312-325.

Zhang, Z., Zhang, Y., Xia, S., Kong, Q., Li, S., Liu, X., . . . Ansara, J. (2020). Gasdermin E suppresses tumour growth by activating anti-tumour immunity. *Nature, 579*(7799), 415-420.

Zhao, P., Wang, M., Chen, M., Chen, Z., Peng, X., Zhou, F., . . . Qu, J. (2020). Programming cell pyroptosis with biomimetic nanoparticles for solid tumor immunotherapy. *Biomaterials, 254*, 120142.

Zhou, B., Zhang, J.-y., Liu, X.-s., Chen, H.-z., Ai, Y.-l., Cheng, K., . . . Wu, Q. (2018). Tom20 senses iron-activated ROS signaling to promote melanoma cell pyroptosis. *Cell research, 28*(12), 1171-1185.

Zhou, J., Tang, Z., Gao, S., Li, C., Feng, Y., & Zhou, X. (2020). Tumor-associated macrophages: recent insights and therapies. *Frontiers in oncology, 10*, 188.

Zong, Z., Zou, J., Mao, R., Ma, C., Li, N., Wang, J., . . . Shi, Y. (2019). M1 macrophages induce PD-L1 expression in hepatocellular carcinoma cells through IL-1β signaling. *Frontiers in immunology, 10*, 1643.