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Disrupting Mismatch Repair Induces Immune-Sensitive Phenotype in Colorectal Cancer

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Abstract. Immune checkpoint blockade (ICB) has transformed cancer therapy by overcoming tumor immune evasion, particularly in advanced solid tumors resistant to conventional treatments. However, clinical responses are highly variable and strongly associated with mismatch repair (MMR) status: patients with MMR-deficient/microsatellite instability-high (dMMR/MSI-H) tumors respond more robustly than those with MMR-proficient/microsatellite stable (pMMR/MSS) tumors. While ICB responsiveness has been attributed to neoantigen-driven immunogenicity or increased susceptibility to inflammatory cell death (e.g., PANoptosis), it remains unclear whether MMR deficiency alone can confer both advantages. Here, we investigated this question by transiently knocking down MLH1 in pMMR/MSS colorectal cancer cells to induce a dMMR/MSI-H–like phenotype. MLH1 knockdown resulted in MSI at 2 of 5 tested loci. When co-cultured with pre-activated $CD8^+$ T cells (E:T = 1:5), reprogrammed cells triggered significant pro-inflammatory cytokine release, with IFN-γ levels approximately double those of the dMMR benchmark, indicating hyper-enhanced immunogenicity. Separately, MLH1-deficient cells displayed intermediate susceptibility to cytokine-mediated inflammatory cell death (50.95%), compared with low responses in pMMR cells (8.68%) and near-complete death in dMMR cells (99.09%). These findings demonstrate that transient MLH1 knockdown can simultaneously enhance tumor immunogenicity and susceptibility to inflammatory cell death, supporting both neoantigen-based and PANoptotic models of ICB responsiveness. This approach offers a controllable, reversible strategy to sensitize "immune-cold" pMMR tumors to ICB, potentially reducing the risk of chronic side effects associated with prolonged interventions. By establishing MLH1 knockdown as a flexible adjunct to multiple therapies, from ICB to cytokine-based treatments, our work lays a foundation for translational strategies that unify treatment paradigms for pMMR and dMMR colorectal cancers.

Keywords: Immune checkpoint blockade, Colorectal cancer, Mismatch repair, Microsatellite instability, Gene knockdown, Inflammatory cell death.

1. Introduction

The immune system plays a decisive role in shaping cancer outcomes, particularly through mechanisms of immune evasion that allow tumor cells to escape $CD8^+$ T-cell recognition despite presenting neoantigens. Immune checkpoint blockade (ICB) was developed to counter this evasion

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and has transformed treatment for several advanced solid tumors that were historically refractory to therapy. Across diverse cancer types—including melanoma, colorectal cancer, non-small cell lung cancer, and triple-negative breast cancer—ICB has induced tumor regression, extended survival beyond chemotherapy benchmarks, and in some cases achieved clinical complete responses [1-4]. Mechanistically, ICB acts by blocking inhibitory receptors such as PD-1/PD-L1 and CTLA-4/CD80, thereby restoring T-cell–mediated recognition and killing of tumor cells [5,6].

Despite these successes, the majority of patients fail to benefit from ICB. Objective response rates remain below 20–40% across most cancers, and many responders ultimately develop resistance [7,8]. In colorectal cancer, where checkpoint inhibitors have generated both landmark responses and complete failures, immune-related objective response rates in unselected cohorts rarely exceed 20% and sometimes fall near zero [9-11]. These limitations underscore the need for predictive biomarkers and novel combination strategies [12].

Deficiency in the DNA mismatch repair (MMR) system has emerged as one of the most reliable biomarkers of ICB responsiveness. MMR proteins such as MLH1, MSH2, MSH6, and PMS2 normally correct replication errors, while their loss generates hypermutation and microsatellite instability (MSI) [13]. Clinically, MLH1 loss—whether through genetic defects or epigenetic silencing—represents the most common MMR deficiency and is closely linked to hereditary cancer syndromes [14,15]. Patients with MMR-deficient/MSI-high (dMMR/MSI-H) tumors, though representing less than 15% of cases, consistently achieve superior outcomes on ICB compared with MMR-proficient/microsatellite stable (pMMR/MSS) patients. Landmark trials demonstrated striking differences: in metastatic colorectal cancer, ICB elicited a 40% response rate in dMMR tumors versus 0% in pMMR tumors [11], while treatment-naïve dMMR rectal cancer patients achieved a 100% clinical complete response with PD-1 blockade alone [3]. With its relatively high prevalence of dMMR (~15% vs. ~3% pan-cancer), extensive clinical evidence, and regulatory approval for ICB, colorectal cancer has become the prototypical model for studying MMR status as a determinant of immunotherapy outcomes [16,17].

The mechanistic basis of this association remains unclear. The prevailing explanation attributes dMMR/MSI-H immunogenicity to the accumulation of somatic mutations and neoantigens, which promote immune recognition [18]. However, mutational burden alone does not consistently predict response, suggesting additional mechanisms. Recent work has highlighted PANoptosis—a form of programmed cell death that integrates apoptosis with inflammatory pyroptosis and necroptosis—as a critical determinant. In dMMR/MSI-H colorectal cancer cell lines, hyperactivation of PANoptotic pathways enhanced tumor susceptibility to cytokines TNF-α and IFN-γ released by $CD8^+$ T cells [19]. Whether MMR deficiency alone drives this dual sensitivity, or whether additional factors are required, remains unclear.

