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EDITED BY
Takashi Karako,
National Center For Global Health and Medicine, Japan

REVIEWED BY
Qian He,
Shenzhen Polytechnic, China
Xin Shao,
Maoming People's Hospital, China

*CORRESPONDENCE
Bo Ning
13053818196@163.com
Xinmin Guo
guoxinmin@ext.jnu.edu.cn

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work

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Macrophages depletion alleviates lung injury by modulating AKT3/GXP4 following ventilator associated pneumonia

Youfeng Zhu[1†], Yang Chen[1†], Di Xie[2†], Dong Xia[1], Huanming Kuang[1], Xinmin Guo[3*‡] and Bo Ning[4*‡]

[1]Department of Intensive Care Unit, Guangzhou Red Cross Hospital, Jinan University, Guangzhou, Guangdong, China, [2]Department of Emergency, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China, [3]Department of Ultrasonography, Guangzhou Red Cross Hospital, Jinan University, Guangzhou, Guangdong, China, [4]Department of Neurosurgery, Guangzhou Red Cross Hospital, Jinan University, Guangzhou, Guangdong, China

Background: AKT3 appears to play a role in lung cancer. However, its role in ventilator-associated pneumonia is still unclear. Therefore, this study aimed to investigate the role of AKT3 in macrophages during ventilator-associated pneumonia.

Methods: The mRNA level of AKT3, Data from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), The data is analyzed using the Xiantao academic analysis tool. Additionally, the roles of AKT3 in ventilator-associated pneumonia (VAP) were investigated through *in vivo* experiments.

Results: AKT3 was differentially expressed in various normal and tumor tissues. Functional enrichment analysis indicated the immunomodulatory function and inflammatory response of AKT3 in lung cancer. Depletion of macrophages protected against lung epithelial cells and significantly decreased MMP9, MMP19, FTH, and FTL expression levels and increased GPX4 expression levels, while partially reversing the changes in macrophage. Mechanistically, macrophage depletion attenuates ferroptosis of lung epithelial cells by modulating AKT3 following VAP.

Conclusion: Collectively, this study suggests the need for further validation of the immunoregulatory function of AKT3 in lung cancer. Additionally, macrophage depletion mitigates lung injury by modulating the AKT3/GPX4 pathway in the context of VAP.

KEYWORDS

lung cancer, Akt3, macrophage, immunomodulation, VAP

1 Introduction

Monocytes and macrophages (MΦs) play a critical role as immediate responders to various types of invading pathogens, primarily of viral and bacterial origins (1). They are important components of the innate immune system and possess essential abilities, including phagocytosis, cytokine production and release, and antigen presentation (2). Monocytes are typically found in the bloodstream, while macrophages (MΦs) are distributed throughout the body's tissues, including immune-privileged regions such as microglia in the central nervous system, ocular macrophages, and those in the testes and placenta (3). The widespread distribution of monocytes/MΦs positions them as one of the initial cell populations to encounter invading pathogens. Pneumonia involves the formation of a complex cytokine signaling network that includes various cell populations, such as airway epithelium, fibroblasts, and macrophages (MΦs) (4).

Ferroptosis is a distinctive form of programmed cell death that relies on iron. It is characterized by iron accumulation, lipid peroxidation, excessive production of reactive oxygen species, and mitochondrial shrinkage (5). This mode of cell death exerts a significant influence on the pathogenesis of several complex diseases, such as cancer, Parkinson's disease, and sepsis (6). Given its crucial involvement in various diseases, ferroptosis has become a central focus of research, with concentrated efforts aimed at identifying small molecules and drugs capable of disrupting this process (7).

Glutathione peroxidases (GPXs) are a group of antioxidant enzymes that are commonly expressed in various human tissues to detoxify peroxides. The GPX enzyme family consists of eight members, but only GPX1 and GPX4 are expressed in the human kiVAPey (8). Among these, GPX4 plays a critical role in ferroptosis.GPX4 is currently the only known enzyme with the ability to directly remove lipid peroxides, converting them from harmful to harmless substances, and ultimately interrupting the process of lipid peroxidation, thereby inhibiting cell ferroptosis. Inhibition of GPX4 activity results in the accumulation of lipid peroxides, which serves as an indicator of ferroptosis in cells (9–11). The phenotypic characteristics of M1 macrophages are determined by the expression of CD86, while M2 macrophages are identified by the presence of CD206. MMP9 serve as functional markers for M1 polarization (12–14).

Despite significant advancements in macrophage research, understanding the molecular mechanisms underlying their development remains elusive. This study aims to establish a foundation for comprehending the developmental mechanisms of lung macrophages and exploring their potential role in VAP.

2 Materials and methods

2.1 Data from public databases Cancer Genome Atlas gene expression and the application of the xiantao tool for analysis

The Cancer Genome Atlas (TCGA) gene expression RNA-seq data, were 1149 patients. The gene expression profile of the GSE215219 dataset, which contains RNA-seq data from 36 lung cancer patients, the TCGA data were used to evaluate diagnostic and prognostic potential of 12 MMPs in different cancer types. Gene expression measured by RNA-seq was analyzed by differential expression, hierarchical clustering. Data processing utilizing Xiantao academic tools (https://www.xiantaozi.com/products) (15, 16).

2.2 Differentially expressed genes analysis and functional enrichment were performed using the Xiantao tools

Functional enrichment analyses were performed on the screened overlapping genes using the xiantao tools. This included Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and tissue enrichment analysis. Gene Set Enrichment Analyses (GSEA) of the screened overlapping genes were conducted using Xiantao academic tools(https://www.xiantaozi.com/products) (17, 18).

2.3 PA VAP-induced lung injury

Model of lung injury induced by ventilator-associated pneumonia (VAP) was established in this study. Wild-type (WT) mice were anesthetized with avertin (15 mg/kg, Sigma) and were intranasally administered either 15 µl of normal saline as the control group, or an equal volume of Pseudomonas aeruginosa (PA) at a concentration. Two days after the administration of PA, the mice underwent mechanical ventilation (MV) with a high tidal volume for a duration of 3 hours (19).

2.4 Histopathological and morphological analysis

At the end of the 3 day, the lungs were removed, fixed with 4% paraformaldehyde, embedded in paraffin, and then were cut into 4µm thick sections. he parafin-embedded kiVAPeys and liver specimens were sectioned at 4 µm and then stained with hematoxylin-eosin (HE) staining. The histopathological changes in the pancreas were measured and classified according to the Schmidt score, including inflammation, the degree of edema, vacuolation, hemorrhage, and necrosis.

