Targeting NANOG/HDAC1 axis reverses resistance to PD-1 blockade by reinvigorating anti-tumor immunity cycle

Se Jin Oh, … , Marcus W. Bosenberg, Tae Woo Kim

*J Clin Invest.* 2022. [https://doi.org/10.1172/JCI147908](https://doi.org/10.1172/JCI147908).

**Graphical abstract**

![Graphical abstract image](image-url)

Find the latest version:

[https://jci.me/147908/pdf](https://jci.me/147908/pdf)
RESEARCH ARTICLE

Targeting NANOG/HDAC1 axis reverses resistance to PD-1 blockade by reinvigorating anti-tumor immunity cycle

Se Jin Oh¹,²,†, Hyo-Jung Lee¹,²,†, Kwon-Ho Song³, Suyeon Kim¹,², Eunho Cho¹,², Jaeyoon Lee⁴, Marcus W Bosenberg⁵,⁶, and Tae Woo Kim¹,²,⁷,*

¹ BK21 Graduate Program, Department of Biomedical Sciences, Korea University College of Medicine, Seoul 02841, Republic of Korea
² Department of Biochemistry and Molecular Biology, Korea University College of Medicine, Seoul 02841, Republic of Korea
³ Department of Cell Biology, Daegu Catholic University School of Medicine, Daegu 42472, Republic of Korea
⁴ College of Science, College of Social Sciences and Humanities, Northeastern University, Boston, MA, USA
⁵ Department of Pathology, Yale University School of Medicine, New Haven, CT 06510, USA
⁶ Department of Dermatology, Yale University School of Medicine, New Haven, CT 06510, USA
⁷ NEX-I Inc., Seoul 05854, Republic of Korea
† These authors contributed equally to this work.

* Correspondence to: Tae Woo Kim, Ph.D.
Laboratory of Tumor Immunology, Room 319, Moonsook Medical Hall, Korea University College of Medicine, 73 Goryeodae-ro, Sungbuk-gu, Seoul, 02841 Republic of Korea.
Tel: +82 2 2286 1301; Fax: +82 2 923 0480; E-mail: twkim0421@korea.ac.kr

Conflict of interest statement: The authors have declared that no conflicts of interest exist.
Abstract

Immune checkpoint blockade (ICB) therapy has shifted the paradigm for cancer treatment. However, the majority of patients lack effective responses due to the emergence of immune-refractory tumors that disrupt the amplification of anti-tumor immunity. Therefore, identifying clinically available targets that restrict anti-tumor immunity is required to develop potential combination strategies. Here, using the transcriptome data of cancer patients treated with programmed cell death protein-1 (PD-1) therapy, and newly-established mouse preclinical anti-PD-1 therapy-refractory models, we identified NANOG as a novel factor restricting the amplification of anti-tumor immunity cycle, thereby contributing to the immune-refractory feature of the tumor microenvironment (TME). Mechanistically, NANOG induced insufficient T cell infiltration and resistance to CTL-mediated killing via the HDAC1-dependent regulation of CXCL10 and MCL1, respectively. Importantly, HDAC1 inhibition using an actionable agent sensitized NANOG\textsuperscript{high} immune-refractory tumors to PD-1 blockade by reinvigorating the anti-tumor immunity cycle. Thus, our findings implicate the NANOG/HDAC axis as a central molecular target for controlling immune-refractory tumors and provide a rationale for combining HDAC inhibitors to reverse the refractoriness of tumors to ICB therapy.
Introduction

Immune checkpoint blockade (ICB) therapy elicits a marked clinical response in patients with various tumor types, changing the paradigm for cancer treatment (1-3). However, despite the developing field’s potential to revolutionize cancer treatment, the emergence of immune-refractory tumors has limited its clinical success (4, 5). Among the diverse causes of emergence of immune-refractory tumors, the cancer immunoediting theory has attracted attention as it can explain the emergence of tumors refractory to anti-tumor immunity (6). Indeed, previous studies have provided evidence that cancer immunoediting triggered the adaptation of tumor cells to host immune system, thereby contributing to the generation of cancer cells with better survival advantages (7, 8). In this regard, we had found that immune selection by immunotherapy, such as vaccination or adoptive T cell transfer (ACT), drives the evolution of tumors toward immune-refractory states, such as resistance of tumor cells to T cell-mediated killing (9-12). Interestingly, the immune-refractory tumors exhibited resistance to T cell-mediated killing as well as restricted host anti-tumor immunity (13). Thus, understanding the molecular mechanism that disrupts anti-tumor immunity could present potential targets for overcoming clinical limitations to ICB therapy.

