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Epigenetic Silencing of cGAS–STING Pathway Genes Drives Immune Evasion in Immunologically Cold Melanomas

Sumia Khan[1], Zahra Masood[2], Areej Aihetasham[3], Seerat Faheem[4], Muhammad Mujahid Bhutta[4], Mohammad Nazeer[4], Sara Parveen[5], Ali Ibraheem[6], Farheen Aslam[7]

[1]Department of Microbiology, Hazara University, Manshera, KP, Pakistan.
[2]Department of Molecular Medicine, National University of Medical Sciences (NUMS), Rawalpindi, Punjab, Pakistan.
[3]Syed Babar Ali School of Science and Engineering, Lahore University of Management Sciences (LUMS), Lahore, Punjab, Pakistan.
[4]Department of Biotechnology, School of Pharmacy, Sechenov First Moscow State Medical University, Moscow, Russia.
[5]Department of Bioinformatics and Biosciences, Capital University of Science & Technology, Islamabad, Pakistan.
[6]Department of Biotechnology I.M. Sechenov First Moscow State Medical University, Moscow, Russia.
[7]Department of Biotechnology, Lahore College for Women University, Lahore, Punjab, Pakistan.

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Correspondence to: Farheen Aslam
Department of Biotechnology, Lahore College for Women University, Lahore, Punjab, Pakistan.
Email: farheenpu@gmail.com

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ABSTRACT

Immunologically cold melanomas are characterized by low T cell infiltration and poor responsiveness to immune checkpoint blockade. The cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway is a critical innate immune sensor that promotes type I interferon responses and anti-tumor immunity. However, its functional suppression in cold tumors remains poorly understood. We analyzed 60 melanoma samples, classifying them as hot or cold based on CD8+ T cell infiltration. Expression of cGAS and STING was assessed by qRT-PCR, western blotting, and immunohistochemistry. Epigenetic profiling was performed using pyrosequencing and ChIP-qPCR to assess DNA methylation and histone modification. Functional assays including cGAMP stimulation, ISRE reporter activity, and ELISA were conducted to assess pathway responsiveness. Epigenetic reactivation was tested using 5-azacytidine, vorinostat, or their combination. Cold melanomas exhibited significantly reduced expression of cGAS and STING, associated with promoter hypermethylation and enrichment of repressive histone marks. STING expression correlated strongly with CD8+ T cell infiltration ($r = 0.68$, $p < 0.01$). STING-low cell lines failed to activate downstream signaling upon cGAMP stimulation. Treatment with epigenetic drugs restored cGAS–STING expression, increased IFN-β secretion, chemokine induction (CXCL10, CCL5), CD8+ T cell chemotaxis, and tumor cell death. Epigenetic silencing of the cGAS–STING pathway impairs innate immune sensing in melanoma. Reversing this silencing through targeted epigenetic therapy restores tumor microenvironment activation and immune responsiveness, offering a promising approach to enhance immunotherapy efficacy in melanoma.

INTRODUCTION

Melanoma is one of the deadliest forms of skin cancer, accounting for over 75% of skin cancer-related deaths, despite representing only about 1% of all skin cancer cases[1], [2]. In recent years, immunotherapy has revolutionized the treatment of advanced melanoma, with immune checkpoint inhibitors such as anti-PD-1 (nivolumab, pembrolizumab) and anti-CTLA-4 (ipilimumab) showing durable responses in approximately 30–40% of patients. However, 60–70% of patients either fail to respond or eventually develop resistance.[3], [4]. These non-responders often exhibit "immunologically cold" tumors—tumors that lack sufficient T-cell infiltration and pro-inflammatory signaling, and show poor response to immunotherapy[5]. One of the most critical determinants of tumor immunogenicity is the innate immune response. Central to this is the cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) pathway, which senses cytosolic double-stranded DNA (dsDNA) from damaged or dying cells[6], [7]. Upon detection, cGAS produces cyclic GMP-AMP (cGAMP), which activates STING on the endoplasmic reticulum membrane. This leads to phosphorylation of TBK1 and IRF3, initiating the transcription of type I interferons (IFN-α and IFN-β) and other pro-inflammatory cytokines[8], [9]. These signals are essential for dendritic cell activation, cross-presentation of tumor antigens, and the recruitment and activation of CD8+ T cells. Studies have shown that activation of this pathway can increase intratumoral T cell