2.5 Macrophage depletion

For this study, a total volume of 300 µl of LC or LV was administered intraperitoneally for two consecutive days, either prior to or 48 hours after ischemia/reperfusion (I/R).In specific experiments, mice were administered an intravenous injection of $1 \times 10^{\wedge 6}$ IFN-γ or IL-4-activated macrophages via the retroorbital sinus immediately after I/R injury. The depletion of

circulating monocytes was evaluated by utilizing a Hemavet 950 automated cell counter (20).

2.6 Immunofluorescence

Lung tissue sections (20 μm) underwent deparaffinization and hydration. Antigen retrieval was performed, followed by blocking with 10% bovine serum albumin at room temperature for one hour. The sections were incubated overnight at 4°Cwith the following primary antibodies (all from AiFang Biological) diluted at 1:100: anti-MMP9 (AF06799), anti-MMP19 (AF02793), anti-SFTA1 (AF10524), anti-FTH (AF02247), anti-FTL (AF301381), and anti-GPX4 (AF11931).Lung epithelial cells were washed three times with PBS, fixed in 4% paraformaldehyde for 30 minutes, rinsed with cold PBS, and subsequently permeabilized with 0.3% Triton X-100 for 30 minutes. After permeabilization, cells were blocked with 10% bovine serum albumin for 1 hour prior to incubation with appropriate primary and secondary antibodies. Cells were observed using a fluorescence microscope (FV3000, Olympus). Co-staining was performed using a Seven color mIHC Fluorescence kit(Recordbio Biological Technology, Shanghai, China) based on the tyramide signal amplification (TSA) technology according to the manufacture's instruction. Mix concentrated fluorescent dye with TSA buffer in a ratio of 1:50-1:200. Apply the TSA fluorescent dye reaction solution evenly onto the tissue section and let it react at room temperature for 1-15 minutes. Place the paraffin section in antigen retrieval solution in a 95-degree water bath for 25-40 minutes. Preheat the mIHC specific antibody elution solution to 37 degrees until completely dissolved, then let it sit at 37 degrees for 5-20 minutes. Discard the elution solution and add a sufficient amount of antibody elution solution to cover the sample, then let it sit at 37 degrees for 5-20 minutes. Discard the elution solution and wash the samples with PBS three times, each time for 5 minutes.

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0.The $\chi 2$ test was used to assess the correlation between AKT3 expression and clinicopathological parameters. The Mann-Whitney U test or Kruskal-Wallis test was applied for other analyses. Two-sided P-values were used, and statistical significance was considered at an alpha level of $P< 0.05$.

3 Results

3.1 Tissue–specific expression patten of AKT3 in pan–cancer

First, the physiological levels of AKT3 mRNA using the TCGA dataset and the xiantao tool was utilized for supplementary analysis. Additionally, AKT3 mRNA levels in TCGA data using the Xiantao tools. Differentially expressed genes associated with AKT3 expression include ACC, BLCA, BRCA, CESC, CHOL, COAD, DLBC, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LAML, LGG, LIHC, LUAD, LUSC, and MESO (Figures 1A, B).

3.2 The predictive value of AKT3 in tumor-associated inflammation and on overall survival in different types of cancers by Xiantao tools

Patients with lung cancer who exhibited higher levels of AKT3 showed poorer overall survival (OS) (Figure 2A).The clinical significance of overall survival (OS, Figure 2B), progression-free interval (PFI, Figure 2C), pathological staging (Figure 2D), and quality of life outcomes (Figure 2E) were assessed.

3.3 Gene expression associated with AKT3 in lung cancer

Initially, the co-expressed genes associated with AKT3 in lung cancer patients from the TCGA dataset were investigated using Xiantao tools (Figures 3A-C). FAM241B, TMEM37, UGT2B7, LRG1, SHCBP1, SOSTDC1, NPTX1, FAM98B, ZNF439, and ZNF440 show differential expression using the Xiantao tools.

3.4 Functional enrichment analysis of AKT3 in lung cancer were performed by Xiantao tool

Figure 4A displays the top 8 enriched sets. The results of the enrichment analysis indicate that AKT3 and its associated partners serve as mediators in immunological modulation, particularly in immunoregulatory interactions associated with cytokine production, immune effector processes, nuclear division, mitotic nuclear division, cell cycle, and microtubule binding (Figures 4B, C). The bubble chart and mountain chart after GSAE analysis can be seen in the figure (Figures 4D, F). The infiltration of various immune cells in lung cancer can be seen in the Supplementary Figures (S1A-S1F).

3.5 Macrophages depletion contribute to the reduction of VAP-induced pulmonary, hepatic, and renal damage, as evidenced by pathological staining

The mice in the control group and the DM group showed transparent glomerular capillaries (Figures 5A, D). The endothelial cells of VAP mice exhibited more severe morphological damage than the VAP+DM group, manifested by severe endothelial cells adhesions and increased capillary collapse (Figures 5B, C). Compared with the VAP group, the collagen fiber content in the

