Exp Biomed Res 2026; 9(1): 50-67 **https://doi.org/ 10.30714/j-ebr.2026.267**

Experimental Biomedical Research *Original article*

Transcriptional regulons of tumor-associated macrophages reveal divergent polarization states across cancer types

Zeynep Akçor[1], Melisa Ekici[1], Yasin Kaymaz[1*]

Ege University, Faculty of Engineering, Bioengineering Department, Izmir, Türkiye

ABSTRACT

Aim: To investigate transcription factor regulons that drive macrophage polarization across multiple cancer types using single-cell RNA sequencing data.

Method: Publicly available datasets from lung, ovarian, pancreatic, and head and neck cancers were retrieved. After standard quality control and normalization, gene regulatory networks were reconstructed using our bioinformatic workflow, which integrates co-expression analysis, motif enrichment, and regulon activity scoring. Transcription factors associated with M1 and M2 macrophage polarization were systematically examined.

Results: Analysis of regulon activity revealed that macrophages in lung, ovarian, and pancreatic cancers predominantly exhibited M1-associated transcriptional programs, suggesting anti-tumor properties. Key active transcription factors in these cancers included *JUND*, *STAT1*, *NFκB*, and *IRF1*. In contrast, head and neck cancers displayed a predominance of M2-associated transcriptional activity, with strong enrichment of *SPI1*, *CEBPB*, and *IRF8*, indicative of pro-tumor macrophage polarization. These findings highlight cancer type–specific heterogeneity in macrophage transcriptional regulation.

Conclusions: This study demonstrates that transcription factor regulon analysis can distinguish tumor-associated macrophage states across cancers and provides insights into their divergent roles in tumor progression. The identification of cancer type–specific transcriptional drivers of macrophage polarization may inform the development of macrophage-targeted immunotherapies and improve prognostic biomarker discovery.

Keywords: Tumor-associated macrophages, regulons, single-cell transcriptome, transcription factors.

✉ *Yasin Kaymaz **
Ege University, Faculty of Engineering, Bioengineering Department, Izmir, Türkiye
E-mail: yasin.kaymaz@ege.edu.tr

Received: 2025-10-02 / Revisions: 2025-12-07
Accepted: 2025-12-24 / Published: 2026-01-01

1. Introduction

Carcinogenesis is the process by which a normal cell acquires mutations at critical checkpoints during cell division, ultimately transforming into a tumor cell through uncontrolled proliferation. Tumor development during carcinogenesis is often driven either by the activation of oncogenes or the inactivation of tumor suppressor genes. For a tumor to be classified as malignant and designated as cancer, it must acquire metastatic properties. Metastasis refers to the dissemination of cancer cells to distant sites within the body. To gain metastatic capacity, a cell typically reduces the expression of genes encoding adhesion molecules while increasing the expression of genes responsible for motility. These processes

occur within the tumor microenvironment (TME) [1].

The TME is a dynamic and complex milieu that arises from physical and cellular changes induced by tumor cells in host tissues, ultimately surrounding and supporting tumor development [2]. It comprises endothelial cells, various immune cell subsets, extracellular matrix, stromal cells, and blood vessels [3]. Tumor–host interactions are largely mediated by the vascular and lymphatic systems, which also facilitate metastatic dissemination [2]. As the tumor enlarges, it exerts pressure on surrounding tissues and progressively invades blood and lymphatic vessels. This invasive process begins when malignant cells penetrate vascular or lymphatic walls and gain access to the circulation. Numerous factors influence metastatic spread, including the intrinsic properties of tumor cells, their ability to breach vascular barriers, immune surveillance responses, and the permissiveness of distant organs [4].

Over the past decades, both experimental and clinical studies have shown that cancer progression depends not only on the presence or absence of an immune response but also on the type of immune response that is mounted [5]. Two central concepts, immune surveillance and immune modulation, define the role of immunity in cancer [6]. The immune surveillance theory postulates that effector immune cells can recognize and eliminate emerging tumor cells, thereby protecting the host [5]. However, some tumor cells evolve mechanisms to escape or evade immune recognition, weakening immune surveillance and thereby increasing metastatic risk. Conversely, immune modulation occurs when cancer cells release factors that suppress immune cell activity, reducing their ability to attack tumors and facilitating metastasis [5]. Recent advances in immunotherapy have demonstrated that enhancing immune cell function can effectively restrain cancer metastasis.

Immune cells are broadly classified into two categories. Adaptive immune cells, including T cells, B cells, and natural killer (NK) cells, are activated upon exposure to specific antigens and utilize immune memory to strengthen subsequent responses. Innate immune cells, on the other hand, provide immediate, non-specific defense within hours of pathogen invasion. These include macrophages, neutrophils, and dendritic cells [2]. Within the TME, macrophages are among the most abundant immune cell populations. As professional phagocytes, macrophages regulate immune responses through phagocytosis and antigen presentation, playing key roles in both tumor progression and suppression. Based on activation states and functions, macrophages exhibit opposing roles [7]. Monocyte-derived macrophages, critical for tissue repair and wound healing, are broadly classified into pro-inflammatory, tumor-suppressive M1 macrophages and immunosuppressive, wound-healing M2 macrophages [2].

M1 macrophages exert pro-inflammatory and anti-tumor effects [7], whereas M2 macrophages suppress inflammatory responses and promote tissue remodeling, thereby fostering tumor growth; hence, M2 macrophages are often described as pro-tumorigenic [8]. Tumor-associated macrophages (TAMs), which often constitute up to 50% of the tumor mass, represent a major component of solid tumors and facilitate invasion and metastasis. Their abundance in the TME is strongly correlated with poor prognosis across many cancer types [7,8]. We have recently demonstrated that pharmacologic agents such as aspirin can modulate the immune

landscape of non-small cell lung cancer (NSCLC), notably by downregulating PD-L1 expression and reprogramming macrophages toward a less immunosuppressive phenotype through inhibition of M2 polarization [9]. Nevertheless, in certain cancers such as ovarian and colorectal carcinoma, TAM infiltration has been associated with favorable outcomes, underscoring the context-dependent nature of macrophage function [10]. Macrophages also exhibit organ-specific diversity to maintain tissue homeostasis, with well-known subsets including Kupffer cells in the liver, alveolar macrophages in the lung, and microglia in the brain [11].

Transcription factors (TFs) are regulatory proteins that bind specific DNA regions to activate or repress gene expression, thereby serving as key controllers of gene "switching" events. By modulating the transcription of inflammatory genes, TFs influence both disease severity and therapeutic responses. Their binding activity can be further shaped by epigenetic modifications such as DNA methylation and DNA polymorphisms [12]. Regulons, defined as sets of genes regulated by a given TF, represent the fundamental units of transcriptional control. Aberrations in immune gene regulation have been implicated in the pathogenesis of multiple cancers, suggesting that immune-related regulons may underlie cancer heterogeneity and immune infiltration patterns [13]. Understanding the interplay between immune gene regulation and cancer biology is therefore crucial for delineating regulatory networks within the TME.

In macrophages, TFs orchestrate polarization states, driving differentiation toward M1 or M2 phenotypes, which in turn profoundly influence tumor immunity. TME-derived signals can alter gene expression through TF activity, shaping macrophage function, proliferation, and polarization. Emerging technologies such as single-cell RNA sequencing (scRNA-seq) have transformed cancer research by enabling transcriptional profiling at the single-cell level, thereby uncovering cellular heterogeneity and regulatory dynamics [14]. scRNA-seq has proven invaluable in cancer subtype identification, differentiation trajectory analysis, and the design of personalized therapeutic strategies. Single-cell transcriptional profiling of the lung cancer microenvironment further supports the dynamic nature of macrophage subtypes, revealing transitions between pro-inflammatory and anti-inflammatory states that are closely linked to disease progression [15]. Furthermore, bioinformatics approaches now play a critical role in reconstructing gene regulatory networks from scRNA-seq data, allowing the identification of TF–target interactions and cancer-relevant regulons [14]. These advances provide opportunities to elucidate the molecular mechanisms of tumor–immune interactions and to identify novel therapeutic targets.

Given the central role of tumor-associated macrophages in shaping the tumor microenvironment, understanding the transcriptional programs that govern their polarization is essential for elucidating cancer progression and therapeutic resistance. Recent advances in scRNA-seq and gene regulatory network inference now make it possible to study these processes at unprecedented resolution. In this study, we applied the SCENIC (Single-Cell rEgulatory Network Inference and Clustering) framework to publicly available scRNA-seq datasets from four cancer types: lung, ovarian, pancreatic, and head and neck cancers, to systematically characterize the transcription factor regulons

associated with macrophage polarization. By comparing regulon activity across cancer types, we aimed to determine whether macrophages adopt predominantly anti-tumor or pro-tumor states within different tumor contexts. The results presented below provide novel insights into the heterogeneity of macrophage responses across cancers and highlight transcription factors that may serve as potential biomarkers or therapeutic targets.