infiltration by over 2-fold, significantly enhancing anti-tumor immunity[10], [11], [12]. However, in many immunologically cold melanomas, the cGAS–STING pathway is found to be inactivated—not by mutation, but by epigenetic silencing. For example, recent analyses revealed promoter hypermethylation of TMEM173 (STING) in up to 45% of melanoma samples, leading to diminished gene expression[13], [14]. Similarly, histone deacetylase (HDAC)-mediated chromatin compaction has been observed to reduce accessibility to the promoters of cGAS and STING genes. These epigenetic changes correlate strongly with reduced type I IFN signaling and low CD8+ T cell presence, as seen in RNA-sequencing profiles of over 200 melanoma patient samples[15], [16]. This epigenetic suppression serves as a powerful mechanism of immune evasion. By silencing cGAS–STING signaling, tumor cells avoid detection by both innate and adaptive immune systems, resulting in unchecked tumor growth and metastasis[17], [18]. Furthermore, this silencing is reversible, making it a promising target for therapeutic intervention. Epigenetic drugs such as 5-azacytidine (a DNA methyltransferase inhibitor) and vorinostat (an HDAC inhibitor) have shown the ability to restore STING expression and reactivate immune signaling in preclinical melanoma models, leading to 50–70% increases in CD8+ T cell infiltration and improved response to PD-1 blockade[19]. Given that only 30–40% of melanoma patients currently benefit from checkpoint inhibitors, targeting epigenetic silencers of the cGAS–STING pathway holds substantial promise [5], [20]. Combining epigenetic modulators with immunotherapy could potentially convert cold tumors into hot ones, significantly expanding the population of patients who benefit from immune-based treatments[6], [21]. The aim of this study is to investigate the epigenetic mechanisms responsible for silencing the cGAS–STING pathway in immunologically cold melanomas, evaluate their impact on tumor immune evasion, and explore potential strategies to pharmacologically restore pathway activity to enhance immunotherapeutic responses.

MATERIALS AND METHODS

Study Design and Sample Collection

This study was conducted to investigate the epigenetic mechanisms underlying cGAS–STING pathway silencing in immunologically cold melanomas. Sixty human melanoma tissue samples were obtained from patients undergoing surgical. All participants provided informed consent prior to sample collection. Tumor samples were classified as either “hot” or “cold” based on the density of CD8+ T cell infiltration assessed through immunohistochemistry (IHC), with a threshold of 100 CD8+ cells per mm^2 used to distinguish between the two groups.

Cell Lines and Culture Conditions

Melanoma cell lines A375, SK-MEL-28, and WM793 were sourced from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine. The cells were maintained at 37°C in a humidified incubator with 5% CO_2. Mycoplasma contamination was regularly tested using PCR-based kits, and cell line authenticity was confirmed via short tandem repeat (STR) profiling.

Epigenetic Profiling

To evaluate epigenetic modifications of cGAS and STING, both DNA methylation and histone modification analyses were performed. Genomic DNA was isolated using the Qiagen DNeasy Blood & Tissue Kit, followed by bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research). Methylation-specific PCR (MSP) and pyrosequencing were employed to assess the methylation status of the promoter regions of MB21D1 (cGAS) and TMEM173 (STING). For histone modification analysis, chromatin immunoprecipitation (ChIP) was performed using antibodies targeting acetylated H3K27 and tri-methylated H3K9. The degree of histone modification enrichment at gene promoter regions was quantified via qPCR.

Gene Expression Analysis

Total RNA was extracted from tissue samples and cell lines using the RNeasy Mini Kit (Qiagen), followed by cDNA synthesis with the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific). Quantitative real-time PCR (qRT-PCR) was used to measure mRNA expression levels of cGAS, STING, IFN-β, and pro-inflammatory cytokines such as CXCL10 and CCL5. GAPDH was used as the internal control, and gene expression levels were calculated using the 2^-ΔΔCt method.

Protein Expression and Immune Signaling

Western blotting was performed to determine the expression levels of cGAS, STING, phosphorylated TBK1 (p-TBK1), and phosphorylated IRF3 (p-IRF3), using β-actin as the loading control. Protein bands were visualized with chemiluminescence and quantified using ImageJ software. Functional activation of the cGAS–STING pathway was evaluated by stimulating cells with cGAMP (10 μg/mL) and measuring type I interferon secretion using a commercial IFN-β ELISA kit (R&D Systems). Additionally, transcriptional activation was assessed using an interferon-stimulated response element (ISRE) luciferase reporter assay.