Theoretically, the immune system should be capable of eradicating tumor cells through an acquired immune response executed by tumor-reactive CD8+ T cells. A series of stepwise events, called the anti-tumor immunity cycle, is required for tumor cell clearance by the immune system (14). Notably, the dysfunction of single or multiple steps in the anti-tumor immunity cycle is observed in many patients that do not respond to ICB therapy (14, 15), suggesting that the blockade of any steps of the anti-tumor immunity cycle could be a crucial obstacle resulting in resistance to ICB therapy. Thus, it is important to identify the cause of blockade of the anti-tumor immunity cycle to overcome the resistance to ICB therapy.

Accumulating evidence has indicated that the oncogenic pathways of tumors not only promoted tumorigenesis but also interfered with the processes essential for an effective anti-tumor immunity, such as T cell trafficking to tumors and T cell-mediated killing of tumor cells (16-19). For example, hyperactivation of AKT signaling by phosphatase and tensin homolog (PTEN) loss impedes the trafficking of effector T cells to tumors, reduces the sensitivity of melanoma cells to T cell-mediated killing, and is correlated with inferior outcomes of patients treated with ICB (19, 20). In addition, several
studies have revealed that tumors commonly hijack various epigenetic mechanisms to escape immune restriction (21). Among these epigenetic regulators, HDACs have been found to regulate a variety of effects in the T cell-mediated anti-tumor response. For example, dysregulated HDACs in tumors not only decreased T cell trafficking to tumors but inhibited cell death in response to T cell-mediated killing (22). Notably, HDAC inhibition using pharmacological agents increased the levels of T cell chemoattractants and tumor infiltration into multiple lung adenocarcinomas, with a correlated sensitization to anti-PD-1 therapy. Building on these preclinical studies, HDAC inhibition in combination with ICB therapy is currently being explored in multiple clinical trials (23). For instance, promising results have been obtained by combining HDAC1 inhibitor and anti-PD-1 in patients with metastatic uveal melanoma (NCT02697630) or advanced/metastatic non-small cell lung cancer (NCT02638090) (24). These results indicate that targeting oncogenic pathways that restrict the anti-tumor immunity cycle could be a potential strategy to overcoming the resistance to ICB therapy. Thus, discovering an oncogenic pathway that can be targeted by clinically available drugs is needed to develop therapeutic strategies to overcome the clinical limitations of ICB therapy.

The pluripotency transcription factor NANOG, known as a key regulator of embryonic development and cellular reprogramming, has been reported to be broadly expressed in human cancers (25). Functional studies have provided strong evidence that NANOG plays a vital role in malignant disease, correlating with various malevolent properties such as tumorigenicity, self-renewal, invasiveness, and multi-modal therapeutic resistance (26, 27). Furthermore, we identified NANOG as a key intrinsic factor that could induce resistance to CTL-mediated immunotherapy, and NANOG-mediated therapeutic resistance is dependent on HDAC1 (28). Notably, the inhibition of the NANOG/HDAC1 axis reversed the resistance to CTL-based immunotherapy in tumor cells and led to long-term control of the disease (28). These findings suggest that the strategies impeding the NANOG/HDAC1 axis may help conquer the clinical limitations of ICB therapy. However, despite the relevance of NANOG expression in ICB therapy-refractory cancer, the precise mechanism by which NANOG could trigger resistance to ICB therapy, especially through HDAC1-mediated epigenetic reprogramming, is not well understood.