Here, we hypothesized that transient inhibition of MLH1 in pMMR/MSS colorectal cancer could phenocopy the immune sensitivity of dMMR/MSI-H tumors, thereby overcoming ICB resistance. We demonstrate that MLH1-inhibited cells acquire enhanced immunogenicity and increased susceptibility to cytokine-mediated killing. Our findings support both the neoantigen and PANoptotic models of ICB responsiveness and point to a convergence therapeutic strategy that could extend the benefits of checkpoint blockade to the majority of colorectal cancer patients who are currently resistant.

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2. Methods

2.1. Cell culture and reagents

Human colorectal carcinoma HT-29 and HCT116, purchased from the China Center for Type Culture Collection, were cultured in McCoy's 5A Medium (Gibco, #16600082) supplemented with 10% fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin at 37°C and 5% CO_2. $CD8^+$ T cells (≥95% purity by flow cytometry) were obtained from IMMOCELL Biotechnology (IMP-M195) and cryopreserved in liquid nitrogen prior to use. All other reagents were purchased from Beyotime Biotechnology unless otherwise specified.

2.2. MLH1 knockdown in HT-29 cells

HT-29 cells were transfected with three MLH1-targeting siRNAs (siMLH1-1, -2, -3; Azenta Life Sciences, #80-1672811117), a non-targeting control siRNA (siCTRL), or left untransfected. Transfection was performed using Lipofectamine 8000 (Beyotime) according to manufacturer instructions. The siRNA sequences are provided in Table 1. Knockdown efficiency was confirmed via Western blot.

Table 1. siRNA sequences for MLH1 knockdown

siRNA	Sequence
siMLH1-1	Sense: 5'- CCAAGUGAAGAAUAUGGGAAATT-3' Antisense: 5'- UUUCCCAUAUUCUUCACUUGGTT-3'
siMLH1-2	Sense: 5'- GCCUGAUCUAUACAAAGUCUUTT-3' Antisense: 5'-AAGACUUUGUAUAGAUCAGGCTT-3'
siMLH1-3	Sense: 5'-CCAUUCCUGUACCUCAGUUUATT-3' Antisense: 5'-UAAACUGAGGUACAGGAAUGGTT-3'
Non-targeting siRNA	Randomly generated and verified by manufacturer

2.3. Western blot assays

Cells were lysed 72 hours post-transfection in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) with protease/phosphatase inhibitors and 1% PMSF. Protein concentrations were determined using the BeyoBCA High Sensitivity Protein Assay Kit (Beyotime, P0399S, Supplementary Figure 1). Equal amounts of protein (15 μg) were resolved on 12.5% SDS–PAGE gels, transferred to PVDF membranes, and probed with MLH1 (rabbit mAb, Beyotime, AF1327, 1:1000) and β-actin (HRP-conjugated mouse mAb, Beyotime, AF2815, 1:1000). Detection was performed with ECL substrate. Band intensities were quantified with ImageJ (v1.54g), and MLH1 expression was normalized to β-actin.

2.4. Microsatellite Instability (MSI) assays

Genomic DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN, #56304) from HT-29 (±siMLH1) and HCT116 cells. MSI was evaluated using the 5D Fluorescent Microsatellite Instability PCR Kit (Beyotime, D8501S), which targets five mononucleotide loci: BAT25, BAT26, NR21, NR24, and NR27. PCR was performed per manufacturer instructions (Tables 2 and 3), and capillary electro`phoresis was conducted on an ABI 3730xl DNA Analyzer. Fragment analysis was

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performed using GeneMapper v5.0. MSI status was categorized as MSI-High (≥2 unstable loci), MSI-Low (1 unstable locus), or microsatellite stable (MSS, 0 unstable loci).

Table 2. Components and respective volumes of PCR reactions

Components of kit	Volume per reaction
MSI PCR Mix (2x)	5 L
MSI Primer Pair Mix (10x)	1 L
Genomic DNA (40 ng)	x L
Nuclease-Free Water	(4-x) L
Total Volume	10 L

Table 3. Steps of each PCR reaction

Step	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	15s	
Annealing	60°C	15s	30
Extension	72°C	15s	
Final extension	72°C	10 min	1

2.5. CD8+ cell activation and CRC-CD8+ co-cultures

$CD8^+$ T cells were cultured in RPMI-1640 medium (Gibco, #11875093) with 10% FBS, 1% penicillin-streptomycin, and 50 μM β-mercaptoethanol. Cells were activated with 2 μg/mL anti-CD3 (Sciprotech, MA1001L-T1) and 1 μg/mL anti-CD28 (Sciprotech, MA1002L) for 24 hours at 37°C. HT-29 (±siMLH1) and HCT116 cells were co-cultured with activated T cells at a 1:5 effector-to-target ratio for 48 hours in McCoy's 5A medium.