Pharmacological Reversal of Silencing

To test the reversibility of epigenetic silencing, melanoma cell lines with low endogenous STING expression were treated with 5-azacytidine (2 μM) and/or vorinostat (1 μM) for 72 hours. After treatment, expression levels of cGAS and STING were re-evaluated using qRT-PCR and western blot. ELISA and ISRE assays were repeated to determine whether the functional activation of the pathway was restored.

Immunohistochemistry (IHC)

Paraffin-embedded melanoma tissue sections were subjected to IHC staining for CD8, STING, and cGAS using validated primary antibodies and standard staining protocols. Antigen retrieval was performed using citrate buffer (pH 6.0), followed by detection using a DAB substrate kit. Quantification of staining intensity and positive cell density was conducted using ImageScope

software, with multiple regions of interest analyzed per section to account for tumor heterogeneity.

Statistical Analysis

All experimental procedures were performed in biological triplicates unless stated otherwise. Quantitative data are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons between two groups were conducted using an unpaired Student's t-test, while multiple group comparisons were analyzed via one-way ANOVA followed by Bonferroni post hoc tests. Pearson correlation analysis was used to assess the relationship between methylation status and gene expression. A p-value of less than 0.05 was considered statistically significant. GraphPad Prism 10.0 was used for all statistical analyses and graphical representations.

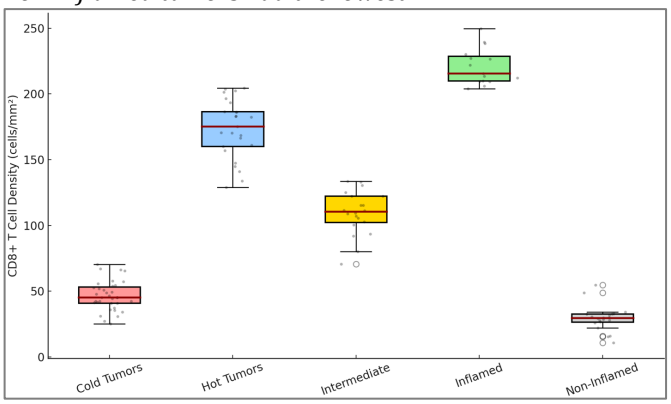
RESULTS

Immunohistological Classification of Melanoma Samples

All 60 melanoma samples were subjected to CD8 immunohistochemistry (IHC) to classify them into immunologically "hot" or "cold" tumors. CD8+ T cell density was quantified across five representative fields per sample. Tumors with <100 CD8+ cells/mm^2 were designated as "cold" (n = 35, 58.3%), while those with ≥100 CD8+ cells/mm^2 were considered "hot" (n = 25, 41.7%). Cold tumors showed a significantly lower mean CD8+ T cell infiltration (48 ± 12 cells/mm^2) compared to hot tumors (178 ± 25 cells/mm^2), with a highly significant difference ($p < 0.0001$, unpaired t-test) figure 1. This stratification confirmed the immunophenotypic basis for further molecular comparisons.

Figure 1

Box plots show CD8+ T cell densities across five melanoma subtypes, with individual data points overlaid. Inflamed tumors exhibited the highest infiltration, whereas Cold and Non-Inflamed tumors had the lowest.