2. Materials and methods

2.1. Datasets and Data Acquisition: A literature survey was conducted to identify publicly scRNA-seq datasets relevant to macrophage activity in selected cancer types. Publications were screened through the PubMed search engine of the NCBI database, and datasets meeting inclusion criteria were retrieved from the Gene Expression Omnibus (GEO) and EMBL-EBI repositories. Datasets were selected based on relevance to tumor microenvironment (TME) studies and open-access availability.

A total of five single-cell RNA sequencing datasets were included in this study, representing four distinct cancer types. For lung cancer, dataset GSE130148 [16] was analyzed. The ovarian cancer dataset was obtained from GSE118127 [17]. Pancreatic cancer samples were represented by GSE154778 [18]. Two independent head and neck cancer datasets were also included: GSE164690 [19] and GSE139324 [20]. These datasets were selected based on their open-access availability in GEO and EMBL-EBI repositories and their relevance to studies of macrophage activity within the tumor microenvironment.

All datasets were subjected to a uniform preprocessing workflow prior to regulatory network inference. Preprocessing followed standard scRNA-seq analysis pipelines. First, raw count matrices were filtered to remove low-quality cells and genes with low expression levels, thereby minimizing technical noise. Quality control criteria included thresholds for minimum and maximum detected genes per cell, mitochondrial gene percentage, and total UMI counts. After filtering, the datasets were normalized to correct for sequencing depth and log-transformed to stabilize variance across expression levels. Highly variable genes were then identified and retained for downstream analysis, while unwanted sources of variation were reduced through scaling procedures.

2.2. Cell Type Annotation and Macrophage Selection: Cell type identification was performed on the integrated single-cell RNA sequencing dataset following quality control, normalization, and dimensionality reduction. Initial clustering was carried out using Leiden clustering from Scanpy python library based on principal component analysis and nearest-neighbor graph construction [21]. Cell clusters were annotated using a combination of canonical marker gene expression and reference-based annotation. Macrophages were identified based on the enriched expression of established myeloid and macrophage marker genes, including *CD68*, *CD163*, *CSF1R*, *LST1*, and *C1QC*, and the absence of lymphoid markers such as *CD3D*, *CD3E*, and *MS4A1*. Macrophage cells were subsequently isolated by selecting clusters exhibiting high expression of macrophage-associated genes and low expression of non-myeloid lineage markers. Only cells meeting these criteria were retained for downstream regulatory network inference. This filtered macrophage subset was then used as input for SCENIC analysis to reconstruct transcription factor–gene regulatory networks specific to macrophages.

2.3. SCENIC Workflow: We applied SCENIC (Single-Cell rEgulatory Network Inference and

Clustering), a bioinformatics framework for reconstructing gene regulatory networks (GRNs) from scRNA-seq data [22]. SCENIC is compatible with both Python (pySCENIC) and R (R-SCENIC) implementations and is available at http://scenic.aertslab.org. Initial quality control and normalization of expression matrices were performed, including gene filtering and correlation analysis. We constructed the gene–TF relationships using GENIE3 (and GRNBoost), which applies a Random Forest (RF) regression model to predict target gene expression levels based on TF activity, capturing both positive and negative correlations. The analysis was performed using default parameters, including (number of trees = 1000). Only transcription factors curated in the SCENIC database were considered as candidate regulators. Positive correlations indicate that TF activity increases with gene expression, whereas negative correlations suggest inhibitory regulation. We identified the enriched transcription factor (TF) binding motifs in target gene regions to refine predicted regulatory links using Regulatory Motif Analysis (RcisTarget), which assesses whether predicted target genes are likely regulated by specific TFs by searching for conserved DNA binding motifs, thereby strengthening biological interpretation. Motif enrichment analysis was carried out by employing the human motif ranking databases corresponding to the hg38 genome assembly (e.g., hg38__refseq-r80__500bp_up_and_100bp_down_tss and hg38__refseq-r80__10kb_up_and_down_tss). These databases were used to refine co-expression modules into high-confidence regulons based on the enrichment of transcription factor binding motifs. Regulon activity was quantified at the single-cell level using AUCell, which computes the Area Under the Curve (AUC) for the expression ranking of regulon target genes in each cell. Binary regulon activity (active vs. inactive) was determined using the AUCell "enforcement" thresholding approach, which identifies activity cutoffs based on the bimodal distribution of AUC scores across cells. This data-driven thresholding method minimizes arbitrary cutoff selection and enables robust identification of active regulons. Together, these modules allowed SCENIC to integrate expression-based inference with motif discovery and cell-level regulon activity scoring, enabling robust reconstruction of context-specific GRNs from scRNA-seq datasets. All SCENIC analyses were performed on three independent computer systems to validate reproducibility. Data normalization and quality control were performed prior to regulatory network reconstruction.

2.4. ***Code availability:*** All scripts used for data preprocessing, SCENIC/pySCENIC–based regulon inference, and figure generation are openly available at our GitHub repository: https://github.com/BMGLab/MacroPanCancer. The repository includes R and Python workflows with example command lines, configuration files for reproducing the analyses, and documentation describing required dependencies and the runtime environment.

3. Results

3.1. Overview of transcription factor activity across cancer types: Using the SCENIC workflow, we identified transcription factors (TFs) associated with M1- and M2-type macrophage polarization across selected cancer datasets. For each dataset, we generated (i) heatmaps of TF expression, (ii) AUCell activity scores, and (iii) histograms of regulon activation frequencies. These analyses allowed

us to assess the regulatory programs underlying macrophage polarization and to compare TF activity across tumor types.

3.2. Macrophage regulons in lung cancer: In the lung cancer dataset (GSE130148), several transcription factors (TFs) associated with macrophage polarization were identified. *FOSB*, *JUNB*, *JUND*, and *STAT1* were linked to M1-type macrophage activation, whereas *CEBPβ*, *STAT6*, and *JUNB* were associated with M2-type activation. The overall distribution of these TFs is shown in Figure 1 as a heatmap.

AUCell analysis revealed that *JUND*, *STAT1*, *STAT6*, and *CEBPβ* exceeded their respective activity thresholds, with *JUND* showing the strongest enrichment (Figure 2). These regulons were therefore considered transcriptionally active within subsets of macrophages. In contrast, *JUNB* and *FOSB* did not surpass threshold activity in any cells, suggesting that their regulons remained inactive in this dataset. Collectively, these findings indicate that lung cancer–associated macrophages were predominantly polarized toward an M1-like, anti-tumor state, with *JUND* and *STAT1* contributing most strongly to this transcriptional program. The recurrent activation of the JUND regulon across tumor types is consistent with AP-1 functioning as a core integrator of chronic inflammatory, hypoxic, and stress-related cues that broadly shape TAM survival and cytokine programs across diverse microenvironments.