2.6. Co-culture cytokine immunoassays

Supernatants from CRC–$CD8^+$ co-cultures were collected, centrifuged at 1000 rpm for 5 minutes, and stored at -80°C. Concentrations of TNF-α and IFN-γ were measured by double-antibody sandwich mouse ELISA (Beyotime; TNF-α: PT512; IFN-γ: P1508) following the manufacturer's protocols. Absorbance was read at 450 nm, and cytokine concentrations were interpolated from standard curves using second-order polynomial fitting (Supplementary Figure 2).

2.7. Inflammatory cell death assays

HT-29 cells were treated with TNF-α (10 ng/mL) and IFN-γ (100 ng/mL) for 96 hours. Cells were then stained with Annexin V-FITC and propidium iodide (Beyotime, C1062M) and analyzed by flow cytometry (CytoFLEX S, Beckman Coulter). Gating was performed in FlowJo (v10.9), excluding debris and doublets. Apoptosis was quantified as Annexin V^+/PI^- (early) and Annexin V^+/PI^+ (late).

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2.8. Cancer genomic analyses with cbioportal

Publicly available pan-cancer and colorectal cancer datasets were analyzed using cBioPortal (https://www.cbioportal.org; accessed May 25, 2025). For pan-cancer analysis, 10,967 TCGA samples across 32 studies were examined for alterations in MMR genes (MLH1, MSH2, MSH3, MSH6, PMS2) and associated MANTIS scores. MSI was quantified using MANTIS, which calculates the mean absolute difference in microsatellite length distributions between tumor and normal samples.

A colorectal-specific cohort (n = 6754) from 19 TCGA studies was separately analyzed using MSIsensor scores due to limited MANTIS data. MSI was defined by the percentage of unstable microsatellites (motif ≤5 bp, ≥3 repeats) identified via χ^2 goodness-of-fit test comparing tumor and matched normal genomes. Detailed study lists are provided in Supplementary Table 1.

2.9. Statistical analysis

Two-tailed Student's t-tests were used for pairwise comparisons; one-way ANOVA followed by Tukey's HSD test was used for multiple group comparisons. For both methods, normality and equal variance were assumed across groups. Genomic data were analyzed using the Wilcoxon rank-sum test. Experimental results are presented as mean ± s.e.m.; genomic MSI scores as five-number summaries due to non-normality. A p-value <0.05 was considered significant. Statistical analyses were performed using GraphPad Prism v8.0.2 and the cBioPortal online platform.

3. Results

3.1. MLH1 alteration is strongly associated with microsatellite instability across cancers and in colorectal cancer

Given positive associations between microsatellite instability (MSI), tumor immunogenicity, and response to immune checkpoint blockade (ICB), we aimed to identify the human DNA MMR gene associated with the highest MSI among the five commonly studied candidates: MSH2, MSH3, MSH6, MLH1, and PMS2. We analyzed 10,967 tumors from 32 cancer types in the TCGA PanCancer Atlas (via cBioPortal), which predominantly consisted of primary tumors (96.6%) with a near-even sex distribution (female: 48.5%, male: 44.4%) and a median age of 60 years.

Among these samples, 1,092 tumors harbored an alteration in at least one of the five MMR genes and had a reported MANTIS score, a validated metric of MSI ranging from 0.0 (stable) to 2.0 (unstable). Tumors with multiple alterations were represented in all relevant groups. A right-skewed distribution was observed in all groups, reflecting the predominance of pMMR/MSS cancers (Figure 1a). Using a five-point summary, we found that MLH1-altered tumors exhibited the highest median MANTIS score (0.34), significantly exceeding all other groups ($P < 0.01$, Wilcoxon signed-rank test). A substantial proportion of MLH1-altered tumors had scores indicative of dMMR/MSI-H, leading us to select MLH1 as the knockdown target for functional validation.

To determine the most clinically relevant experimental model, we examined the distribution of MLH1 alterations across tumor types. The highest frequencies were seen in endometrial (5.8%), bladder (4.62%), and colorectal cancer (CRC, 3.87%; Figure 1b). Considering CRC's high incidence, well-characterized dMMR/MSI-H subtype, and its consistent responsiveness to ICB, we selected colorectal cancer as the experimental focus.

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To validate that the MSI association persists within CRC, we analyzed a 19-study CRC dataset (n = 487; see Supplementary Table 1), which included but was not limited to TCGA samples. Available metadata showed representation across primary (43.5%) and metastatic (20.7%) tumors, a near-even sex distribution (male: 51.8%, female: 45.0%), and a median age of 65 years. MSI status was quantified by the MSIsensor score, which measures the proportion of unstable microsatellite loci. MLH1 and MSH3 alterations both yielded significantly higher MSIsensor scores relative to other MMR genes ($P < 0.01$, Wilcoxon signed-rank test; Figure 1c). Given MLH1's strong pan-cancer signal and clinical relevance, we prioritized it as the experimental target.