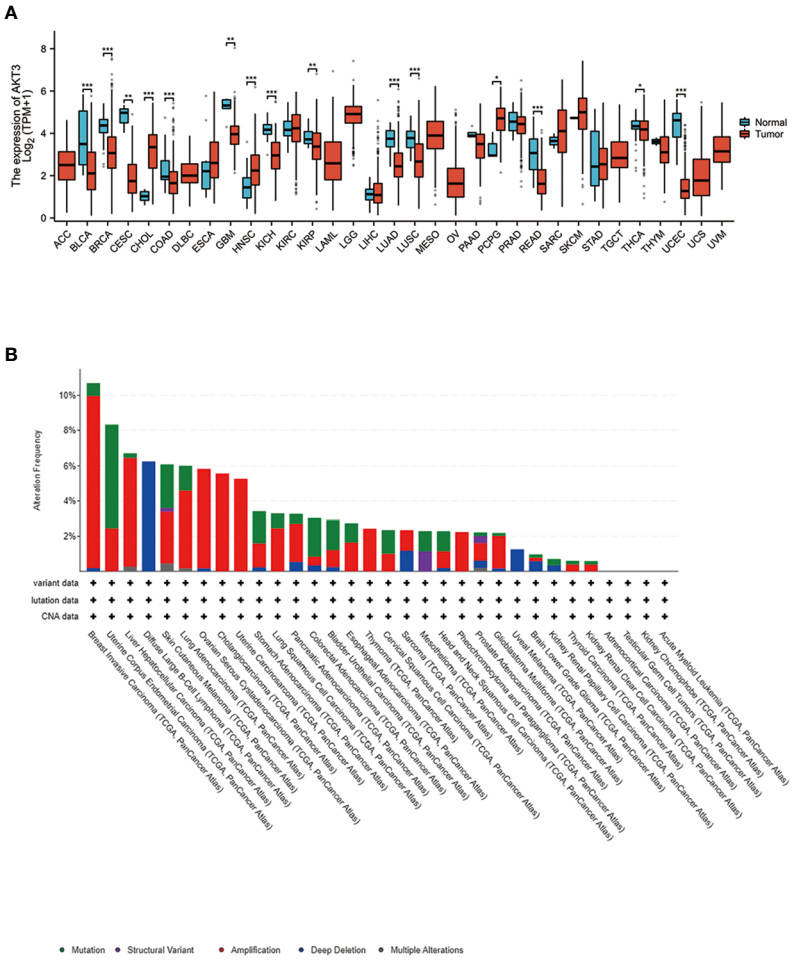

FIGURE 1
Tissue–specifc expression patten of AKT3 in pan–cancer. **(A)** Expression of AKT3 in multiple tumors. **(B)** Differentially expressed genes associated with AKT3 expression include ACC, BLCA, BRCA, CESC, CHOL, COAD, DLBC, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LAML, LGG, LIHC, LUAD, LUSC, and MESO. *P< 0.05, **P<0.01, *** P <0.005.

VAP+DM group was significantly reduced after HE staining (Figures 5E, F). Compared with the control group, the content of collagen fiber was not significantly increased in the liver of DM mice (Figures 5G, H). Here, we further investigated the effects of DM on kidney in VAP mice. Compared with the control group, the kidney injuries in VAP group included steatosis, edema, hyperemia, hepatocyte ballooning, and focal necrosis with inflammatory cell infiltration, whereas DM ameliorated the pathological damage (Figures 5I-L). In summary, these results demonstrate that DM has protective effects on organ injury of lung, liver and kidney.

3.6 Macrophage depletion inhibited the secretion of MMP9 and MMP19 of mice with VAP

To evaluate the potential impact of macrophage in VAP mice, we conducted an immunofluorescence assay to measure the protein expression secretion of inflammatory factors MMP9 and MMP19 in the lung tissues of each group. Immunofluorescence examination revealed a significant induction in the expressions of MMP9 and MMP19 in the tissue of VAP mice when compared to the control group, which was then promoted in the VAP+DM group, as demonstrated in Figures 6A-Z.

3.7 Macrophages depletion increased iron-induced cell death in lung epithelial cells

The production of FTH significantly increased in the lung epithelial cells of VAP mice, as shown in Figures 7A-L. Immunofluorescence examination demonstrated a significant induction of FTH and FTL expression in the tissue of VAP mice compared to the control group. This induction was decrease in the VAP+DM group, as shown in Figures 7M-Z.

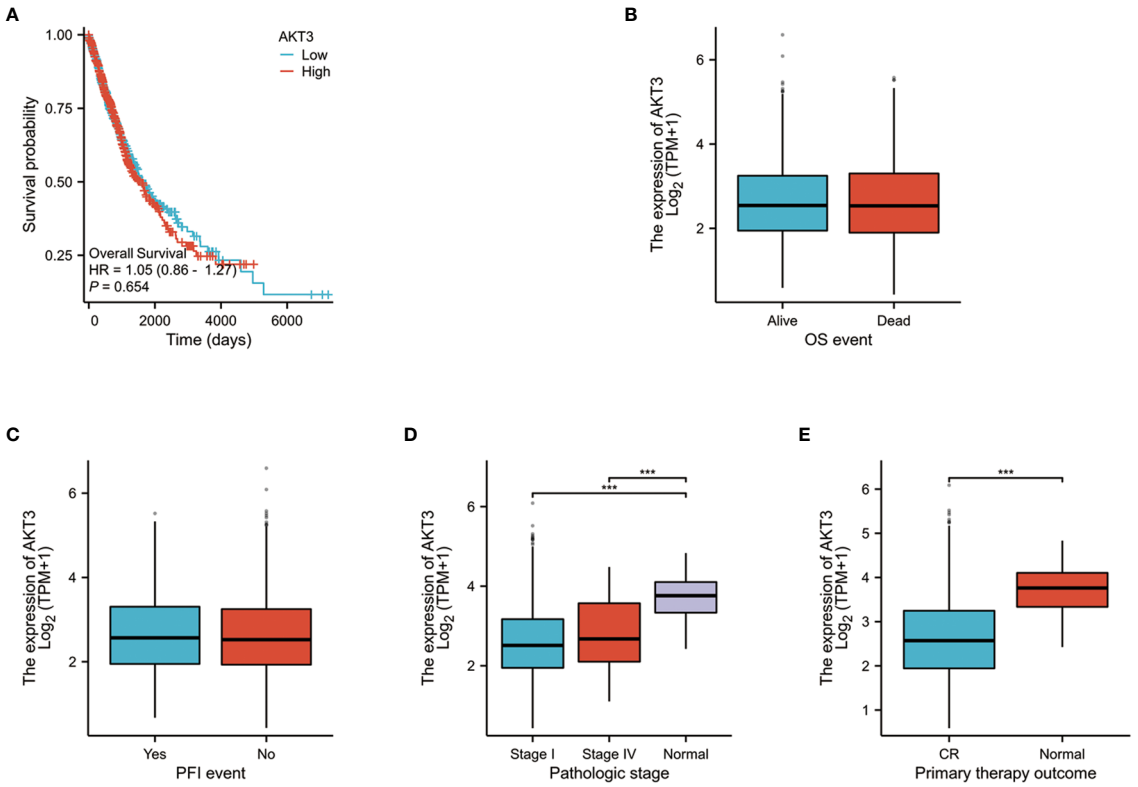

FIGURE 2
The predictive value of AKT3 in tumor-associated inflammation and on overall survival (OS) in diferent types of cancers by Xiantao tools. **(A)** Survival analysis by Xiantao tools. **(B–E)**. There is no difference in the survival pathological staging, clinical treatment efficacy, overall survival (OS), and progression-free interval (PFI) associated with AKT3. *** P <0.005.