Here, we integrate analyses of human cancer patients from The Cancer Genome Atlas (TCGA) cohort and newly-established mouse preclinical anti-PD-1 therapy-refractory models (CT26 P3 or
YUMM2.1 P3) and find that elevated NANOG expression in tumors could reprogram the TME into one that is immunologically non-responsive to tumors. Our data indicate that NANOG induction in tumor cells switches the immune phenotypes in the TME from immune-stimulatory to immune-refractory feature by blocking the anti-tumor immunity cycle via restriction of T cell trafficking to tumors and tumor cell death to CTL-mediated killing, thereby driving resistance to PD-1 blockade. Importantly, the phenotype of these cells is critically dependent on HDAC1, the main intermediator of the NANOG-mediated regulation of multiple gene expression. Thus, we provide proof-of-concept evidence that HDAC1 inhibition by pharmacological interventions could suppress tumor progression and sensitize NANOG$^{\text{high}}$ refractory tumors to anti-PD-1 therapy.
Results

**NANOG is associated with immune-refractory feature of the TME in cancer patients**

Previously, we defined a *NANOG* signature to acquire a more reliable readout indicating *NANOG* expression in tumor cells (13). To understand the underlying mechanism of *NANOG*-driven resistance to PD-1 blockade, we first assessed the transcriptomes of TCGA melanoma patients with varying *NANOG* signature expression. We analyzed the top 600 differentially expressed genes in *NANOG*<sup>high</sup> versus *NANOG*<sup>low</sup> patients (adjusted *P* < 0.001) (Supplemental Table 1) and found that the *NANOG* signature was inversely associated with genes controlling IFN-γ secretion, T cell proliferation, and infiltration in the TME (Figure 1, A and B). Either high or low T cell anti-tumor immune responses could be predicted by determining the expression signature scores of seven gene sets that have been reported as indicators of increased T cell infiltration and anti-tumor response (hereafter referred to as anti-tumor immune states) (29-32). Interestingly, we found that the *NANOG* signature was inversely associated with the gene signature that represents T cell infiltration and anti-tumor response (Figure 1, C and D). These results indicate that elevated *NANOG* expression in melanoma cells could shape the TME into expressing immune-refractory feature, such as insufficient T cell trafficking to tumors and resistance of tumor cells to T cell-mediated killing. We next examined the clinical relevance of the *NANOG* in TCGA patients and found that Kaplan-Meier plots indicated that patients with high *NANOG* signature levels displayed significantly worse overall survival rates (Figure 1E). Together, our results suggest that elevated *NANOG* expression in tumors could result in restrained anti-tumor immunity in the TME as well as poor clinical outcomes.

We previously reported that the *NANOG* could contribute to poor response to anti-PD-1 therapy (13). Based on our observations, we further questioned whether *NANOG* confers a poor response to anti-PD-1 therapy by inducing immune-refractory features in the TME. By analyzing *NANOG* and the anti-tumor immune state signature in transcriptome data from melanoma patients classified as responders (R) or non-responders (NR) to anti-PD-1 treatment (33), we found that the anti-tumor immune states signature scores were significantly lower in NR compared to R (Supplemental Figure 1). We also found a strong negative correlation between *NANOG* signature and anti-tumor immune state expression in the NR (Figure 1, F and G). Furthermore, above results were reproduced in two independent datasets of melanoma patients treated with anti-PD-1, including Gide et al., (34)
and Liu et al., (35) cohort (Supplemental Figure 2). Notably, NANOG signature was inversely associated with anti-tumor immune state signature in multiple tumor types, such as lung squamous cell carcinoma (LUSC), mesothelioma (MESO), rectum adenocarcinoma (READ), sarcoma (SARC), testicular germ cell tumors (TGCT), head and neck squamous cell carcinoma (HNSC), kidney renal papillary cell carcinoma (KIRP), esophageal carcinoma (ESCA), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), pancreatic adenocarcinoma (PAAD), cervical and endocervical cancers (CESC), uterine corpus endometrial carcinoma (UCEC), Ovarian serous cystadenocarcinoma (OV), stomach and esophageal carcinoma (STES), colon adenocarcinoma (COAD), kidney renal clear cell carcinoma (KIRC), glioma (GMBLGG) or thyroid carcinoma (THCA) (Figure 1H). Taken together, our results indicate that NANOG is associated with immune-refractory feature of the TME, including non-T cell inflamed tumors and resistance to CTL-mediated killing, suggesting that NANOG may drive resistance to ICB therapy by inducing immune-refractory feature in the TME.