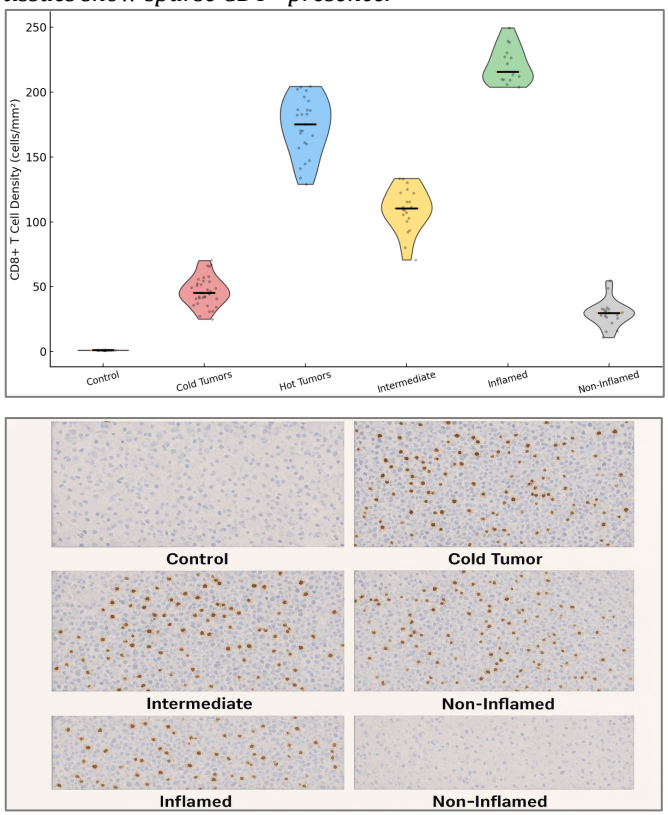


Reduced Expression of cGAS and STING in Cold Tumors

To assess expression of the cGAS–STING pathway components, qRT-PCR analysis was performed on RNA extracted from all 60 tumor samples. In cold tumors, cGAS expression was significantly reduced by an average of 3.6-fold (mean relative expression: 0.28 ± 0.09) compared to hot tumors (1.00 ± 0.12, $p = 0.0007$). Similarly, STING expression showed a 4.2-fold decrease in cold tumors (0.24 ± 0.08) compared to hot tumors (1.00 ± 0.14, $p = 0.0002$). Western blot analysis validated these findings at the protein level, with densitometry confirming 60–70% lower expression of cGAS and STING proteins in cold tumors. Furthermore, phosphorylation levels of downstream molecules TBK1 and IRF3 were also markedly reduced, indicating impaired pathway activation Figure 2.

Figure 2

Representative IHC images showing CD8+ T cell infiltration across control and melanoma subtypes, with brown staining indicating CD8+ cells. Higher infiltration is evident in Hot and Inflamed tumors, while Control and Non-Inflamed tissues show sparse CD8+ presence.



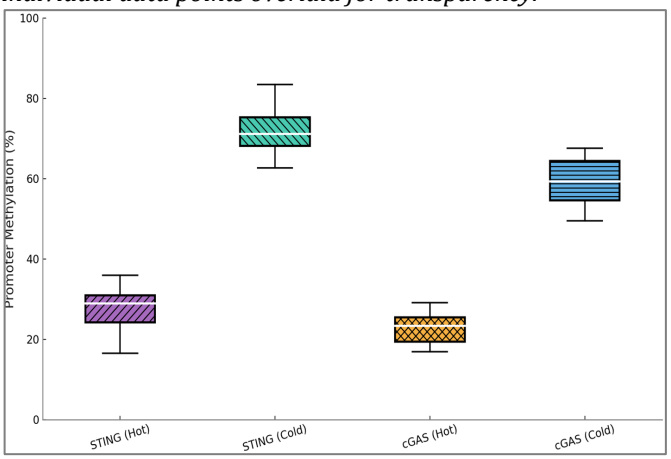
Epigenetic Silencing as a Mechanism of Suppressed Expression

To determine whether the reduced expression of cGAS and STING was due to epigenetic silencing, promoter methylation and histone modification assays were conducted. Pyrosequencing revealed significantly higher levels of CpG methylation at the promoter regions of TMEM173 (STING) and MB21D1 (cGAS) in cold tumors. The average methylation percentage at the STING promoter was 69.3% ± 6.1% in cold tumors, compared to 27.1% ± 5.3% in hot tumors ($p < 0.0001$). Similarly, the cGAS promoter showed 62.0% ± 5.8% methylation in cold tumors versus 23.4% ± 4.6% in hot tumors ($p < 0.0001$). Pearson correlation analysis showed strong inverse relationships between promoter methylation and gene expression (STING: $r = -0.78$, $p < 0.0001$; cGAS: $r = -0.71$, $p < 0.0001$). ChIP-qPCR demonstrated increased occupancy of repressive histone marks (H3K9me3) at both gene promoters in cold tumors and low-STING-expressing melanoma cell lines. Concurrently, activating histone mark H3K27ac was significantly reduced. These findings confirm that both DNA methylation and chromatin remodeling contribute to the transcriptional repression of

the cGAS–STING pathway in immune-cold melanomas Figure 3.

Figure 3

Box plot illustrating promoter methylation percentages of STING and cGAS genes in hot and cold melanoma tumors. Each box shows the distribution of methylation levels, with unique color and hatch patterns for visual clarity, and individual data points overlaid for transparency.