3.8 Macrophage depletion inhibited the lung epithelial cells of ferroptosis by inhibiting the AKT3/GPX4 pathway

The VAP group exhibited a significant decrease in fluorescence intensity levels of GPX4 compared to the control group, as shown in Figures 8A-D. DM treatment partially attenuated the VAP-induced induction in fluorescence intensity levels of MMP9 and p-AKT, the effect of DM was attenuated by the additional AKT activator, as shown in Figures 8E-Z.

4 Discussion

Monocytes and macrophages (MΦs) play a crucial role as immediate responders to various types of invading pathogens, primarily of viral and bacterial origins. Monocytes/MΦs are integral components of the innate immune system and possess essential abilities such as phagocytosis, cytokine production and release, and antigen presentation. Monocytes are typically found in the bloodstream, whereas macrophages (MΦs) are distributed throughout the body's tissues, including immune-privileged regions such as microglia in the central nervous system, ocular macrophages, and those in the testes and placenta. The widespread distribution of monocytes/MΦs positions them as one of the initial cell populations to encounter invading pathogens. Pneumonia involves the formation of a complex cytokine signaling network comprising various cell populations, such as airway epithelium, fibroblasts, and macrophages (MΦs).This study aims to investigate the roles of lung resident macrophages (MΦs) and monocytes in the cytokine network within the context of the cellular microenvironment, disease history, and genetic factors (21–25).

It is noteworthy that MMP9 potentially plays a crucial role in mediating viral invasion. In the case of West Nile virus, MMP9 was found to be partially localized in the blood vessels, and its expression was increased in the brains of murine models. Additionally, in MMP9 knockout mice, there was a significant reduction in brain viral loads, blood-brain barrier permeability, inflammatory cytokines, and leukocyte infiltration. MMP19 expression has also been reported in macrophages, and it is upregulated under inflammatory conditions like arthritis and multiple sclerosis. Additionally, it has become evident that MMPs, Our study found increased expression of MMP9 and MMP19 in the lungs after VAP modeling. Decreased expression of MMP9 and MMP19 after macrophage clearance (26, 27).

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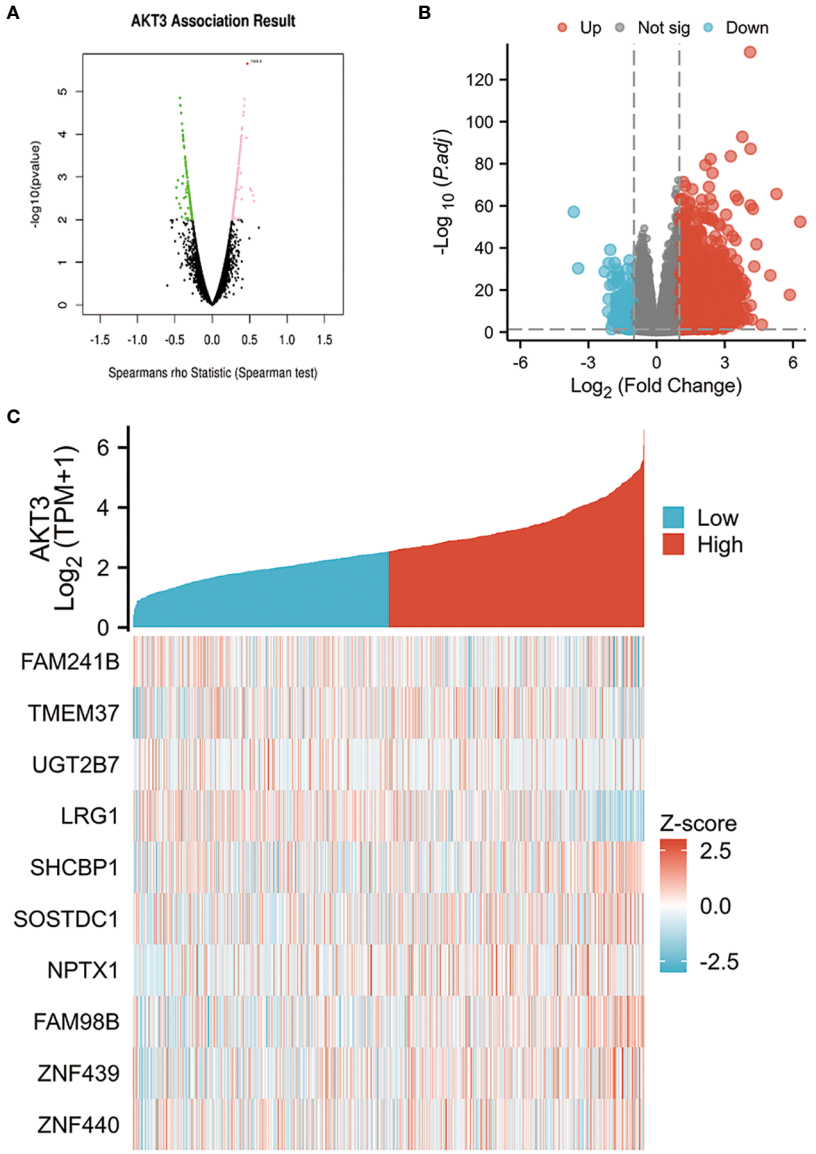

FIGURE 3
Gene expression associated with AKT3 in lung cancer. **(A)** Comparison of AKT3 expression level among different subtypes of lung cancer analyzed by Xiantao tools. **(B)** Comparison of AKT3 level in lung cancer patients by Xiantao tools. **(C)**. FAM241B, TMEM37, UGT2B7, LRG1, SHCBP1, SOSTDC1, NPTX1, FAM98B, ZNF439, and ZNF440 show differential expression.

Ferroptosis dysregulation adversely affects immune system functioning. Our studies show that the administration of dimethyl (DM) significantly reduces expression levels of Ferritin heavy chain (FTH) and Ferritin light chain (FTL) in mice with VAP. These findings suggest that DM inhibits ferroptosis in VAP, although the specific mechanism remains unclear (28–30).

Glutathione peroxidases (GPXs) are a group of antioxidant enzymes commonly expressed in various human tissues to detoxify peroxides. The GPX enzyme family comprises eight members, but only GPX1 and GPX4 are expressed in the human kiVAPey. Among these, GPX4 is critical in ferroptosis. GPX4 is currently the only known enzyme capable of directly removing lipid peroxides, converting them from harmful to harmless substances, and ultimately interrupting the process of lipid peroxidation, thereby inhibiting cell ferroptosis. Inhibition of GPX4 activity leads to the accumulation of lipid peroxides, which is an indicator of ferroptosis in cells. Our investigation found that administering DM significantly elevated GPX4 expression levels in mice with VAP (31, 32).