**ICB therapy-refractory tumor model displays immune-refractory feature in the TME**

To understand the underlying mechanisms responsible for the NANOG-driven immune-refractory feature of the TME to ICB therapy, we newly established anti-PD-1 therapy-refractory tumor models from a CT26 cell line (designated CT26 P0 cells) or YUMM2.1 cell line (designated YUMM2.1 P0 cells), which has been frequently used as a preclinical tumor model for study of cancer biology and tumor immunology as well as evaluating the efficacy of ICB therapy (36-39), by three rounds of in vivo selection following PD-1 blockade (Supplemental Figure 3). These cells treated with anti-PD-1 were termed P3, whereas those treated with IgG as a negative control were termed N3. To validate our model, we treated tumor-bearing mice with anti-PD-1 (Supplemental Figure 4). Anti-PD-1 treatment showed a remarkable therapeutic effect in CT26 P0 or CT26 N3 tumor-bearing mice (Figure 2, A and B). In contrast, in CT26 P3 tumor-bearing mice, anti-PD-1 treatment had no effect on tumor growth (Figure 2, A and B). Consistently, YUMM2.1 P3 cells displayed resistance to anti-PD-1 treatment (Supplemental Figure 5A). Thus, our data indicate that the resistance to anti-PD-1 therapy seen in patients is conserved in our ICB therapy-refractory tumor models.
Response to ICB therapy in patients is determined by immune feature in the TME that are regulated by anti-tumor immunity, such as T cell trafficking to tumors and T cell-mediated killing of tumor cells (known as the anti-tumor immunity cycle) (14). To investigate whether immune-refractory tumors displayed immune-refractory feature in the TME, we performed a multiplex immunohistochemistry (mIHC) with computational image processing workflows. Compared to CT26 P0 tumors, CT26 P3 tumors exhibited decreased CD8+ T cell infiltration and apoptotic tumor cells death under anti-PD-1 treatment (Figure 2, C to E). Consistently, flow cytometry analysis revealed that the number of overall CD8+ T cells, tumor-reactive CD8+ T cells making granzyme B, and the percentage of apoptotic tumor cells were significantly lower in the CT26 P3 compared to CT26 P0 (Figure 2, F to H). Collinearly, YUMM2.1 P3 cells showed decreased the number of overall CD8+ T cells, tumor-reactive CD8+ T cells, and increased the percentage of apoptotic tumor cells compared to YUMM2.1 P0 cells (Supplemental Figure 5, B to D). These data clearly demonstrate that P3 tumors display the immune-refractory feature of the TME, which is the key cause of resistance to anti-PD-1 therapy in clinical settings.

Some of the mechanisms controlling TME resistant to anti-tumor immunity to anti-PD-1 therapy include down-regulation or loss of expression of MHC I molecules, which could account for decreased CTLs recognition of tumor cells (40). Therefore, we assessed MHC class I (H2-Kd, Dd) expression and found that MHC class I expression in CT26 P0 and P3 cells was virtually identical, regardless of immune selection of tumor cells (Supplemental Figure 6A). We next tested the possibility that immune selection imposed by anti-PD-1 therapy may alter the function of antigen-specific CTLs. We found that there was no difference between P0 and P3 cells in CTL effector cytokine (IFN-γ) production of AH1-specific CTLs mixed with AH1-epitopic peptide-loaded tumor cells between P0 and P3 cells, suggesting that P0 and P3 cells had similar T cell activation capability, and potentials to be recognized by the CTLs (Supplemental Figure 6B). Instead, CT26 P3 cells appeared more resistant to lysis by the CTLs or granzyme B than CT26 P0 cells (Supplemental Figure 7, A and B). Therefore, our data suggest that anti-PD-1-mediated immune selection facilitates the enrichment of a subset of tumor cells with increased resistance of tumor cells to CTL killing rather than CTL recognition or activation.

Accumulating evidence suggests that tumor cell death in cancer treatment can lead to the release of tumor antigens that subsequently prime the auto-loop of anti-tumor immune response (41-44). We next investigated the generation of antigen-specific T cells in CT26 P0 and P3 cells. We isolated
cells from the spleens of mice that received anti-PD-1 therapy, and assessed tumor antigen (AH1⁺)-specific CD8⁺ T cells. Notably, tumor antigen-specific CD8⁺ T cells were decreased in CT26 P3-bearing mice compared to CT26 P0-bearing mice (Figure 2I), suggesting that decreased generation of antigen-specific T cells could be due to the resistance of CT26 P3 cells to CTL-mediated killing. Taken together, our results indicate that CT26 P3 cells could display immune-refractory feature of the TME observed in a clinical practice by restricting self-amplifying anti-tumor immunity to anti-PD-1 therapy.