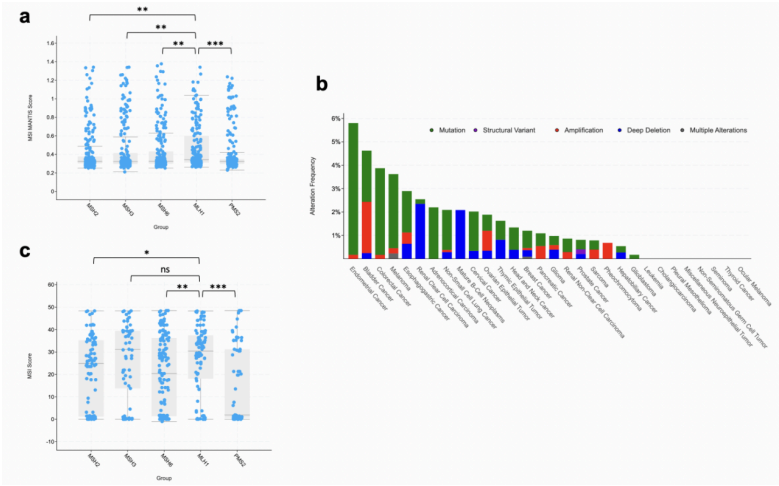


Figure 1. Alterations in mismatch repair (MMR) genes and their association with microsatellite instability (MSI) and cancer type. (a) MSI MANTIS scores from 1092 tumor samples with MMR gene alterations (MSH2, MSH3, MSH6, MLH1, and/or PMS2), extracted from the TCGA PanCancer Atlas dataset (n = 10967; 32 cancer types involved; see Supplementary Table 1). Samples with co-occurring alterations are shown in multiple groups. (b) MLH1 alteration frequency across cancer types in the TCGA PanCancer Atlas dataset. (c) MSIsensor scores from 487 colorectal cancer samples with MMR gene alterations, extracted from a curated cBioPortal dataset (n = 6754; see Supplementary Table 1). Samples with co-occurring alterations are shown in multiple groups. Significance between every two groups was determined through a two-sided Wilcoxon signed-rank test. *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; ns, not significant

3.2. SiRNA-mediated MLH1 knockdown induces an MSI-H phenotype in HT-29

To disrupt the MMR pathway and thus convert pMMR/MSS colorectal cancer into dMMR/MSI-H, we silenced MLH1 using small interfering RNA (siRNA). We tested three MLH1-targeting siRNAs alongside a sequence-verified control. Western blotting 72 hours post-transfection revealed that siMLH1-1 achieved the most efficient knockdown (relative expression: 0.49 normalized to β-actin), followed by siMLH1-2 (0.84; Figure 2a, b). siMLH1-1 was selected for all subsequent experiments, hereafter referred to as siMLH1.

Knockdown of MLH1, a functional component of the MMR pathway, would theoretically result in MSI. To test this, we examined five quasimonomorphic mononucleotide repeats (BAT25, BAT26, NR21, NR24, and NR27), a panel with superior sensitivity and specificity compared to the Bethesda panel. [20,21]. Samples were classified as MSI-H (≥2 unstable loci), MSI-L (1 unstable locus), or MSS (no unstable loci). Baseline HT-29 and K562 controls displayed stable microsatellite profiles, confirming an MSS phenotype (Figure 2c). In contrast, HT-29 + siMLH1 displayed instability at 2-3

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loci, consistent with MSl-H status, comparable to naturally dMMR/MSI-H HCT1 16 cells. Thus, siRNA-mediated MLH1 knockdown successfully induced an MSl-H phenotype in HT-29.