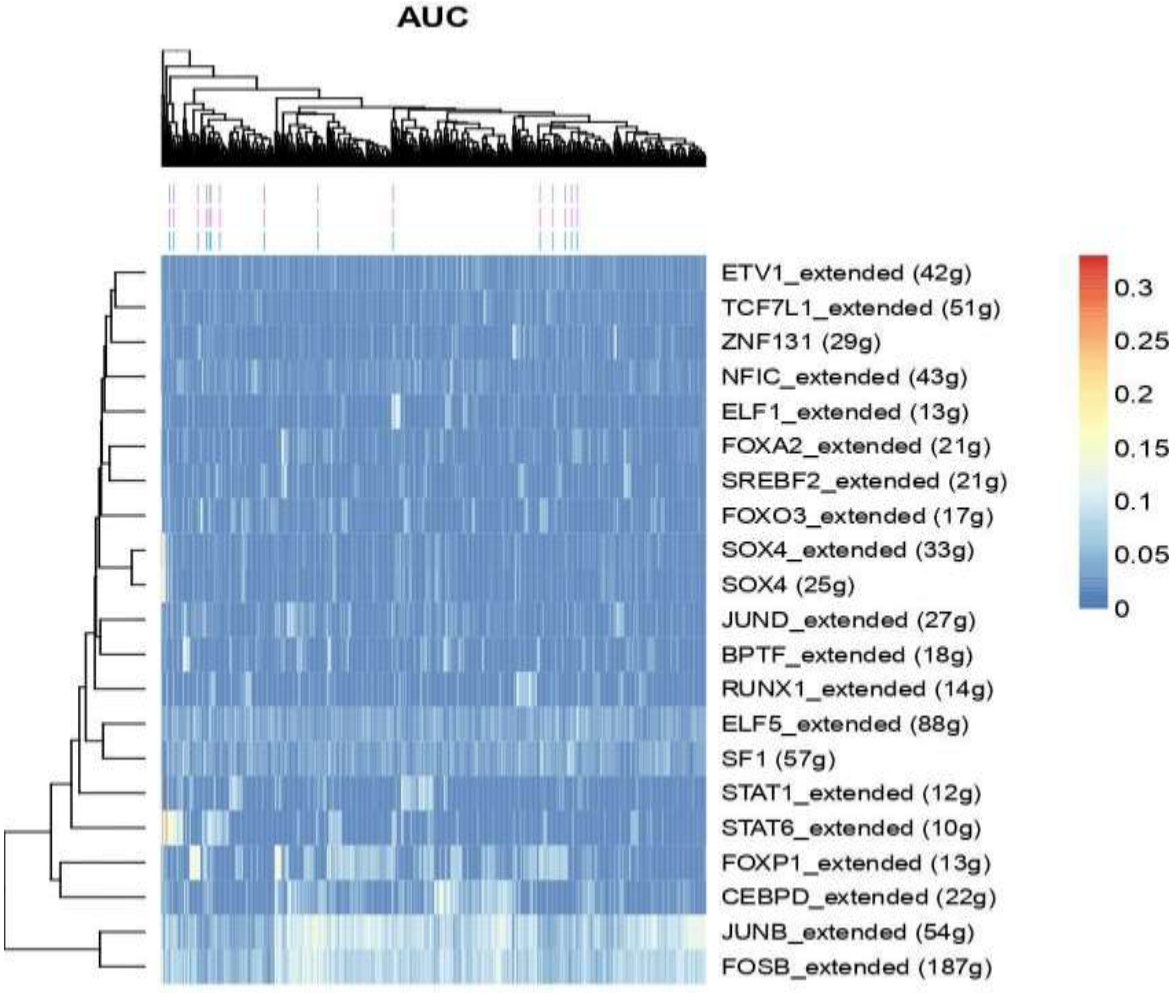

Figure 1. Heatmap of transcription factor activity identified in the lung cancer dataset (GSE130148). The map illustrates expression patterns of M1- and M2-associated macrophage regulators across single cells.

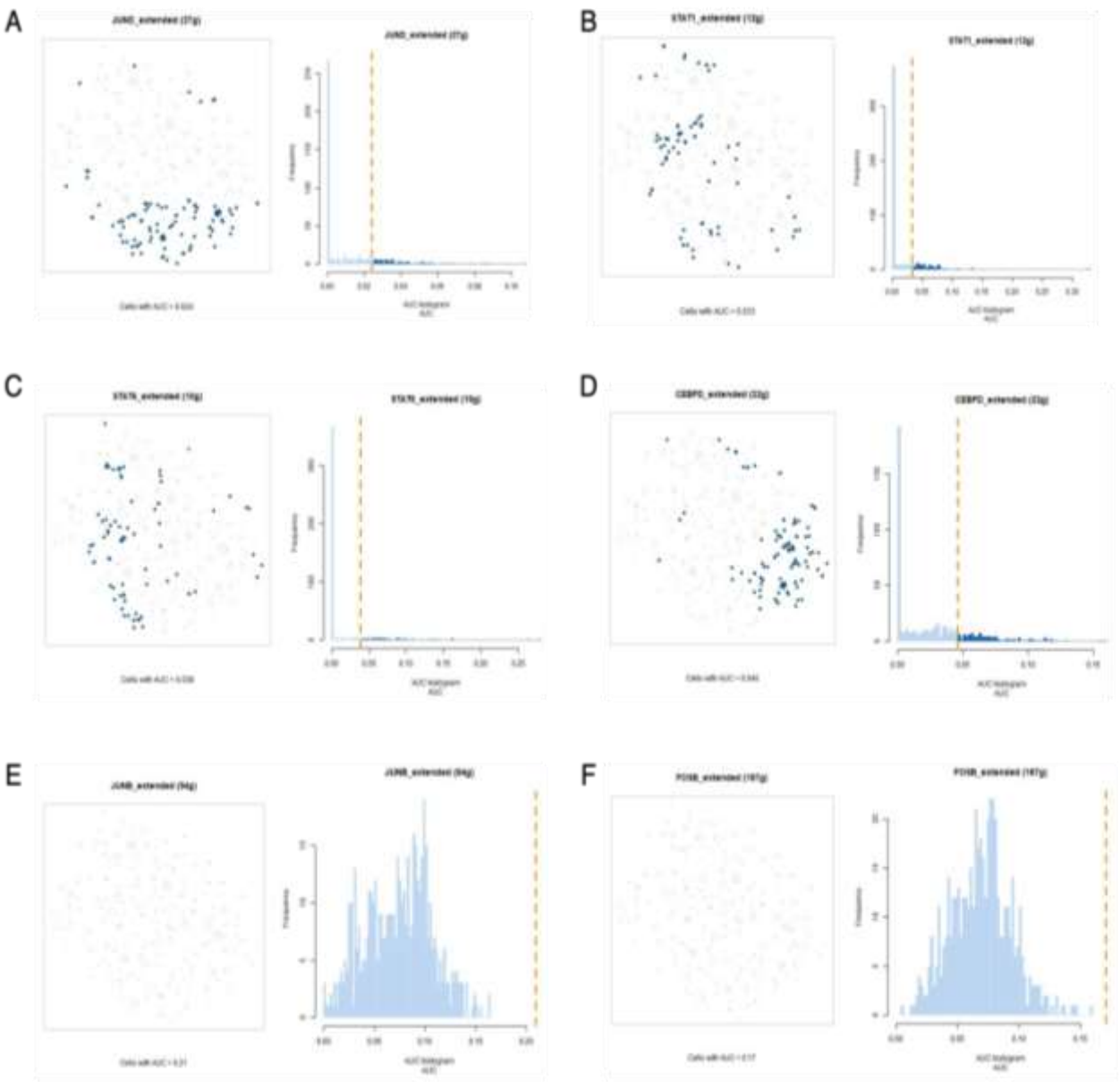

Figure 2. AUCell activity distributions and histograms for macrophage-associated transcription factors in the lung cancer dataset. Shown are *JUND* (AUC > 0.024), *STAT1* (AUC > 0.033), *STAT6* (AUC > 0.038), *CEBPβ* (AUC > 0.046), *JUNB* (AUC > 0.21), and *FOSB* (AUC > 0.17). Regulons exceeding AUC thresholds (*JUND, STAT1*, *STAT6,* and *CEBPβ*) were classified as transcriptionally "open," whereas *JUNB* and *FOSB* remained below threshold, suggesting "did not surpass the activity threshold" regulon states.

3.3. Macrophage regulons in ovarian cancer: In the ovarian cancer dataset (GSE118127), the identified TFs included *JUNB*, *ATF3*, *IRF1*, *KLF6*, and *NFκB*. Most of these regulators, particularly *ATF3*, *IRF1*, *KLF6*, and *NFκB*, were associated with M1-type macrophage polarization, consistent with a pro-inflammatory program. *JUNB*, however, was observed as a driver of M2-type activation (Figure 3).

The observation of *JUNB* in both M1- and M2-associated contexts suggests that its role in ovarian cancer macrophages may be context dependent. This highlights the heterogeneity of the tumor microenvironment, where macrophage populations with both pro-inflammatory and immunosuppressive features coexist.

To further examine regulon activity, AUCell analyses were performed for the ovarian cancer dataset. The results demonstrated that *NFκB*, *IRF1*, *KLF6*, and *JUNB* exceeded their respective threshold values, with *NFκB* showing the strongest enrichment. These findings indicate that the regulons associated with these TFs were transcriptionally active, or "open," within subsets of macrophages in ovarian cancer (Figure 4).