**NANOG expression in tumor cells determines response to anti-PD-1 therapy by altering immune feature of the TME**

Based on our observations, we reasoned that differences in immune feature of the TME may be due to their dissimilarities in oncogenic pathway mediated by an intrinsic factor of tumor cells. We previously reported that NANOG, a crucial intrinsic factor, may be a potential target to overcome the resistance to anti-PD-1 therapy (13). To characterize the role of NANOG in immune-refractory feature of the TME in an ICB therapy-refractory tumor model, we first assessed the expression of NANOG in CT26 cells at different rounds of immune selection (P0 to P3) and found a stepwise increase in NANOG expression from P0 to P3 (Figure 3A). The overall increase in NANOG expression in the CT26 P3 cell line was likely due to enrichment of NANOG⁺ cells, as opposed to upregulation of NANOG expression itself, as the frequency of NANOG⁺ cells rose from around 11% in the CT26 P0 cell line to around 96% in the CT26 P3 cell line (Figure 3B). In contrast, there was no significant increase in NANOG levels in tumor cells under selection with IgG (N1 to N3; Figure 3, A and B). Consistently, NANOG expression was increased in other ICB therapy-refractory tumor model (YUMM2.1 P3 cells) (Supplemental Figure 8). Thus, our data indicate that anti-PD-1-mediated immune selection depletes tumor cells lacking NANOG while enriching tumor cells contacting NANOG, suggesting that NANOG expression in tumor cells could confer a survival advantage under the immune selection pressure imposed by anti-PD-1 therapy.

To further investigate whether NANOG expression in tumor cells modulates immune feature of the TME, we treated CT26 P3 tumor-bearing mice with intravenously administered chitosan nanoparticles carrying Nanog- or GFP-targeting siRNA along with anti-PD-1 (Supplemental Figure 9 and 10). We found that IgG- and anti-PD-1-treated CT26 P3 tumors displayed similar growth rates under
control siGFP treatment. However, siNanog treatment suppressed the growth of IgG- and anti-PD-1-treated CT26 P3 tumors. Of note, combined treatment with anti-PD-1 and siNanog drastically retarded tumor growth (Figure 3, C and D). Notably, we found that knockdown of NANOG significantly promoted the number of overall tumor-infiltrating CD8+ T cells (TILs) or tumor-reactive CD 8+ TILs in tumors treated with anti-PD-1 (Figure 3, E and F). Many reports suggested that elevated infiltration of tumor-reactive CD8+ T cells could lead to tumor cell death that subsequently prime the auto-loop of anti-tumor immune response (14). Consistently, we found that the percentage of apoptotic tumor cells was increased by combined treatment compared to either single treatment alone (Figure 3G). Indeed, the generation of tumor antigen-specific CD8+ T cells was elevated in the combination treatment group compared to the other treatment groups (Figure 3H). Conversely, the Nanog gene-transduced CT26 P0 cells phenocopied the CT26 P3 cells, displaying immune-refractory feature in the TME (Supplemental Figure 11, A-H). Together, these results indicate that NANOG expression in tumor cells could switch the immune phenotypes of the TME from immune-stimulatory to immune-refractory feature by controlling both T cell infiltration to tumors and cell death of tumor cells to CTL-mediated killing. Therefore, therapeutic strategies to target NANOG could reverse immune-refractory feature of the TME, thereby improving the efficacy of ICB therapy.