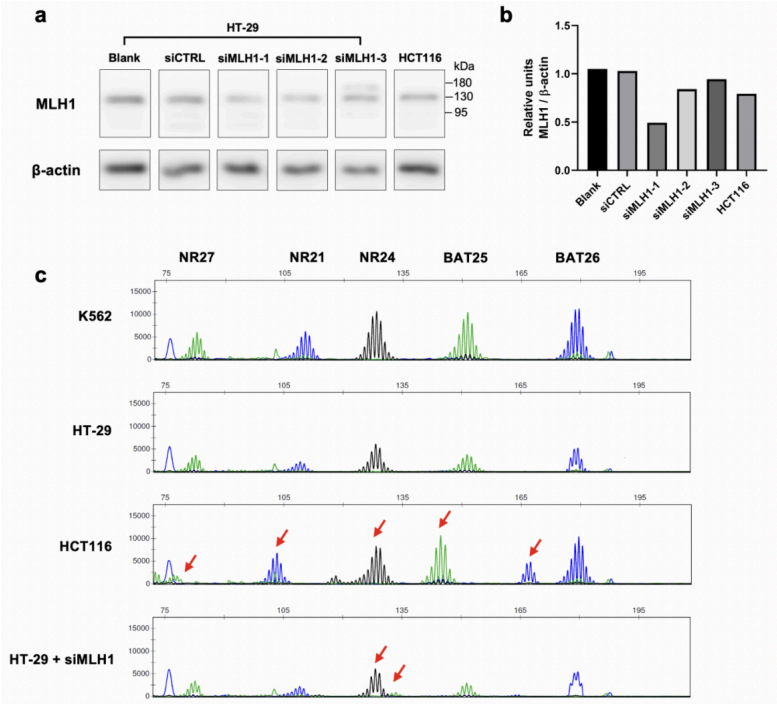


Figure 2. Validation of MLH1 knockdown and MSI induction in HT-29 cells. (a) Western Blot for MLH1 expression in HT-29 transfected with a MLH1-targeting siRNA (siMLH1-1, -2, or -3), a non-targeting control siRNA (siCTRL), or left untransfected, compared to MLH1-deficient HCT116 cells. (b) Densitometric quantification of MLH1 expression in Western Blot, normalized to β-actin. (c) MSI profiles of HT-29 + siMLH1 (siMLH1-2), HT-29, and HCT116 cells, assessed at five mononucleotide loci (BAT25, BAT26, NR21, NR24, NR27). K562, a microsatellite-stable (MSS) human myelogenous leukemia cell line, was used as an additional negative control after HT-29. Red arrows indicate MSI events

3.3. MLH1 knockdown enhances pro-inflammatory cytokine production by CD8+ t cells

dMMR/MSI-H colorectal cancer is associated with higher tumor immunogenicity and sensitivity to treatment. Higher immunogenicity connotes the release of more pro-inflammatory cytokines, which promote necrosis, the inflammatory form of cell death. Therefore, we investigated whether MLH1 knockdown in pMMR/MSS colorectal cancer enhances pro-inflammatory cytokine production, resembling the naturally dMMR/MSI-H cases. To simulate the tumor microenvironment in vitro, we co-cultured HT-29 + siMLH1, parental HT-29, and HCT116 with activated CD8+ T cells (5:1 ratio) for 48 hours. Subsequently, we measured the concentration of Tumor Necrosis Factor-alpha (TNF-α) and Interferon-gamma (IFN-γ) in the supernatant with ELISA , as these cytokines are hallmarks of cytotoxic T cell activity.

MLH1 knockdown significantly increased TNF-α secretion from 3.38 ± 0.23 pg/mL to 5.04 ± 0.29 pg/mL ($P < 0.01$, Tukey's HSD test after one-way ANOVA), comparable to HCT116 (Figure 3a). Even more strikingly, IFN-γ secretion from CD8+ T cells co-cultured with HT-29 + siMLH1 reached 9.8 ± 0.45 pg/mL, doubling HCT116 levels (4.05 ± 0.59 pg/mL, $P < 0.0001$, Student's t-test; Figure 3b). IFN-γ levels for parental HT-29 were below assay sensitivity in all five biological

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replicates. These data confirm that MLH1 knockdown increases the immunogenicity of pMMR/MSS CRC cells.

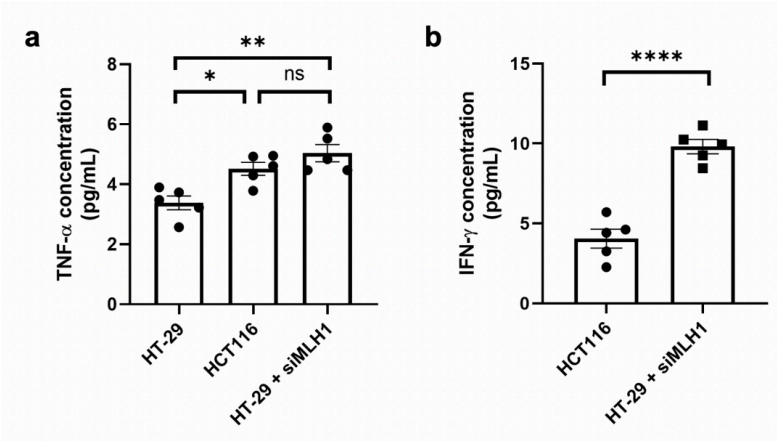


Figure 3. Pro-inflammatory cytokine production by CD8+ T cells in co-cultures with colorectal cancer. (a) TNF-α concentrations measured by ELISA in co-cultures of CD8+ T cells with HT29 + siMLH1, HT-29, or HCT116 cells (n = 5 per group). (b) IFN-γ concentrations in co-cultures with HT29 + siMLH1 and HCT116 (n = 5 per group). IFN-γ production in the HT-29 co-culture was below assay sensitivity and thus is not represented. Significance in (a) was determined through a one-way ANOVA followed by Tukey's HSD test; in (b), through a two-tailed Student's t-test. Data are presented as mean ± s.e.m. **** $P \leq 0.0001$; ** $P \leq 0.01$; * $P \leq 0.05$; ns, not significant

3.4. MLH1 knockdown increases susceptibility to cytokine-induced inflammatory cell death

Having confirmed higher tumor immunogenicity, we then evaluated whether MLH1 knockdown enhances tumor sensitivity to immune effector cytokines. Previous studies have demonstrated that dMMR/MSI-H colorectal cancer undergoes inflammatory cell death when exposed to TNF-α and IFN-γ. Anticipating the same in HT-29 + siMLH1, we co-treated HT-29 + siMLH1, HT-29, and HCT116 with TNF-α (10 ng/mL) and IFN-γ (100 ng/mL) for 96 hours, as previously described [19]. Viability was assessed by flow cytometry with Annexin V-FITC and PI staining (Figures 4a–d). The unstained control using cytokine-treated HT-29 + siMLH1 defined gating thresholds (Figure 4a), and compensation was unnecessary due to minimal spillover. PI+ cells were classified as non-viable, with double-positive Annexin V+/PI+ staining indicating late apoptosis or necrosis, while Annexin V-FITC$^+$/PI$^-$ indicating early apoptosis.