Importantly, the association between AKT3 and lung cancer has therapeutic implications. Targeting the AKT3 pathway has emerged as a potential therapeutic strategy to enhance treatment outcomes for lung cancer patients. Inhibitors of the AKT pathway, such as AKT-specific inhibitors or agents targeting upstream regulators of

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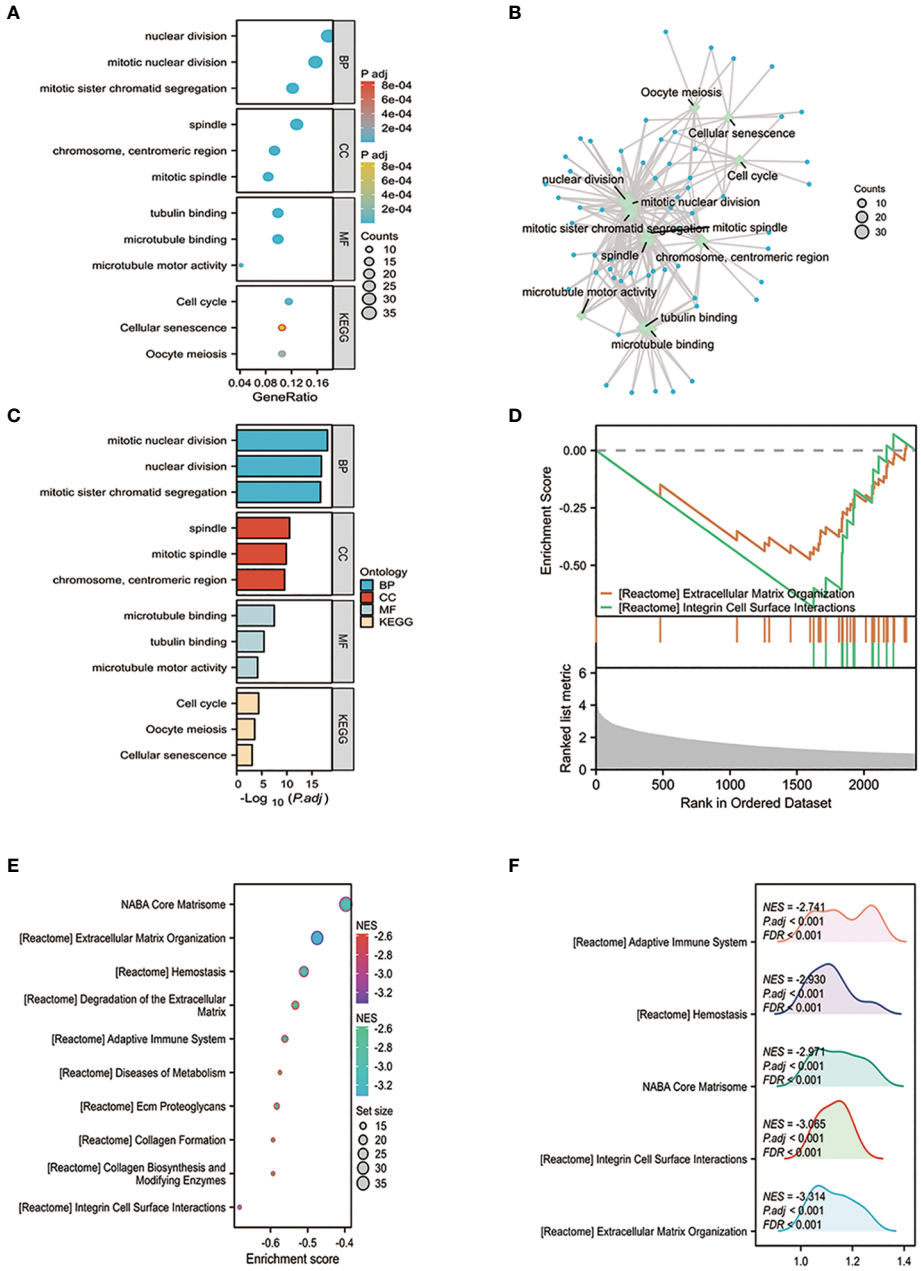

FIGURE 4
Genome-wide genes associated with AKT3 expression in lung cancer. **(A–F)** The enrichment analysis indicates that AKT3 and its associated partners play a significant role as mediators in immunological modulation. Specifically, they have implications in immunoregulatory interactions linked to cytokine production, immune effector processes, nuclear division, mitotic nuclear division, cell cycle, and microtubule binding.

AKT3 activation, have shown promise in preclinical studies and clinical trials. Combining AKT inhibitors with standard therapies, such as chemotherapy or targeted therapies, may provide a synergistic effect and improve treatment response.

In addition to its role in lung cancer, AKT3 has also been implicated in ventilator-associated pneumonia (VAP), a common nosocomial infection in critically ill patients on mechanical ventilation. AKT3 activation has been linked to the regulation of innate and adaptive immune responses that contribute to the pathogenesis of VAP. Targeting the AKT3 pathway in the context of VAP may help modulate the immune response and reduce the incidence and severity of this infectious complication. In conclusion, AKT3 plays a significant role in lung cancer development and progression. Dysregulation of the AKT3 signaling pathway contributes to tumor cell survival, proliferation, resistance to therapy, and metastatic potential. Targeting AKT3 or