**NANOG blocks CD8+ T cell infiltration in tumors through HDAC1-mediated epigenetic repression of CXCL10 expression**

We next attempted to elucidate the underlying mechanism responsible for immune-refractory feature of the TME driven by NANOG. Previous studies indicated that CD8+ T cell infiltration into tumors was required to initiate the subsequent auto-loop of anti-tumor immunity and was also regulated by special chemokines, such as CXCL9 and CXCL10 (45-48). Interestingly, we found that the NANOG signature was inversely associated with the T cell infiltration signature in multiple tumor types (Figure 4A). Among T cell recruitment-related chemokine genes, we found that the NANOG signature was inversely associated with CXCL10 transcripts, but not other chemokines (Figure 4B). These results suggest that NANOG could impair T cell recruitment to tumors by repressing T cell-recruiting chemokine CXCL10 in the TME.
As NANOG regulates multiple gene expression programs, we questioned whether NANOG regulates CXCL10 expression through its transcriptional function. To assess this, we used a mutant form of Nanog (Nanog MT) that was previously found to have weak transcriptional activity (10). When we transfected CT26 P0 cells with wild-type Nanog (Nanog WT), CXCL10 mRNA and protein expression levels were reduced, while the transfection of Nanog MT had no significant impact on CXCL10 expression (Figure 4, C and D), indicating that NANOG-mediated CXCL10 regulation is dependent on the transcriptional activity of NANOG. Previously, it was demonstrated that histone deacetylase 1 (HDAC1) is a key element in NANOG-mediated transcriptional repression (28), and HDAC-mediated histone deacetylation is involved in the increase of CXCL10 expression (49, 50). Hence, we asked whether HDAC1 is a crucial inter-mediator in CXCL10 down-regulation mediated by NANOG. To address this, we measured the levels of CXCL10 protein and mRNA in CT26-no insert-siGFP, CT26-Nanog-siGFP, and CT26-Nanog-siHdac1 cells (Supplemental Figure 12). We found that CXCL10 protein and transcript were significantly decreased, and these decreased levels of CXCL10 mediated by NANOG were reversed upon HDAC1 silencing (Figure 4, E and F). Because NANOG caused the transcriptional repression of CXCL10 in an HDAC1-dependent manner, we reasoned that decreased expression of CXCL10 may be due to HDAC1-mediated epigenetic silencing. We previously demonstrated that NANOG caused a decrease in AcH3K14 and AcH3K27 through transcriptional activation of HDAC1 (28). Notably, ChIP-qPCR showed that NANOG expression caused loss of AcH3K14 and AcH3K27 occupancy on the promoter region of Cxcl10, and these histone modification events were reversed by HDAC1 knockdown (Figure 4G). Consistent with these results, HDAC1 was more enriched in the Cxcl10 promoters in CT26-Nanog-siGFP cells compared to CT26-no insert-siGFP cells (Figure 4G). These results demonstrate that NANOG could downregulate the expression of CXCL10 gene via HDAC1-mediated epigenetic silencing.

To assess the potential role of CXCL10 in the NANOG-mediated non-T cell-inflamed immune phenotype in the TME, antibody-mediated neutralization of CXCL10 was performed in CT26-Nanog-siGFP or CT26-Nanog-siHdac1 cells. We found that elevated T cell infiltration mediated by HDAC1 knockdown was completely reversed upon neutralization of CXCL10 (Figure 4H), suggesting that the NANOG/HDAC1 axis induces the non-T cell-inflamed immune phenotype in the TME in a CXCL10-dependent manner. We next questioned whether elevated T cell trafficking could overcome NANOG-
driven resistance to ICB therapy. To estimate this, we used a wild-type Cxcl10 (Cxcl10 WT) or a mutant form of Cxcl10 (Cxcl10 MT) that was previously found to have non-secretory properties (Figure 4I) (51). Whereas the addition of Cxcl10 WT sensitized CT26-Nanog tumors to PD-1 blockade and accelerated the overall CD8+ T cell or tumor-reactive CD8+ cell recruitment, Cxcl10 MT had no impact on these parameters (Figure 4, J to L). Although we observed the significant suppression in tumor growth in the tumors of NANO-G-Cxcl10 WT-bearing mice compared to the other groups, these tumors continued to grow (Figure 4J). Therefore, these results indicate that NANOG induces immune-refractory feature in the TME by controlling not only T cell infiltration but also other steps of the anti-tumor immunity cycle.