HT-29 + siMLH1 exhibited a higher proportion of PI$^+$ cells (50.93%) compared to parental HT-29 (8.68%), but lower than HCT116 (99.09%; Figure 4e). Among PI$^+$ HT-29 + siMLH1 cells, 80.19% were also Annexin V-FITC$^+$, indicative of late-stage cell death. These findings indicate that MLH1 knockdown not only enhances immunogenicity but also partially recapitulates the cytokine sensitivity observed in naturally MSI-H CRC.

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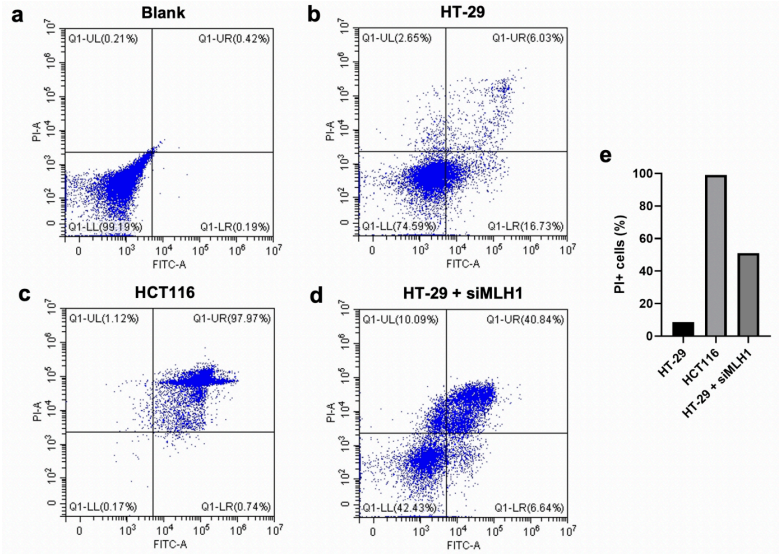


Figure 4. Assessment of inflammatory cell death following cytokine treatment. HT-29 cells were co-treated with TNF-α (10 ng/mL) and IFN-γ (100 ng/mL) for 96 hours. With the gating strategy established from (a) untreated control (n = 9633), flow cytometry was performed on (b) HT-29, (c) HCT116, and (d) HT29 + siMLH1 (n = 10000 per group), stained with Annexin V-FITC and propidium iodide (PI). (e) Percentage of late-apoptotic or necrotic (PI+) cells across all groups

4. Discussion

Colorectal cancers with proficient mismatch repair (pMMR) and microsatellite stability(MSS) remain largely unresponsive to immune checkpoint blockade (lCB), representing a major clinical gap. These "immune-cold" tumors lack both sufficient neoantigen presentation and susceptibility to infammatory effector pathways, limiting durable immunotherapy benefit.Here, we show that transient knockdown of the mismatch repair protein MLH1 induces adMMR/MSl-H phenotype in pMMR/MSS colorectal cancer cells, enhancing both their recognition and inflammatory cytotoxicity by CD8*T cells. Our findings establish a mechanistically grounded approach to sensitize otherwise resistant tumors toimmunotherapy.

Current strategies for pMMR/MSS tumors highlight the need for alternative approaches. Neoadjuvant checkpoint blockade and fecal microbiota transplantation have shown potential but delivered variable outcomes, with mechanisms of benefit that remain incompletely understood [22-24]. Moreover, both approaches carry practical challenges: neoadjuvant checkpoint blockade increases perioperative risk through immune-related adverse events, while fecal microbiota transplantation introduces compliance and infection concerns [25-27]. In contrast, MLH1 knockdown directly targets the immunological deficits of pMMR/MSS tumors. Because the process can be tracked via MSI, it offers a potentially more controllable and mechanistically guided intervention.

Cytoplasmic degradation prevents sustained maintenance of the dMMR/MSI-H state. While this may necessitate repeated dosing, it also creates a reversible therapeutic window sufficient to sensitize tumors to ICB without incurring long-term off-target effects or chronic immune activation. This transient nature further allows dynamic, dose-adjustable modulation of mismatch repair and immune status, enabling personalization based on patient response.