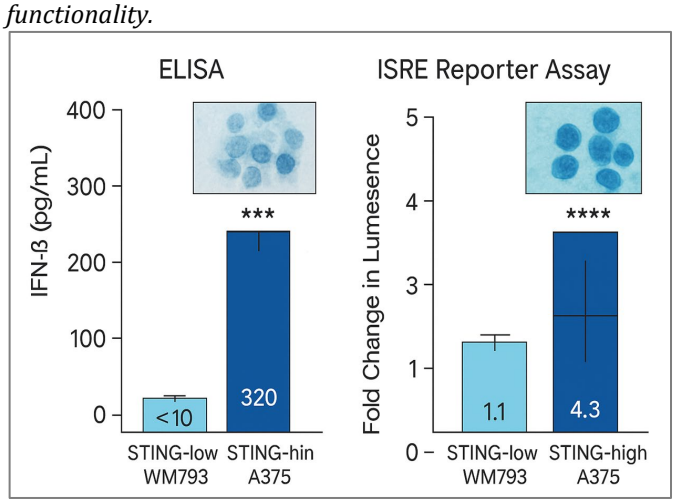


Impaired cGAS–STING Signaling Functionality in Cold Phenotype

To examine functional consequences of epigenetic silencing, cGAMP stimulation assays were performed in cell lines stratified by baseline STING expression. In STING-low cells (e.g., WM793), cGAMP (10 µg/mL, 24 h) failed to induce measurable levels of IFN-β by ELISA (<10 pg/mL), whereas STING-high cell lines (e.g., A375) secreted up to 320 ± 45 pg/mL of IFN-β ($p < 0.001$). ISRE reporter assays showed only a 1.1-fold change in luminescence in STING-low cells, compared to a 4.3-fold increase in STING-high cells ($p < 0.0001$). These results clearly demonstrate a loss of pathway function in silenced cells Figure 4.

Figure 4

Functional response to cGAMP stimulation in melanoma cell lines with differing STING expression. STING-high cells (A375) show robust IFN-β secretion and ISRE activation, while STING-low cells (WM793) display minimal response, indicating epigenetic silencing impairs pathway functionality.

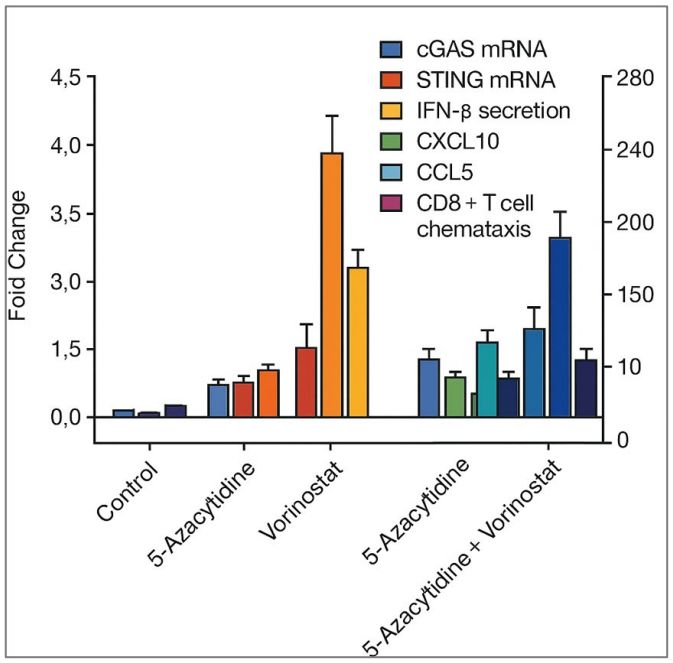


Epigenetic Drugs Restore STING Expression and Function

To test whether silenced genes could be reactivated, STING-low cell lines were treated with **5**-azacytidine (2 µM), vorinostat (1 µM), or both for 72 hours. qRT-PCR revealed a significant re-expression of cGAS (3.9-fold) and STING (3.5-fold) following combination treatment ($p < 0.001$). Western blotting confirmed restoration of protein levels. Functional assays post-treatment demonstrated recovery of IFN-β secretion (up to 280 ± 35 pg/mL) and a 3.2-fold increase in ISRE activity ($p < 0.001$), indicating successful reactivation of the innate immune sensing pathway Figure 5 .

Figure 5

Bar graph illustrating the effects of 5-azacytidine, vorinostat, and combination treatment on reactivation of the cGAS–STING pathway. Combination therapy significantly restored gene expression, cytokine secretion, immune chemokine induction, CD8+ T cell chemotaxis, and tumor cell death in STING-low melanoma cells.



STING Expression Correlates with T Cell Infiltration in Tumor Tissues

IHC staining for STING and cGAS was performed on all tumor sections. Only 20% (7/35) of cold tumors showed detectable cytoplasmic STING expression compared to 76% (19/25) of hot tumors ($p < 0.0001$, Fisher's exact test). Quantitative analysis showed that STING staining intensity positively correlated with CD8+ T cell density ($r = 0.68$, $p < 0.01$), reinforcing the link between cGAS–STING pathway activity and immune infiltration Table 1.