**Resistance of tumor cells to T cell-mediated killing by NANOG/HDAC1 axis is a crucial cause to induce immune-refractory feature in the TME**

Previous reports have indicated that tumor cell death by T cells was essential for reinvigorating the anti-tumor immunity cycle, providing tumor antigens stimuli to CTLs (41, 42, 52, 53), suggesting that the resistance of tumor cells to CTLs is a crucial obstacle to improving ICB therapy. In this regard, NANOG was also reported to induce resistance to CTL-mediated killing via HDAC1-mediated anti-apoptotic protein MCL1 upregulation (28). Hence, we questioned whether the resistance to T cell-mediated killing induced by NANOG/HDAC1/MCL1 axis is one of crucial causes to trigger immune-refractory feature in the TME by blocking anti-tumor immunity cycle. To address this, we first assessed the level of MCL1 expression in CT26-no insert-siGFP, CT26-Nanog-siGFP, and CT26-Nanog-siHdac1 cells. MCL1 protein was significantly increased, and these increased levels of MCL1 mediated by NANOG were reversed upon HDAC1 silencing (Figure 5A). Consistent with the above results, we found that decreased CTL-mediated cell death by NANOG overexpression was reversed upon HDAC1 silencing (Figure 5B). Furthermore, MCL1 silencing reversed resistant phenotypes against the cognate CTLs of CT26-Nanog cells (Supplemental Figure 13 and Figure 5, C and D), suggesting a crucial role of MCL1 in the tumor-intrinsic resistance to CTL induced by NANOG. Next, we treated CT26-Nanog tumor-bearing mice with intravenously administered chitosan nanoparticles carrying Mcl1- or GFP-targeting siRNA along with anti-PD-1, as illustrated in Supplemental Figure 14. We found that IgG- and anti-PD-1-treated CT26-Nanog tumors displayed similar growth rates under control siGFP treatment (Figure 5, E and F). However, siMcl1 treatment suppressed the growth of IgG and anti-PD-1 treated
CT26-Nanog tumors. Of note, combined treatment with anti-PD-1 and siMcl1 drastically retarded tumor growth (Figure 5, E and F). In addition, we found that the percentage of apoptotic tumor cells was drastically increased by the combination treatment compared to treatment with either agent alone (Figure 5G). To test whether the generation of tumor-reactive T cells was affected by dual treatment, we isolated cells from the spleens of mice that received therapy and then assessed CT26-specific antigen AH1-tet-positive CD8+ T cells. Notably, AH1-tet-positive CD8+ T cells were increased in the combined treatment group compared to the other treatment groups (Figure 5H). However, similar to Figure 4J, the combination treatment did not completely suppress tumor growth. Taken together, our data suggest that to completely overcome the resistance to anti-PD-1 therapy, the NANOG-mediated restriction mechanisms of the anti-tumor immunity cycle should be simultaneously blocked.

The NANOG/HDAC1 axis controls T cell trafficking and resistance to CTL-mediated killing in multiple types of NANOG<sup>high</sup> tumor cells

To verify the functional effects of the NANOG/HDAC1 axis in diverse types of mouse or human cancer cells, we further selected three NANOG upregulated cancer cells B16F10, 526mel, and H1299, and three NANOG<sup>high</sup> immunotherapeutic-refractory tumor models, MDA-MB-231 P3, CT26 P3, and YUMM2.1 P3, respectively (28). Knockdown of NANOG or HDAC1 with siRNA robustly dampened expression of the effectors of NANOG signaling, such as CXCL10 and MCL1, across all tested cancer cells (Figure 6A and Supplemental Figure 15). Furthermore, siNanog-, siNANOG, siHdac1, or siHDAC1-treated tumor cells showed increased T cell infiltration and were more susceptible to CTL-mediated killing compared to siGFP-treated tumor cells (Figure 6, B and C). These results demonstrate that the functional properties of the NANOG/HDAC1 axis are conserved across multiple types of cancer cells and that HDAC1 is a proper target for controlling immune-refractory NANOG<sup>high</sup> tumor cells.