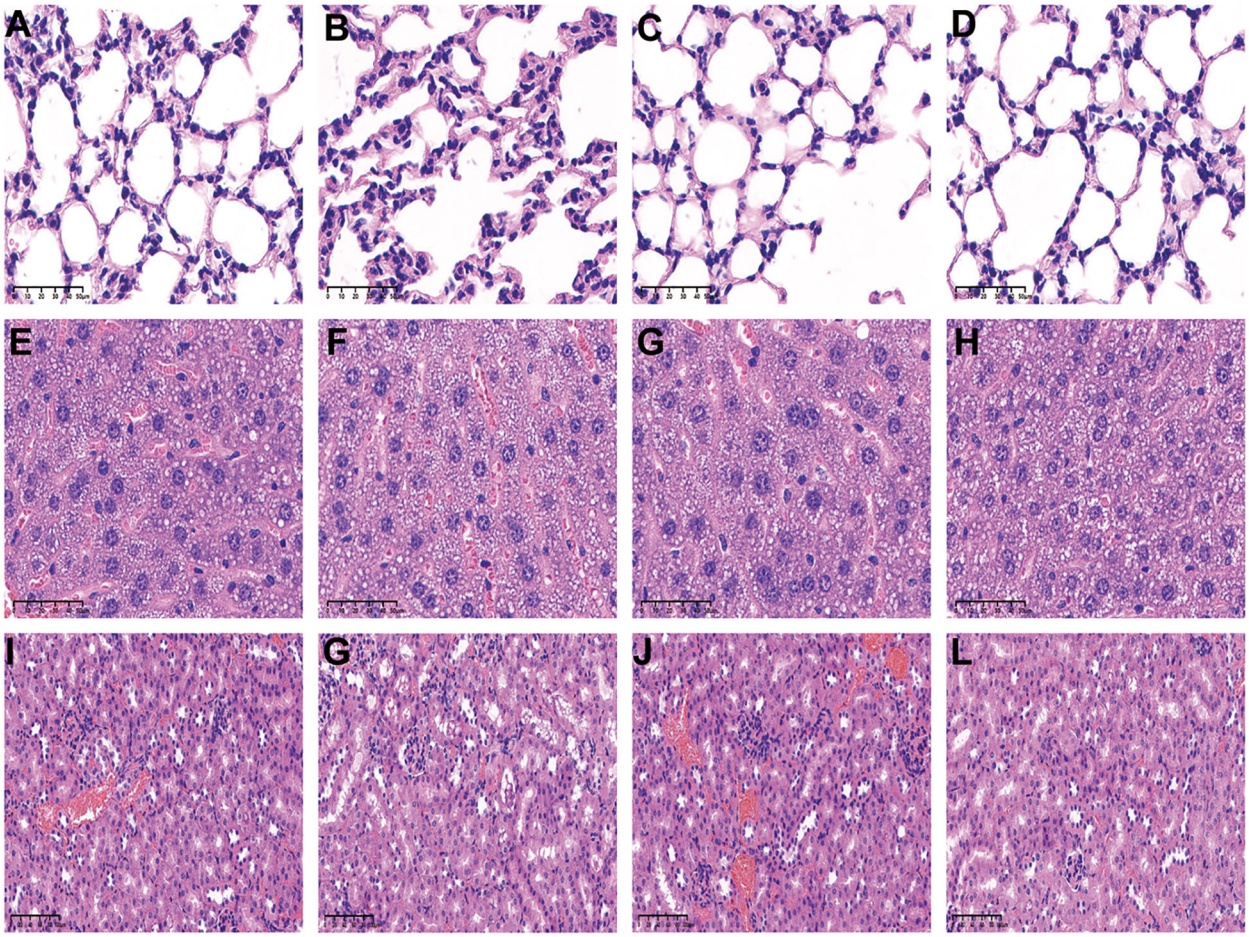

FIGURE 5
Macrophages depletion contribute to the reduction of VAP-induced pulmonary, hepatic, and renal damage, as evidenced by pathological staining. **(A-D)** HE staining of glomerulus under inverted fluorescence microscopy. **(E-H)** HE staining of liver in each group. **(I-L)** HE staining of kidney sections in each group. N=4, A-P scar bar =50um.

its downstream effectors holds promise as a therapeutic approach for lung cancer. Additionally, the involvement of AKT3 in VAP highlights its potential as a target for modulating the immune response in this infectious condition. Further research and clinical trials are warranted to fully explore the therapeutic potential of AKT3 inhibition in both lung cancer and VAP. AKT3, a member of the AKT protein kinase family, has been implicated in the regulation of matrix metalloproteinases (MMPs), which are a group of enzymes responsible for extracellular matrix (ECM) degradation and remodeling. The interplay between AKT3 and MMPs plays a crucial role in various physiological and pathological processes, including cancer metastasis, tissue repair, and inflammation.

In cancer, dysregulation of the AKT3-MMP axis has been associated with tumor invasion, angiogenesis, and metastasis. AKT3 promotes the expression and activation of MMPs, facilitating ECM degradation and enabling cancer cells to invade surrounding tissues and disseminate to distant sites. Moreover, AKT3-induced MMP activation can promote the release of growth factors and cytokines from the ECM, promoting tumor cell proliferation, angiogenesis, and immune evasion (33–35).

The phenotypic characteristics of M1 and M2 macrophages are differentiated by CD86 and CD206, respectively. Functional markers for M1 polarization include MMP9. Our research findings suggest that eliminating macrophages can alleviate the infiltration of inflammatory factors in the lungs. This elimination also reduces iron-induced death of alveolar epithelial cells through the AKT pathway.

5 Conclusion

This study investigates the expression of AKT3 in pulmonary tumors and reveals its association with inflammatory factors and immune function. Additionally, an animal model was established to understand the role of AKT3 in ventilator-associated pneumonia. Our study reveals that depletion of macrophages alleviates lung injury by modulating the AKT3/GXP4 signaling pathway in ventilator-associated pneumonia.