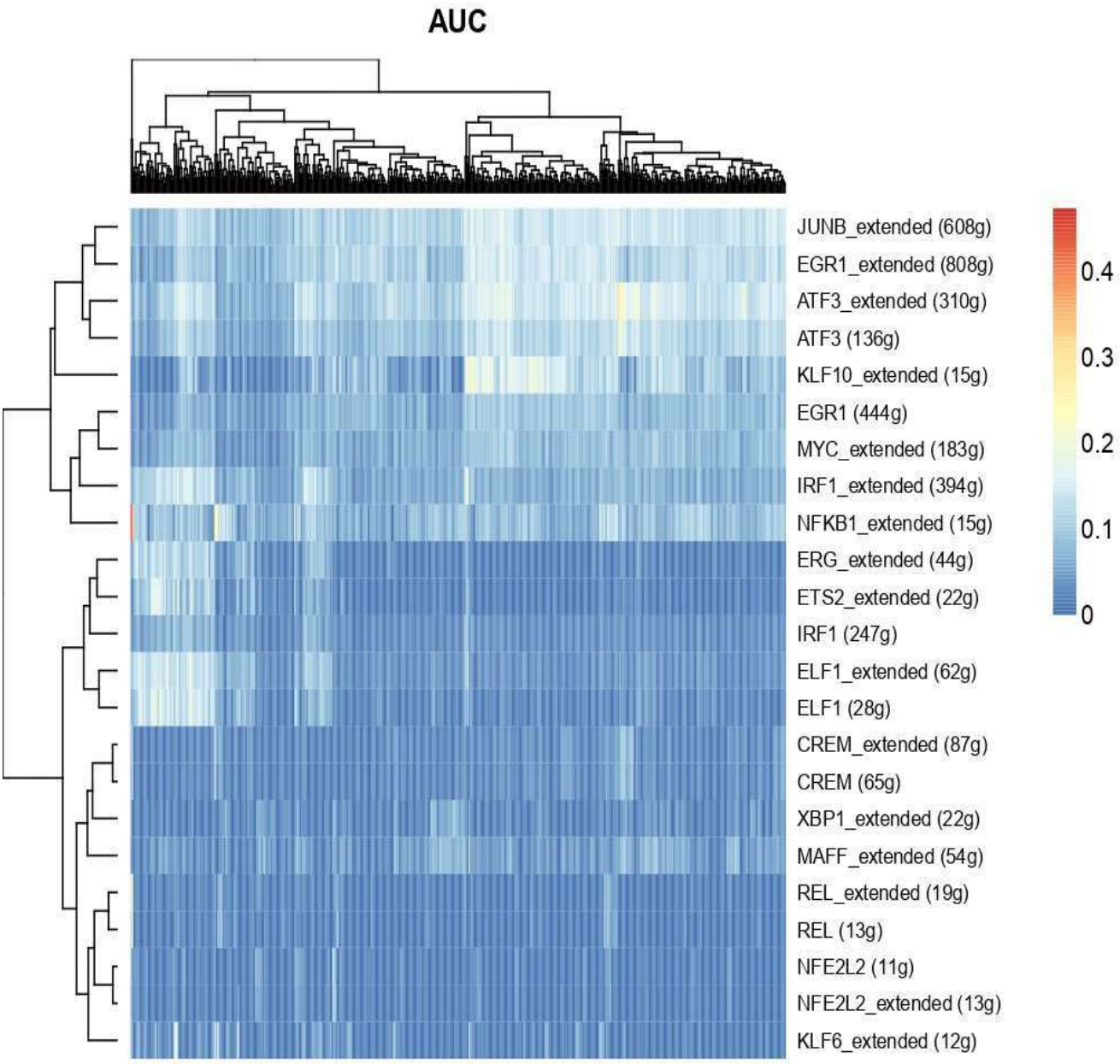

Figure 3. Heatmap of transcription factor activity identified in the ovarian cancer dataset (GSE118127).

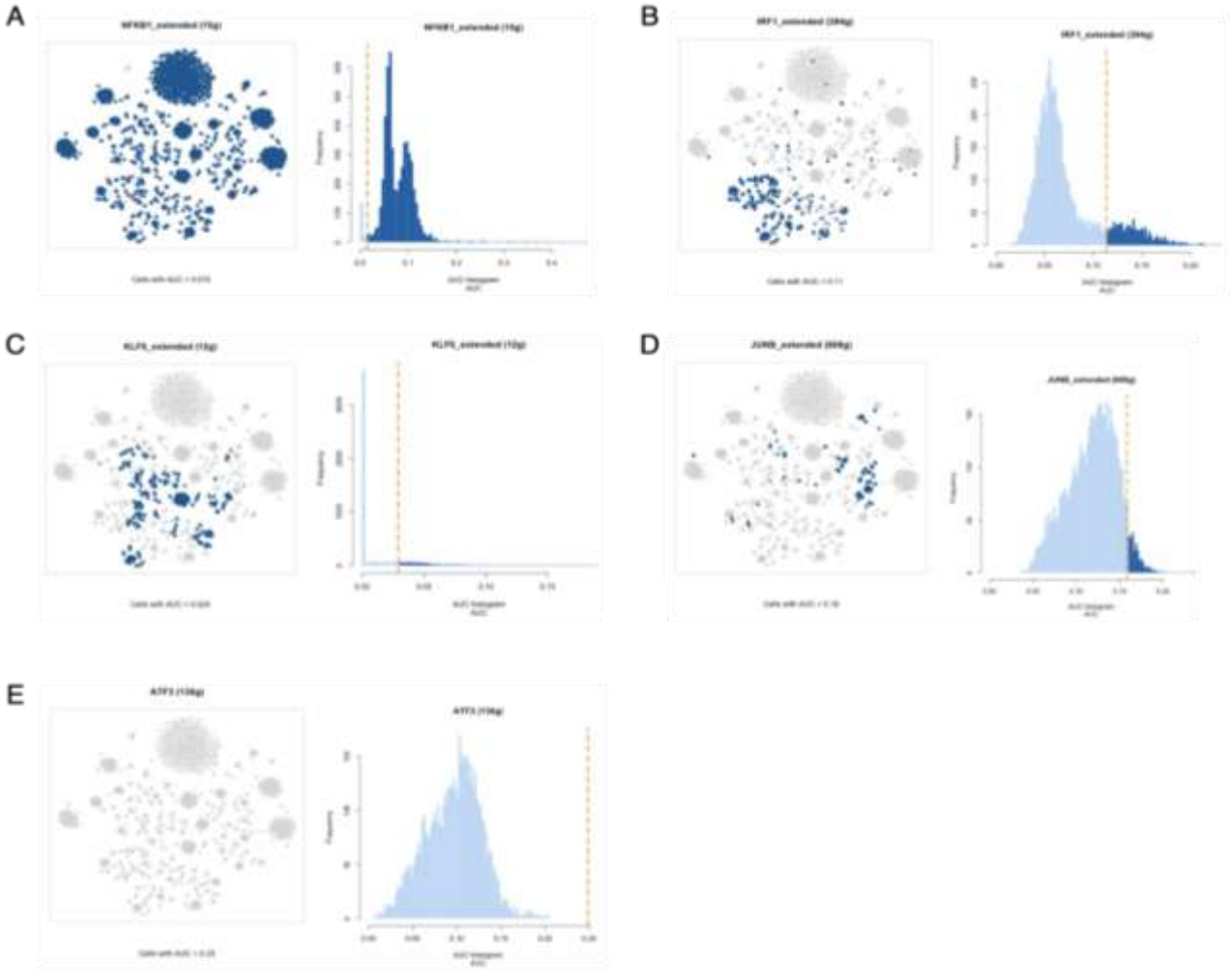

Figure 4. AUCell activity distributions and histograms of transcription factors in the ovarian cancer dataset (GSE118127). *NFκB* (AUC > 0.015), *IRF1* (AUC > 0.11), *KLF6* (AUC > 0.029), and *JUNB* (AUC > 0.16) surpassed their respective thresholds, indicating transcriptionally active regulons in subsets of macrophages. In contrast, *ATF3* (AUC > 0.25) did not surpass the threshold, suggesting an inactive, or "did not surpass the activity threshold," regulon state.

SCENIC analysis of the ovarian cancer dataset revealed that the majority of the identified transcription factors were associated with M1-type macrophage activation. This suggests that macrophages in this dataset predominantly exhibited anti-tumor properties.

3.4. Macrophage regulons in pancreatic cancer: In the pancreatic cancer dataset (GSE154778), SCENIC analysis identified a wide range of transcription factors associated with macrophage polarization (Figure 5). Among these, *ATF3*, *ATF4*, *ATF7*, *BATF*, *FOSB*, *FOS*, *FRA2*, *IRF1*, *IRF3*, *IRF9*, *JUNB*, and *JUND* were linked to M1-type macrophage activation, whereas *FOSB*, *FRA1*, and *JUNB* were associated with M2-type activation.

Given the large number of transcription factors identified in this dataset, only the most representative member of each TF family, defined as the one surpassing the AUCell threshold in the highest number of cells, was selected for detailed analysis. This approach ensured a more focused evaluation of the functional regulons most relevant to macrophage activity in pancreatic cancer.