Mechanistically, our results provide support for both major models of ICB responsiveness. By functionally separating immune recognition (cytokine production) from inflammatory cell death, we

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found that MLH1 loss enhanced tumor immunogenicity and simultaneously increased susceptibility to cytokine-induced killing. These dual effects align with the neoantigen hypothesis as well as recent evidence implicating PANoptosis [19]. Importantly, this versatility positions MLH1 knockdown as a flexible adjunct: it could compensate for the paucity of neoantigens in pMMR tumors when paired with ICB or amplify tumor susceptibility in combination with cytokine-based therapy.

This translational insight is also built upon earlier work that supports and complements our conclusions. Our success in inducing the MSI phenotype through transient MLH1 knockdown aligns with existing in vitro and murine studies, which showed that artificial MLH1 loss generates somatic frameshift mutations—the mutation type known to yield more immunogenic neoantigens [28-30]. Immunoassay results reaffirmed earlier findings that MLH1 knockdown triggers twofold cytokine production; yet, while we exclusively focus on TNF-α and IFN-γ, MLH1-deficient tumor microenvironments are also characterized by profuse cytolytic effectors (e.g. perforin and granzymes), which may cause more extensive cancer apoptosis [19,31]. Eventually, besides consistency with previous in vivo research, we further revealed that inflammatory death in MLH1 knockdown cells is elevated but intermediate between “immune-hot” and “-cold” profiles, extending the significance of this observation in the context of phenocopying [19].

Our study has several limitations that warrant further refinement. First, experiments were limited to two colorectal cancer cell lines, and the impact of inherent line-to-in evariability cannot be excluded. Replicating this strategy in additional pMMR/MSS and dMMR/MSI-H cell lines will be essential. Second, knockdown efficiency did not consistently reach the ≥70% reduction benchmark by 72 hours, suggesting that optimization of timing or dosing may improve efficacy. However, the robust effects observed even with partial suppression suggest that therapeutic benefit may not require complete loss of MLH1, though dosing optimization will be critical. Finally, distinguishing necroptosis, apoptosis, and PANoptosis more precisely will require refined live-cell imaging and molecular interrogation of mediators such as GSDMD, caspase-8, and p-MLKL.

Future studies should extend this work into in vivo systems, where immune-tumor interactions can be assessed in the context of the tumor microenvironment. Syngeneic murine models such as CT26 (MSS) and MC38 (MSl) offer platforms to test efficacy, durability, and synergy with ICB. A major translational challenge will be safe and tumor-specific delivery of siMLH1, with nanoparticle formulations, exosomes, and viral vectors emerging as promising options. lmportantly, MSl provides a unique biomarker to dynamically monitor therapeutic effect in real time. Dose-ranging studies and biomarker-driven stratification will be critical for balancing efficacy with safety.

Taken together, our study advances the concept that pMMR/MSS colorectal cancers can be reprogrammed into an ICB-responsive state through transient, reversible MLH1 knockdown. By enhancing both immunogenicity and susceptibility, this approach may broaden the reach of immunotherapy to the majority of colorectal cancer patients who currently derive little benefit. These findings lay the groundwork for translational studies aimed at optimizing delivery, safety, and clinical durability.

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Appendices

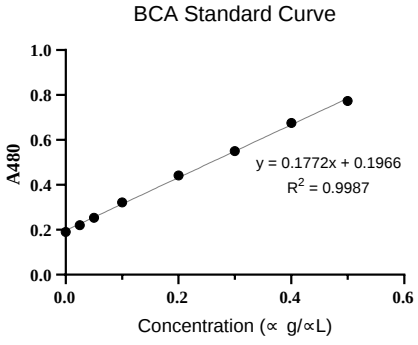
Supplementary Table 1. Studies used in pan-cancer and CRC genomic analysis

	Pan-cancer (32 distinct cancers)	Colorectal cancer
# of samples	10967	6754
# of studies	32	19
Sources	All from TCGA, PanCancer Atlas	See “Studies involved” for elaboration