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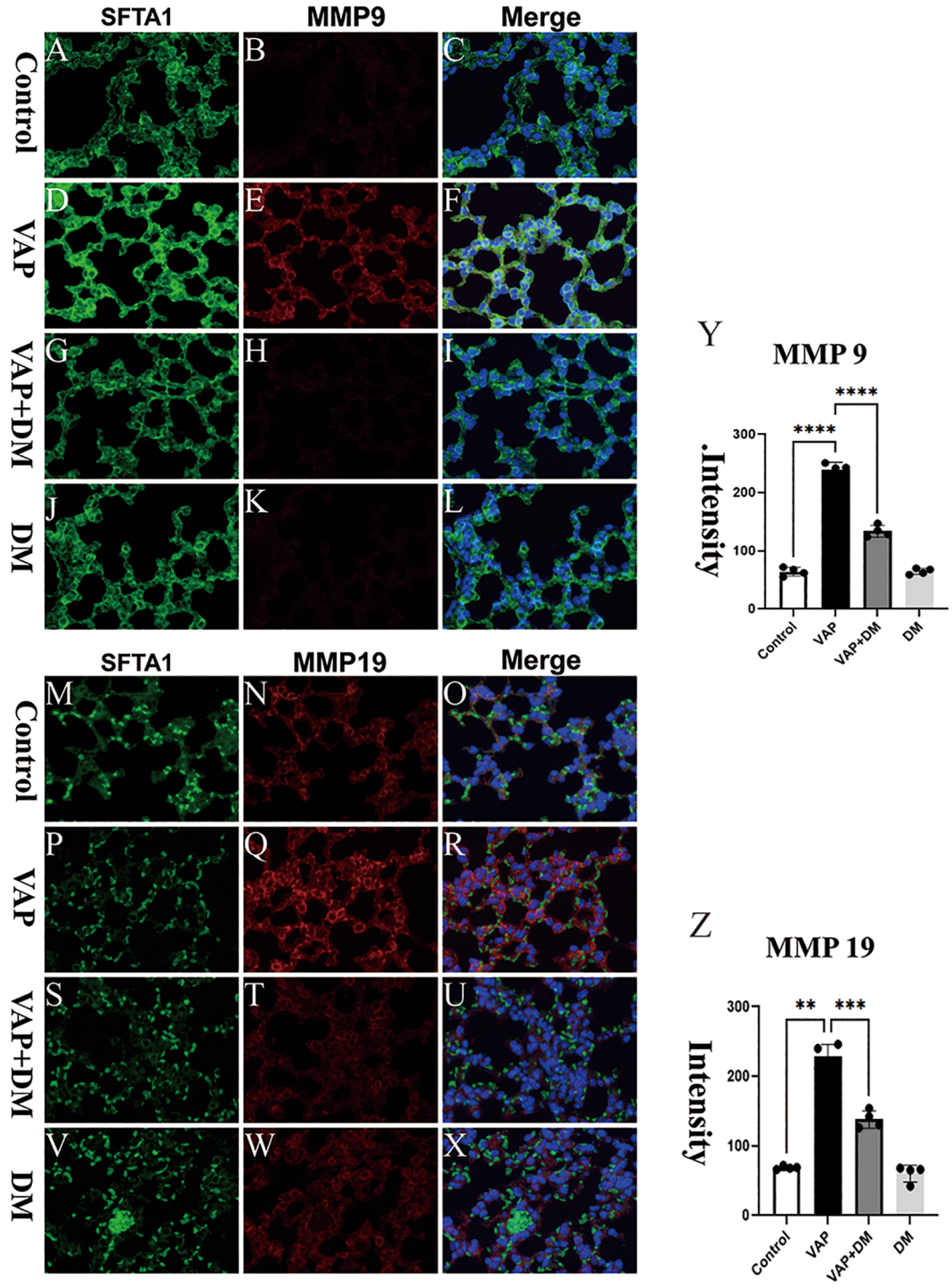

FIGURE 6
Macrophage depletion inhibited the secretion of MMP9 and MMP19 of mice with VAP. Immunofluorescence was used to show the distribution of MMP9 (yellow, **B, E, H, K**) and SFTA1 (green, **A, D, G, J**). The co localization images of the MMP9, DAPI, and SFTA1 are shown in **(C, F, I, L)**. Immunofluorescence was used to show distributions of SFTA1 (green, **M, P, S, V**) and MMP19 (yellow, **N, Q, T, W**) **(O, R, U, X)** with merge pictures. **(Y, Z)** displayed MMP9 and MMP19 bands. The sample size was N=4 with significance set at **P < 0.05,***P < 0.01, ****P < 0.005. Scale bars for **(A–X)** were set at 50 μm.

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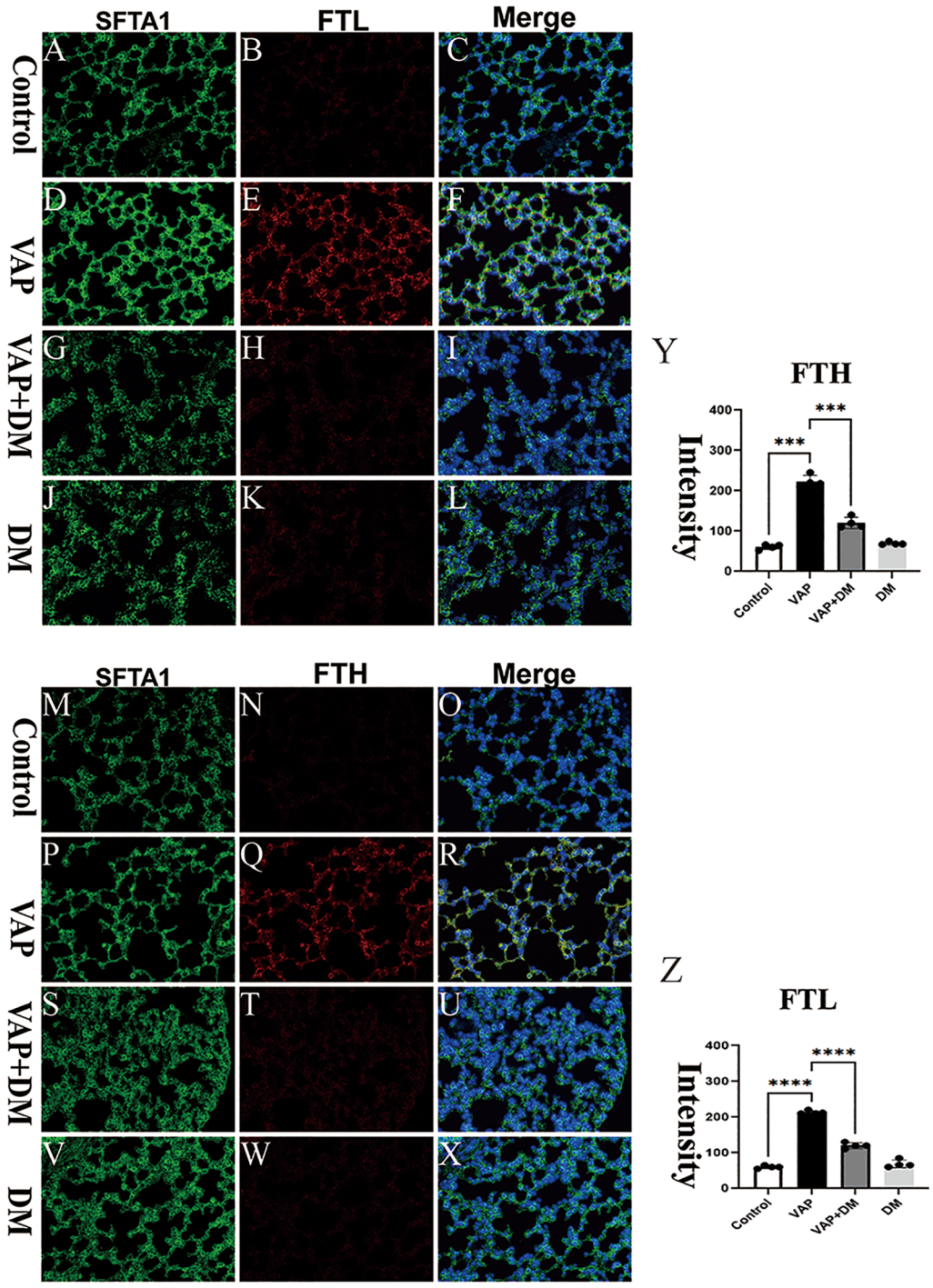