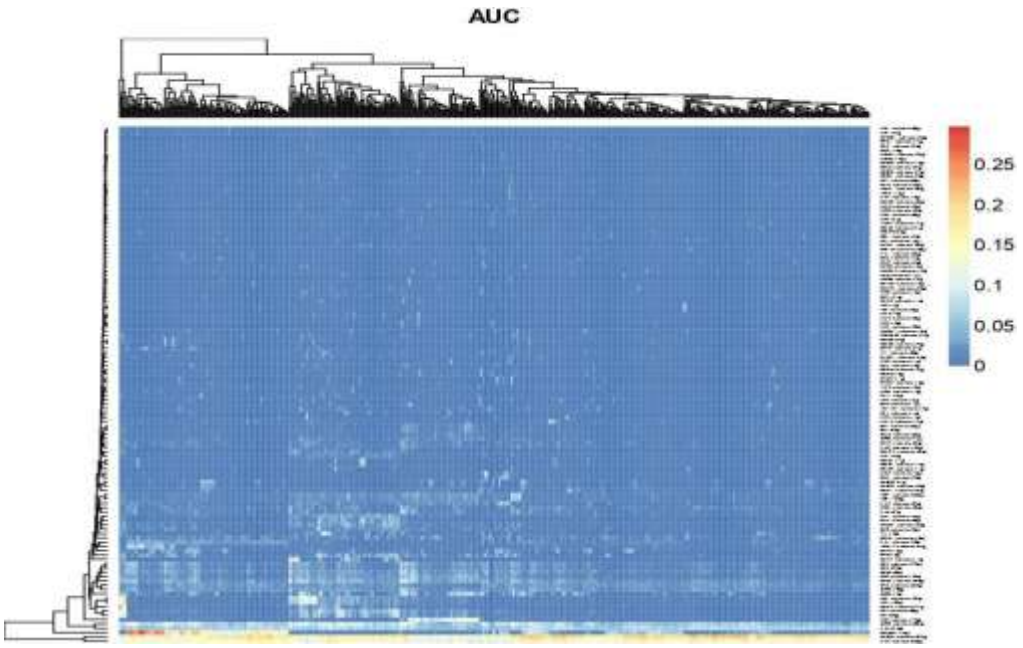

Figure 5. Heatmap of transcription factor activity identified in the pancreatic cancer dataset (GSE154778).

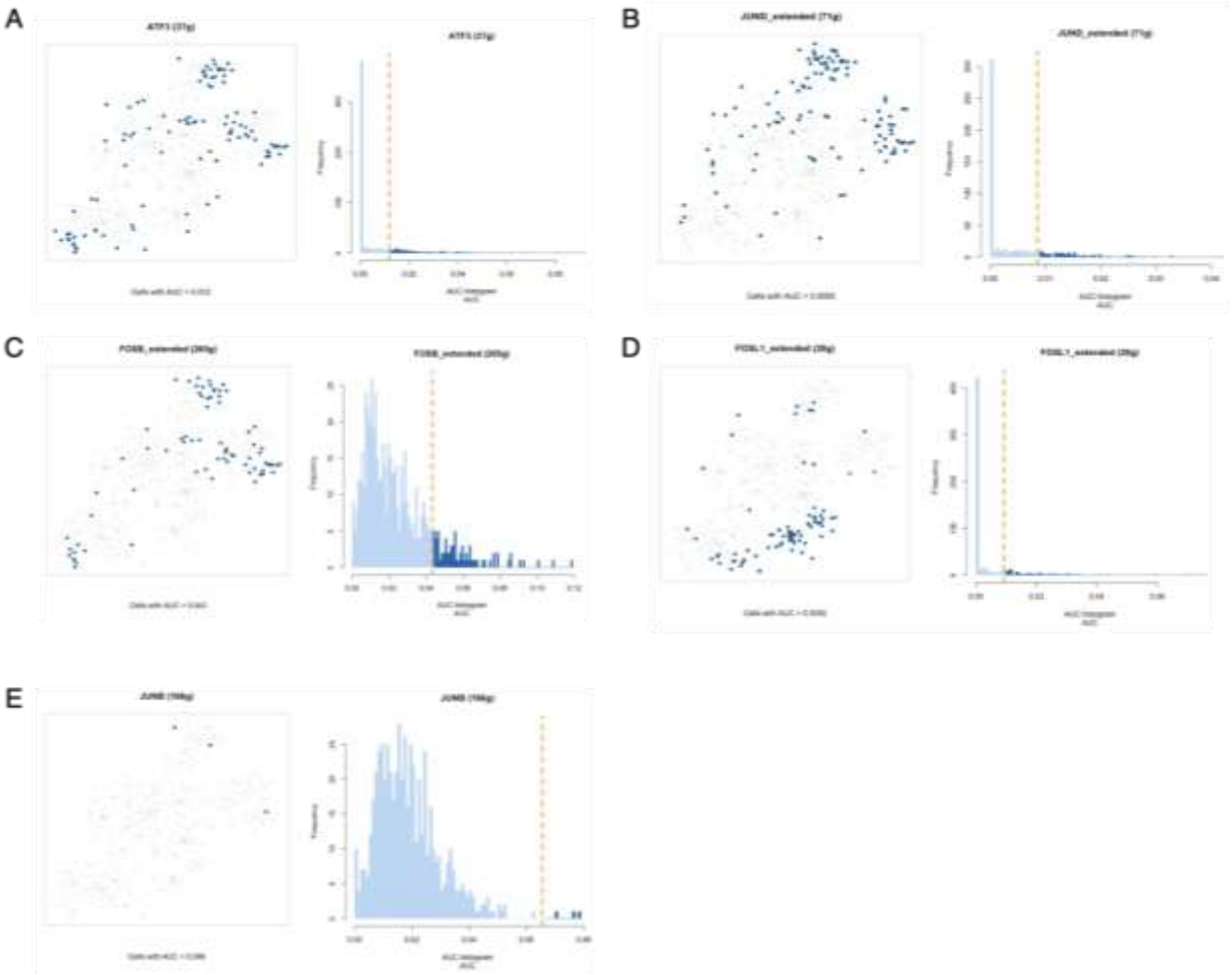

Figure 6. AUCell activity distributions and histograms of transcription factors in the pancreatic cancer dataset (GSE154778). *ATF3* (AUC > 0.012), *JUND* (AUC > 0.0085), *FOSB* (AUC > 0.043), *FOSL1/FRA1* (AUC > 0.0092), and *JUNB* (AUC > 0.066) each surpassed their respective thresholds, indicating transcriptionally active regulons within subsets of macrophages.

To assess regulon activity, AUCell analyses were performed for the pancreatic cancer dataset. The results showed that several transcription factors surpassed their respective thresholds, indicating transcriptionally active, or "open," regulons in subsets of macrophages (Figure 6).

When examining the cells that surpassed the AUCell thresholds, M1-associated transcription factors (*ATF3*, *JUND*, and *JUNB*) were found at higher levels compared to M2-associated factors (*FOSB* and *FOSL1/FRA1*). This suggests that macrophages in the pancreatic cancer dataset predominantly exhibited anti-tumor properties.

3.5. Macrophage regulons in head and neck cancer: Two independent head and neck cancer datasets (GSE164690 and GSE139324) were analyzed using the SCENIC workflow. The transcription factors identified in these datasets are summarized in the heatmaps shown in Figures 7 and 8. These figures illustrate the distribution of TF activity across single cells, highlighting regulators potentially linked to macrophage polarization in the tumor microenvironment.