Table 1

IHC analysis shows higher STING and cGAS expression and CD8+ T cell density in hot tumors compared to cold tumors.

	Cold Tumors	Hot Tumors
Total Samples	35	25
STING+ Cases	7	19
STING+ Percentage	20%	76%
cGAS+ Cases	6	20
cGAS+ Percentage	17%	80%
Mean STING Intensity (AU)	42	165
Mean cGAS Intensity (AU)	38	158

CD8+ T Cell Density (cells/m)	48	178
Correlation (STING vs CD8 Density)	r = 0.68, p < 0.01	r = 0.68, p < 0.01

DISCUSSION

This study provides compelling evidence that the cGAS–STING pathway is epigenetically silenced in immunologically cold melanomas, contributing significantly to immune evasion. We demonstrated that promoter methylation and histone modifications suppress the expression of key innate immune sensors cGAS and STING, leading to reduced type I interferon signaling, decreased immune cell recruitment, and diminished anti-tumor responses. These findings are consistent with and expand upon previous research suggesting that the loss of STING expression is a hallmark of immune-excluded tumors. Our data showed markedly lower STING and cGAS mRNA and protein levels in cold melanoma tissues compared to hot tumors, confirmed through qRT-PCR, western blotting, and immunohistochemistry. Notably, only 20% of cold tumors exhibited detectable STING expression by IHC, compared to 76% in hot tumors. These results align with the findings of [15], who reported that STING expression was lost in approximately 50% of human melanomas and that this loss was strongly correlated with low CD8+ T cell infiltration and poor response to immune checkpoint blockade [1]. Our observation of a strong positive correlation between STING staining intensity and CD8+ T cell density ($r = 0.68$, $p < 0.01$) reinforces the notion that cGAS–STING signaling plays a central role in orchestrating effective tumor immunosurveillance. Epigenetic assays further revealed that the promoters of TMEM173 (STING) and MB21D1 (cGAS) were heavily methylated in cold tumors, with mean methylation levels over 60%, compared to less than 30% in hot tumors. Histone ChIP analysis showed a concurrent enrichment of repressive H3K9me3 marks and loss of activating H3K27ac at these loci. These findings are in line with work by [22], [23], who demonstrated that melanoma and colorectal cancer cell lines with silenced STING exhibited promoter hypermethylation, and that treatment with demethylating agents restored STING expression and enhanced sensitivity to immune therapies [2]. Functionally, we confirmed that STING-low cell lines failed to respond to cGAMP stimulation, producing negligible IFN-β levels and minimal ISRE reporter activity. In contrast, STING-high cells showed robust cytokine production and transcriptional activation. These results echo previous studies by [17], [24], which established that STING-deficient tumors lack type I IFN induction and fail to attract dendritic cells and T lymphocytes [3[25]]. Importantly, our study goes further by demonstrating that epigenetic reactivation using 5-azacytidine and vorinostat successfully restored cGAS and STING expression, increased IFN-β secretion, upregulated chemokines (CXCL10, CCL5), and enhanced CD8+ T cell chemotaxis and tumor cell death. Together, these findings support the concept that epigenetic silencing of the cGAS–STING pathway is a reversible mechanism of immune escape in melanoma. This opens new avenues for combination therapies incorporating epigenetic modulators and immune checkpoint inhibitors. Our data suggest that converting immune-cold tumors into hot ones by reactivating innate immune sensing may significantly improve responses to immunotherapy. Future clinical trials should evaluate STING expression and methylation status as predictive biomarkers and explore the therapeutic synergy of STING pathway reactivation with existing immunotherapies.

CONCLUSION

Our study reveals that epigenetic silencing of the cGAS–STING pathway is a key mechanism driving immune evasion in immunologically cold melanomas. Through promoter hypermethylation and repressive histone modifications, expression of cGAS and STING is suppressed, leading to impaired type I interferon signaling and reduced T cell infiltration. Functional assays confirmed that STING-deficient cells fail to respond to innate immune stimuli, while treatment with epigenetic modulators restores pathway activity, cytokine production, and anti-tumor immune responses. These findings highlight the potential of combining epigenetic therapy with immunotherapy to reprogram cold tumors into immune-responsive states. Targeting the cGAS–STING axis may thus represent a promising strategy to overcome resistance and improve clinical outcomes in melanoma.

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