FIGURE 7
Macrophage depletion inhibited the lung epithelial cells of ferroptosis. Immunofluorescence was used to show distributions of FTH (red, **B, E, H, K**) and SFTA1 (green, **A, D, G, J**) with the merge pictures shown in **(C, F, I, L)**. Double labeled immunofluorescence showed the expression of SFTA1 (green, **M, P, S, V**) and FTL (red, **N, Q, T, W**), and the co-localization was shown in **(O, R, U, X)**. The FTH and FTL is shown in **(Y, Z)**, with a sample size of N=4 and significance level of ***P < 0.01, ****P < 0.005. Scale bars for **(A–X)** were set at 50 μm.

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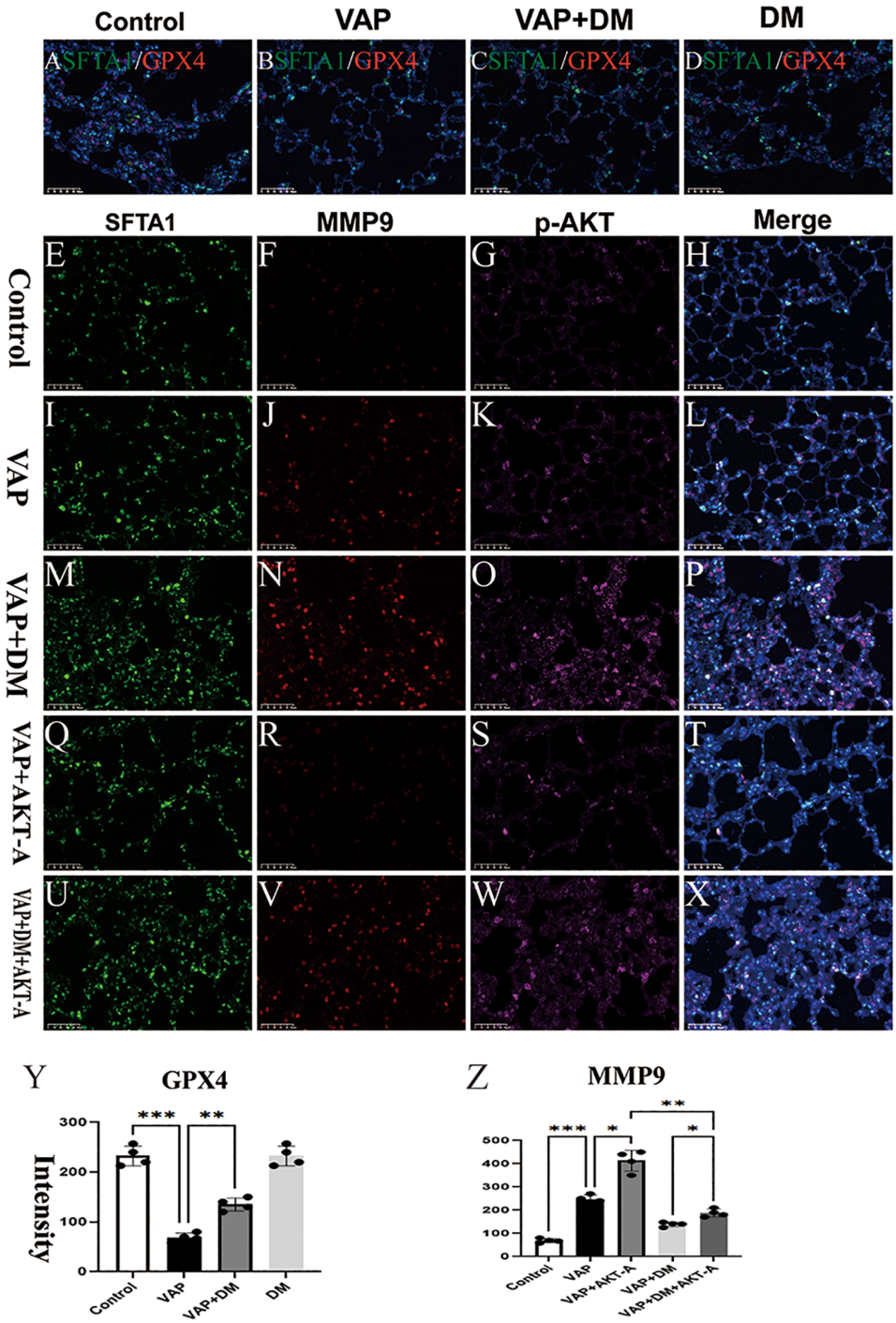

FIGURE 8
Macrophage depletion inhibited the lung epithelial cells of ferroptosis by inhibiting the AKT3/GPX4 pathway. The fluorescence intensities of GPX4 was measured for each group *in vivo* 3h after VAP treatment, as shown in **(A–D)**. MMP9 and p-AKT VAP as shown in **(E–X)**. Caused a significant increase in MMP9 protein expression levels **(J)**. Conversely, treatment with DM decreased MMP9 expression in pulmonary epithelial cells **(R)**. Furthermore, adding AKT-A, an AKT activator, increased the effect of VAP **(N, V)**, VAP significantly increased the levels of p-AKT protein expression **(K)**. In contrast, treatment with DM reduced p-AKT expression **(S)**. Additionally, the addition of AKT-A, an AKT activator, enhanced p-AKT and MMP9 with VAP **(H, L, P, T, X)**. The MMP9 and p-AKT is shown in **(Y, Z)**, N = 4 and *P < 0.05, **P < 0.05, ***P < 0.01, indicating statistical significance. **(A–P)** features a scale bar of 50 μm.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the ethics committee of Guangzhou Red Cross Hospital (approval number 2019-145-01). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YZ: Funding acquisition, Methodology, Writing – original draft. YC: Data curation, Writing – original draft, Writing – review & editing. DXie: Writing – original draft, Writing – review & editing. DXia: Writing – original draft, Writing – review & editing. HK: Writing – original draft, Writing – review & editing. XG: Writing – original draft, Writing – review & editing. BN: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1260584/full#supplementary-material

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