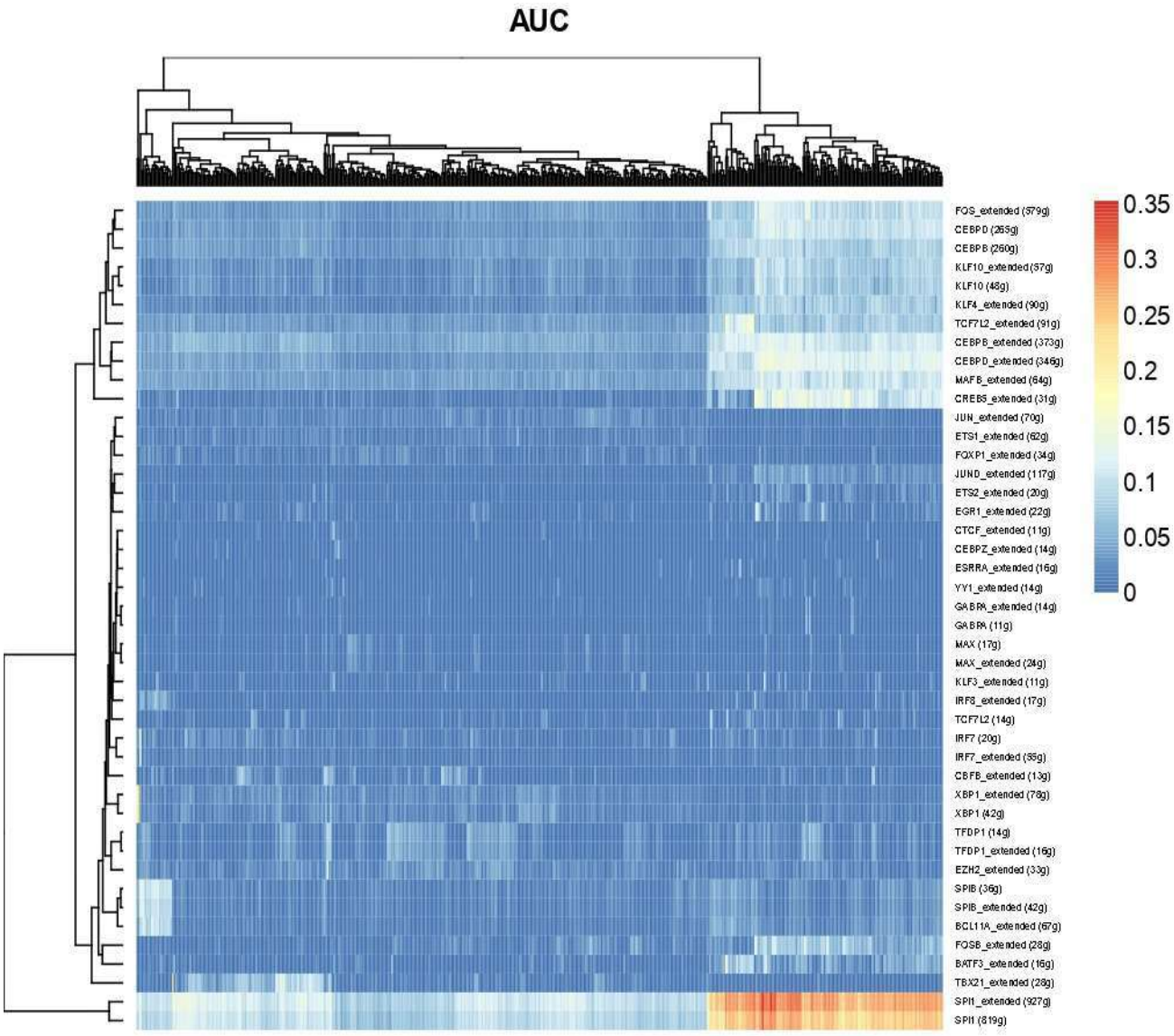

Figure 7. Heatmap of transcription factor activity identified in the first head and neck cancer dataset (GSE164690).

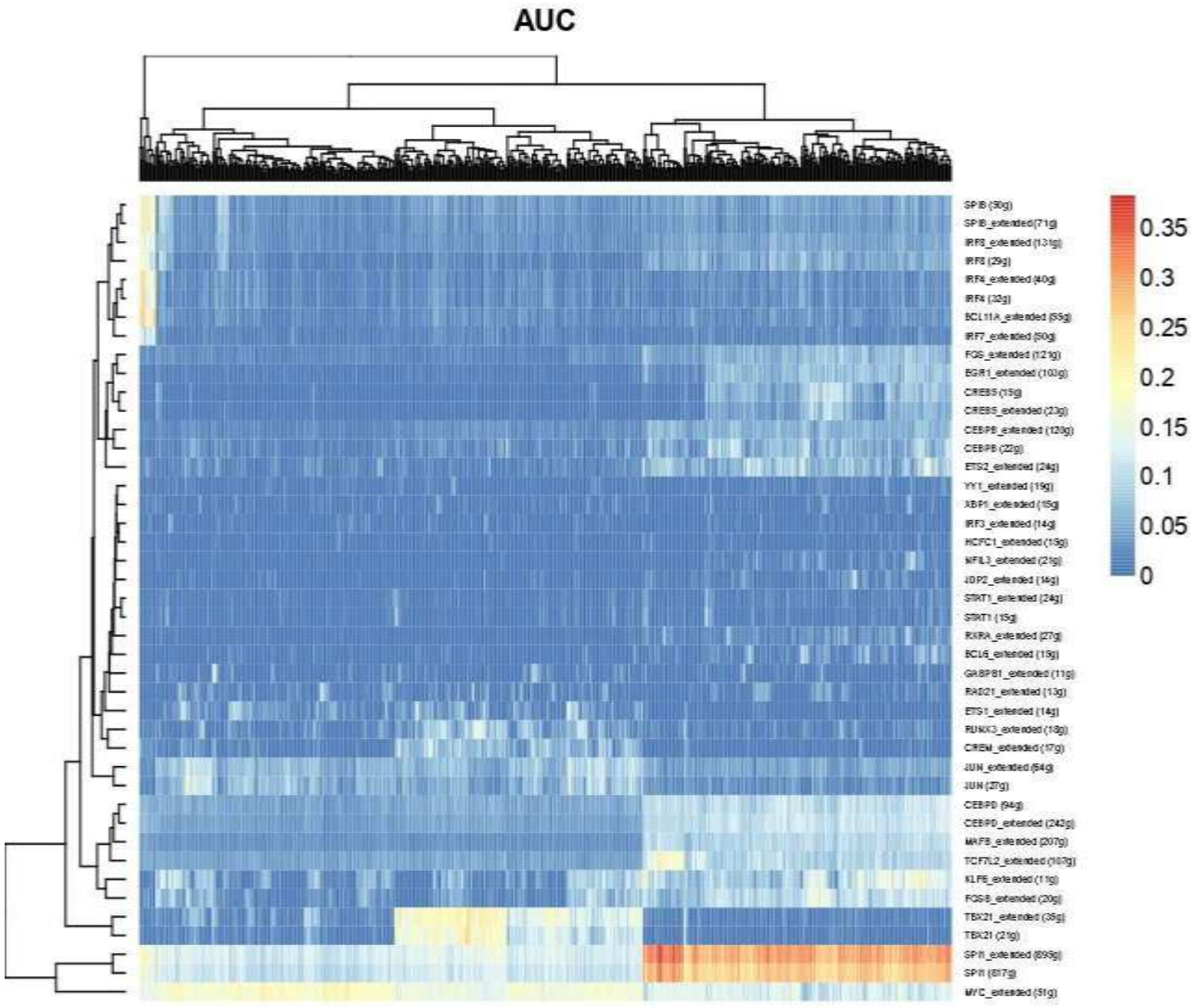

Figure 8. Heatmap of transcription factor activity identified in the second head and neck cancer dataset (GSE139324).

Based on the heatmaps shown in Figures 7 and 8, several transcription factors associated with macrophage polarization were identified in the head and neck cancer datasets. Among these, *FOS*, *FOSB*, *IRF3*, *IRF7*, *IRF8*, *JUND*, *JUN*, *KLF6*, and *STAT1* were linked to M1-type macrophage activation, while *CEBPβ*, *FOSB*, *KLF4*, *IRF4*, and *SP1* were associated with M2-type activation.

Given the large number of transcription factors identified in these datasets, only the five factors surpassing AUCell thresholds in the highest number of cells were selected for detailed analysis. This approach enabled a focused evaluation of the most biologically relevant regulons within the tumor-associated macrophages. Examination of the AUCell results revealed that *SPI1* had the highest overall activity score across all analyses, with values exceeding 0.1 in both datasets. The AUCell distributions further showed that *SPI1*, *JUND*, *FOS*, *CEBPβ*, and *IRF8* all surpassed their respective thresholds, indicating that the regulons associated with these transcription factors were transcriptionally active, or "open," in subsets of macrophages (Figure 9).