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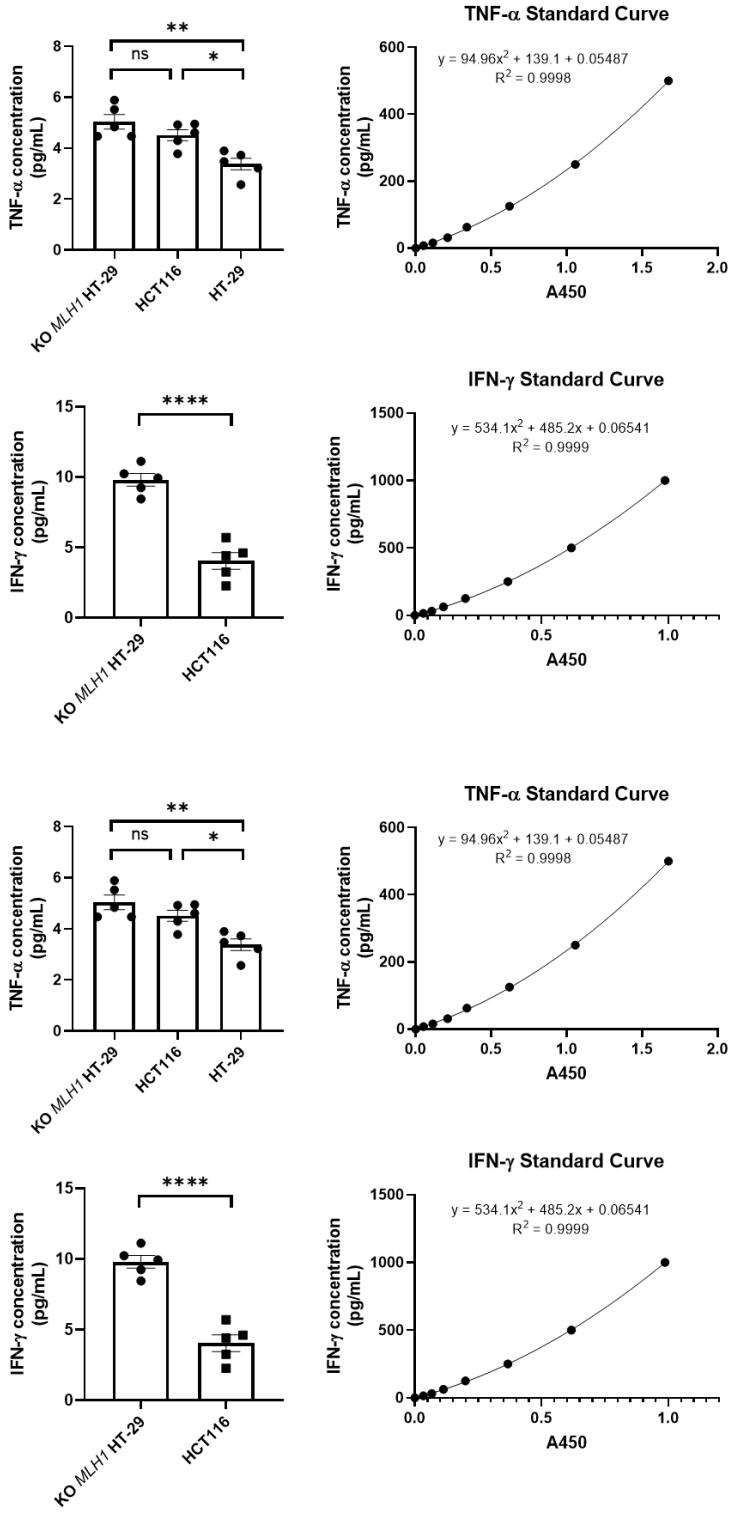
Supplementary Table 1. (continued)

Studies involved	Acute Myeloid Leukemia Adrenocortical Carcinoma Bladder Urothelial Carcinoma Brain Lower Grade Glioma Breast Invasive Carcinoma Cervical Squamous Cell Carcinoma Cholangiocarcinoma Colorectal Adenocarcinoma Diffuse Large B-Cell Lymphoma Esophageal Adenocarcinoma Glioblastoma Multiforme Head and Neck Squamous Cell Carcinoma Kidney Chromophobe Kidney Renal Clear Cell Carcinoma Kidney Renal Papillary Cell Carcinoma Liver Hepatocellular Carcinoma Lung Adenocarcinoma Lung Squamous Cell Carcinoma Mesothelioma Ovarian Serous Cystadenocarcinoma Pancreatic Adenocarcinoma Pheochromocytoma and Paraganglioma Prostate Adenocarcinoma Sarcoma Skin Cutaneous Melanoma Stomach Adenocarcinoma Testicular Germ Cell Tumors Thymoma Thyroid Carcinoma Uterine Carcinosarcoma Uterine Corpus Endometrial Carcinoma Uveal Melanoma	Colorectal Adenocarcinoma (DFCI, Cell Reports 2016) Colorectal Adenocarcinoma (Genentech, Nature 2012) Colorectal Adenocarcinoma (TCGA, PanCancer Atlas) Colorectal Adenocarcinoma Triplets (MSK, Genome Biol 2014) Colorectal Cancer (CAS Shanghai, Cancer Cell 2020) Colorectal Cancer (MSK, Cancer Discovery 2022) Colorectal Cancer (MSK, JCO Precis Oncol 2022) Colorectal Cancer (MSK, Gastroenterology 2020) Disparities in metastatic colorectal cancer between Africans and Americans (MSK, 2020) Metastatic Colorectal Cancer (MSK, Cancer Cell 2018) Rectal Cancer (MSK,Nature Medicine 2019) Colon Cancer (Sidra-LUMC AC-ICAM, Nat Med 2023) Colon Cancer (CPTAC-2 Prospective, Cell 2019) Colon Adenocarcinoma (CaseCCC, PNAS 2015) Colorectal Cancer Radiation (MSK, 2024) Rectal Cancer (MSK, Nature Medicine 2022) Colorectal Cancer (MSK, JNCI 2021) Colorectal Adenocarcinoma (MSK, Nat Commun 2022) Colorectal Adenocarcinoma (DFCI/Orion, 2024)



Supplementary Figure 1. BCA standard curve for MLH1 Western Blotting

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Supplementary Figure 2. TNF-α and IFN-γ cytokine standard curve fitting for ELISA