Unlike other cancer datasets analyzed in this study, all transcription factors examined in the

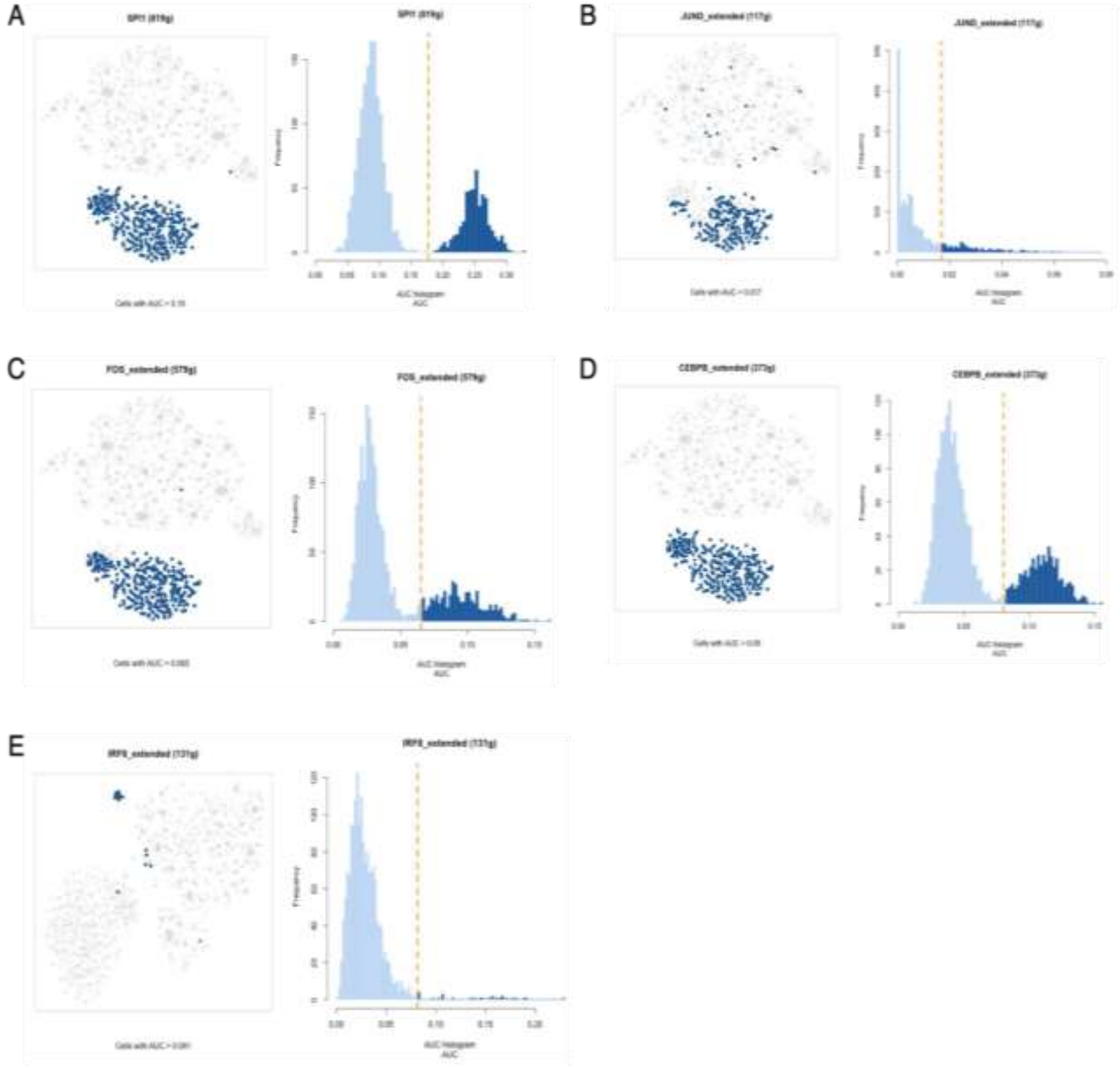

Figure 9. AUCell activity distributions and histograms of transcription factors in the head and neck cancer datasets (GSE164690 and GSE139324). *SPI1* (AUC > 0.18), *JUND* (AUC > 0.017), *FOS* (AUC > 0.065), *CEBPβ* (AUC > 0.08), and *IRF8* (AUC > 0.081) each surpassed their respective thresholds, indicating transcriptionally active regulons within macrophages.

head and neck cancer datasets exceeded their AUCell thresholds; thus, no regulons were classified as "did not surpass the activity threshold." Although M1-associated transcription factors were more numerous overall, the highest activity was observed for *SPI1*, and in general, M2-associated transcription factors showed stronger activity.

This suggests that macrophage polarization in the head and neck cancer datasets was skewed toward an M2-like state, consistent with pro-tumor properties of tumor-associated macrophages. *SPI1* is a master regulator of myeloid identity that primes macrophage enhancer landscapes, enabling robust transcriptional responses to polarization cues.

In tumors, *SPI1* may facilitate coherent activation of tissue-repair and immunoregulatory gene modules consistent with M2-like TAM states [23]. To provide additional context for transcription factors identified as inactive or absent in the SCENIC analysis, we examined the expression levels of these TFs and their putative target genes across the macrophage population. Several transcription factors exhibited detectable expression at the mRNA level but did not form regulons that passed the SCENIC filtering criteria. This indicates that while these TFs are transcriptionally expressed, their downstream target genes do not display sufficiently coordinated expression patterns to support confident regulon inference under SCENIC's co-expression and motif-enrichment constraints.

4. Discussion

Cancer remains one of the leading global health challenges, imposing a significant burden on healthcare systems and economies worldwide. The high costs of diagnosis, treatment, and long-term management represent not only an individual financial strain but also a substantial societal challenge. Despite advances in biotechnology and improved understanding of cancer biology, the economic burden of cancer persists, necessitating strategies that integrate more precise diagnostic tools and effective, targeted therapies. A critical step toward this goal is elucidating the molecular mechanisms underlying tumor progression and therapeutic resistance. Gene expression analyses have emerged as essential tools for exploring these mechanisms, offering insights into transcriptional regulation and cellular heterogeneity within the tumor microenvironment [24].

In this study, we applied regulon-based transcriptional analysis to tumor-associated macrophages derived from single-cell RNA sequencing data across four cancer types: lung, ovarian, pancreatic, and head and neck cancers. The comparative analysis revealed distinct patterns of macrophage polarization. Most transcription factors that surpassed AUCell thresholds were associated with M1-type polarization in the lung, ovarian, and pancreatic cancer datasets. These findings suggest that macrophages in these tumor types tend to adopt anti-tumor properties, consistent with a pro-inflammatory phenotype. Particularly in the lung cancer dataset, strong activity of *JUND* and *STAT1* underscored the predominance of M1-associated regulons. Previous studies offer transcriptomic evidence of TAM diversity in NSCLC, emphasizing differences between tumor and peritumor macrophage states [25]. Similarly, ovarian cancer macrophages showed activation of *IRF1*, *NFκB*, and *KLF6*, whereas pancreatic cancer macrophages exhibited activation of *ATF3*, *JUND*, and *JUNB*, again indicating M1-like polarization. Together, these results suggest that in certain tumor contexts, TAMs may retain or acquire tumor-suppressive functions, potentially contributing to more favorable immune responses. There is accumulated evidence regarding the link between TAM polarization (M1 vs. M2) to metabolic and signaling changes in the TME, supporting a mechanistic rationale for therapeutic reprogramming [26].

In contrast, both head and neck cancer datasets demonstrated a distinct trend toward M2-like macrophage polarization. *SPI1*, *CEBPβ*, and *IRF8*, along with other M2-associated TFs, showed strong transcriptional activity, indicating the predominance of pro-tumorigenic macrophage states in this cancer type. This polarization shift is consistent with a

microenvironment that promotes tumor progression, immune evasion, and therapy resistance. Studies demonstrate that TF regulon shifts during TAM differentiation and links specific subsets to prognosis, underscoring prognostic biomarker potential [27]. The observation that both independent head and neck datasets converged on M2 polarization strengthens the biological relevance of these findings.

Taken together, these results highlight the heterogeneity of macrophage polarization across tumor types, with potential implications for cancer prognosis and therapeutic design. The predominance of M1-associated macrophages in lung, ovarian, and pancreatic cancers suggests opportunities for harnessing or reinforcing anti-tumor immune responses. Conversely, the strong M2 signature in head and neck cancers underscores the need for therapeutic strategies aimed at reprogramming TAMs toward an anti-tumor state. Immunotherapies that target macrophage plasticity, such as *CSF1R* inhibitors, *CD47* blockade, or modulators of *STAT* and *IRF* signaling, may therefore hold promise in altering the immune landscape of tumors characterized by pro-tumor macrophage activity [28].

It is important to note that SCENIC infers transcriptional regulatory activity based on co-expression patterns and motif enrichment, rather than transcription factor expression alone. As a result, transcription factors that are expressed at the mRNA level may not be identified as active regulons if their predicted target genes do not exhibit coordinated expression across cells or fail to meet the AUCell activity thresholds. Conversely, lowly expressed transcription factors may also be underrepresented due to limited detection sensitivity in single-cell RNA-seq data. Therefore, the absence of certain transcription factors in the final regulon set should not be interpreted as evidence of biological inactivity, but rather as a limitation inherent to co-regulatory network inference approaches. Complementary experimental validation or orthogonal computational methods would be required to fully assess the regulatory roles of these transcription factors.

The observed enrichment of an M2-like macrophage regulatory program in head and neck cancers, compared to other tumor types analyzed, likely reflects unique features of the head and neck tumor microenvironment. Several non-mutually exclusive biological factors may contribute to this cancer-type–specific macrophage polarization. First, head and neck tumors arise in tissues that are continuously exposed to a diverse oral and upper aerodigestive tract microbiota [29]. Chronic microbial stimulation has been shown to promote immunoregulatory and tissue-repair–associated macrophage phenotypes, which are closely aligned with M2 polarization. Persistent exposure to microbial-derived signals may therefore bias macrophages toward anti-inflammatory and pro-tumorigenic states in this anatomical context. Second, a substantial subset of head and neck squamous cell carcinomas is associated with human papillomavirus (HPV) infection, which is known to actively modulate host immune responses [30]. HPV-positive tumors have been reported to exhibit altered cytokine landscapes, including increased *IL-10* and *TGF-β* signaling, both of which are potent drivers of M2 macrophage polarization [31]. Viral immune evasion strategies may thus contribute to the establishment of an immunosuppressive macrophage compartment. Third, head and neck cancers display distinct stromal and extracellular matrix compositions,

characterized by dense fibroblast populations and enhanced angiogenic signaling. Cancer-associated fibroblasts and endothelial cells can secrete cytokines and growth factors such as *CSF1*, *VEGF*, and *IL-6*, which are known to support macrophage survival and M2-like functional states [32]. Differences in stromal architecture may therefore indirectly shape macrophage regulatory programs. Finally, tumor-intrinsic differences in cytokine and chemokine expression profiles across cancer types may further contribute to macrophage heterogeneity. Together, these factors provide a biologically plausible framework for understanding the cancer-type–specific enrichment of M2-polarized macrophage regulatory networks observed in our analysis. While our study does not directly test these mechanisms, the findings are consistent with established immunological features of head and neck cancers and highlight the importance of tissue context in shaping tumor-associated macrophage states.

In addition to their biological implications, these findings emphasize the utility of single-cell regulatory network inference in dissecting the complexity of the TME. By focusing on regulon activity rather than gene expression alone, this approach enables a more nuanced understanding of the transcriptional programs that shape macrophage function in cancer. Future work should extend these analyses to larger patient cohorts and additional cancer types, integrating multi-omics data to validate transcription factor–regulon interactions and their impact on clinical outcomes. Such efforts could ultimately contribute to the development of macrophage-targeted therapeutic interventions and more accurate prognostic biomarkers.

Acknowledgments

The authors gratefully acknowledge the support of the Scientific and Technological Research Council of Turkey (TÜBİTAK) – Directorate of Science Fellowships and Grant Program (BİDEB). We also thank our colleagues at Ege University for their valuable discussions and technical assistance throughout this study.

Funding: *This study was supported by the TUBITAK BIDEB-2209 program.*

Conflict of Interest: *The authors declared no conflict of interest.*

Ethical Statement: *This study used sample datasets that did not require an Ethical approval statement.*

AI Use Disclosure: *We declare that the main text was edited and grammatically corrected using the ChatGPT 5.2 model. The authors approve all changes and take full responsibility.*

References

[1]Sarkar S, Horn G, Moulton K, et al. Cancer development, progression, and therapy: an epigenetic overview. Int J Mol Sci. 2013;14(10):21087-113.

[2] Anderson NM, Simon MC. The tumor microenvironment. Curr Biol. 2020;30(16):R921-r925.
[3] Chew V, Toh HC, Abastado JP. Immune microenvironment in tumor progression: characteristics and challenges for therapy. J Oncol. 2012;2012:608406.
[4] Lambert AW, Pattabiraman DR, Weinberg RA. Emerging Biological Principles of Metastasis. Cell. 2017;168(4):670-691.
[5] Ichim CV. Revisiting immunosurveillance and immunostimulation: Implications for cancer immunotherapy. J Transl Med. 2005;3(1):8.
[6] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423-1437.
[7] Poh AR, Ernst M. Targeting Macrophages in Cancer: From Bench to Bedside. Front Oncol. 2018;8:49.
[8] Cendrowicz E, Sas Z, Bremer E, Rygiel TP. The Role of Macrophages in Cancer Development and Therapy. Cancers (Basel). 2021;13(8).
[9] Unver N, Uluturk S, Tavukcuoglu E, et al. The impact of aspirin on PD-L1 expression and alteration of M2 polarization in non-small cell lung cancer. Inflamm Res. 2025;74(1):124.
[10] Wu K, Lin K, Li X, et al. Redefining Tumor-Associated Macrophage Subpopulations and Functions in the Tumor Microenvironment. Front Immunol. 2020;11:1731.
[11] Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496(7446):445-55.
[12] Badeaux AI, Shi Y. Emerging roles for chromatin as a signal integration and storage platform. Nat Rev Mol Cell Biol. 2013;14(4):211-24.
[13] Jiang T, Zhou W, Chang Z, et al. ImmReg: the regulon atlas of immune-related pathways across cancer types. Nucleic Acids Res. 2021;49(21):12106-12118.
[14] Van de Sande B, Flerin C, Davie K, et al. A scalable SCENIC workflow for single-cell gene regulatory network analysis. Nat Protoc. 2020;15(7):2247-2276.
[15] Keremitçi D, Tuna Ö, Houdjedj A, Kazan H, Kaymaz Y. Transcriptional states of lung cancer microenvironment reveal macrophage subtype dynamics linked to disease progression. J Immunol. 2025;214(12):3273-3282.
[16] Vieira Braga FA, Kar G, Berg M, et al. A cellular census of human lungs identifies novel cell states in health and in asthma. Nat Med. 2019;25(7):1153-1163.
[17] Fan X, Bialecka M, Moustakas I, et al. Single-cell reconstruction of follicular remodeling in the human adult ovary. Nat Commun. 2019;10(1):3164.
[18] Lin W, Noel P, Borazanci EH, et al. Single-cell transcriptome analysis of tumor and stromal compartments of pancreatic ductal adenocarcinoma primary tumors and metastatic lesions. Genome Med. 2020;12(1):80.
[19] Kürten CHL, Kulkarni A, Cillo AR, et al. Investigating immune and non-immune cell interactions in head and neck tumors by single-cell RNA sequencing. Nat Commun. 2021;12(1):7338.
[20] Cillo AR, Kürten CHL, Tabib T, et al. Immune Landscape of Viral- and Carcinogen-Driven Head and Neck Cancer. Immunity. 2020;52(1):183-199.e9.
[21] Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. Genome Biol. 2018;19(1):15.
[22] Aibar S, González-Blas CB, Moerman T, et al. SCENIC: single-cell regulatory network

inference and clustering. Nat Methods. 2017;14(11):1083-1086.

[23] Yao J, Duan L, Huang X, et al. Development and Validation of a Prognostic Gene Signature Correlated With M2 Macrophage Infiltration in Esophageal Squamous Cell Carcinoma. Front Oncol. 2021;11:769727.

[24] Ma R-Y, Black A, Qian B-Z. Macrophage diversity in cancer revisited in the era of single-cell omics. Trends in Immunol. 2022;43(7):546-563.

[25] Li A, Wang H, Zhang L, et al. A single-cell RNA-seq dataset describing macrophages in NSCLC tumor and peritumor tissues. Scientific Data. 2024;11(1):1064.

[26] Shen L, Liu J, Hu F, et al. Single-cell RNA sequencing reveals aberrant sphingolipid metabolism in non-small cell lung cancer impacts tumor-associated macrophages and stimulates angiogenesis via macrophage inhibitory factor signaling. Thoracic Cancer. 2024;15(14):1164-1175.

[27] Ji T, Fu H, Wang L, et al. Single-cell RNA profiling reveals classification and characteristics of mononuclear phagocytes in colorectal cancer. PLOS Genetics. 2024;20(2):e1011176.

[28] Zhao JL, Chan J, Land M, et al. Abstract 1728: Single cell RNA-seq analysis reveals a role of pro-inflammatory tumor-associated macrophages in driving prostate cancer progression. Cancer Res. 2022;82(12_Supplement):1728-1728.

[29] Mascitti M, Togni L, Troiano G, et al. Beyond Head and Neck Cancer: The Relationship Between Oral Microbiota and Tumour Development in Distant Organs. Front Cell Infect Microbiol. 2019;9:232.

[30] Husain N, Neyaz A. Human papillomavirus associated head and neck squamous cell carcinoma: Controversies and new concepts. J Oral Biol Craniofac Res. 2017;7(3):198-205.

[31] Echevarria-Lima J, Moles R. Monocyte and Macrophage Functions in Oncogenic Viral Infections. Viruses. 2024;16(10):1612.

[32] Raudenska M, Balvan J, Hanelova K, et al. Cancer-associated fibroblasts: Mediators of head and neck tumor microenvironment remodeling. Biochim Biophys Acta Rev Cancer. 2023;1878(5):188940.