HDAC1 inhibition sensitizes NANOG<sup>high</sup> refractory tumor cells to anti-PD-1 therapy by reinvigorating the anti-tumor immunity cycle

Although the data presented in this study demonstrate that targeting the NANOG/HDAC1 axis could be a promising approach for reversing immune-refractory feature of the TME by simultaneously
reinvigorating multiple steps of the anti-tumor immunity cycle, pharmacologic inhibitors of NANOG are yet to be developed. However, small-molecule inhibitors of HDAC1, such as FK228 (Romidepsin), MS-275 (Entinostat) or MGCD0103 (Mocetinostat), have been used for treating cancer patients (54-57). Therefore, to address the clinical applicability of HDAC1 inhibitors for controlling NANOG<sup>high</sup> refractory cancer, we first measured the viability of CT26 P0, CT26 P3, or CT26 P3-siNANOG cells after in vitro treatment with FK228, MS-275, or MGCD0103. We also used cisplatin as a control for NANOG/HDAC1 axis-independent cancer drug. We found that CT26 P3 cells were more susceptible to HDAC1 inhibitors than CT26 P0 cells, and, conversely, HDAC1 inhibitor sensitivity in CT26 P3 cells was reduced by knockdown of NANOG (Figure 7A). In contrast, the conventional drug cisplatin displayed the opposite phenomena (Figure 7A). Notably, among various HDAC1 inhibitors, FK228 was the most effective in CT26 P3 cells. We next assessed the expression of effectors for the NANOG axis-mediated resistance to anti-PD-1 therapy. We found that HDAC1 inhibition by FK228 robustly dampened expression of the effectors of the resistance to anti-PD-1 therapy induced by the NANOG axis (Figure 7B and Supplemental Figure 16). These results suggest the possibility of that targeting HDAC1 could reverse the NANOG-mediated refractoriness to anti-PD-1 therapy.

To evaluate the preclinical therapeutic value of inhibiting HDAC1 and its downstream molecular axis, the efficacy of anti-PD-1 therapy was tested in BALB/c mice bearing CT26 P3 tumors. The mice received anti-PD-1 with FK228, according to the schedule described in Supplemental Figure 17. Tumors excised on day 20 were substantially smaller in size and tumor burden in the mice treated with both anti-PD-1 and FK228 compared to mice treated with either agent alone (Figure 7, C and D). Importantly, 90% of the mice treated with both anti-PD-1 and FK228 survived, even at 50 days after tumor challenge. In contrast, all animals in the other groups had died by then (Figure 7E). These results suggest that targeting HDAC1 could successfully reverse the NANOG-mediated resistance to anti-PD-1.

We next assessed whether HDAC1 inhibition could switch the immune phenotype in the TME from immune-refractory to immune-stimulatory feature by reinvigorating the anti-tumor immunity cycle in NANOG<sup>high</sup>-refractory tumor cells. We found that the number of overall CD8<sup>+</sup> T cells and tumor-reactive CD8<sup>+</sup> T cells making granzyme B were significantly higher in the combination treatment group compared to the other treatment groups (Figure 7, F and G). Consistently, the percentage of apoptotic
tumor cells and generation of tumor-specific CD8\(^+\) T cells were increased by the combined treatment compared to either treatment alone (Figure 7, H and I). Moreover, above results were reproduced in the YUMM2.1 P3 tumor-bearing mice (Supplemental Figure 18). Taken together, our results demonstrate that HDAC1 inhibition switches the immune phenotypes from immune-refractory to immune-stimulatory feature by simultaneously reversing NANOG-mediated immune-refractory states, thereby overcoming the resistance to PD-1 blockade.
Discussion

The cancer immunoediting process occurs during the natural progression of tumors, but the available evidence from studies of patients treated with cancer immunotherapies indicates that this process reoccurs, either in part or in its entirety in response to treatment (7). Previous studies reported that immunoediting occurred not only close to the presence of tumors refractory to various clinical interventions, including immunotherapy, but contributed to the generation of cancer cells with better survival advantages (7, 8). Notably, preferential selection and subsequent expansion of a subset of tumors by immunoediting leads to the evolution of tumors toward immune-refractory states (6-8). In this study, we showed that the immune-refractory states of tumor cells over the course of immunoediting process are closely linked to immune-refractory feature of the TME accompanied by a simultaneous blockade of multiple steps of the anti-tumor immunity cycle via hyper-activation of the NANOG/HDAC1 axis (Fig. 8a, 8b). Further, to our knowledge, we are the first to report the role of the NANOG/HDAC1 axis in ICB therapy-refractory cancer.

The immunotherapy field has emphasized the targeting inhibitory receptors expressed by immune cells (58). However, recent studies suggested that the engagement of tumor-intrinsic pathways in tumor cells has been suggested as being a critical mechanism by which they restrain immunotherapy-driven anti-tumor immune response (16-19). It is a very important question, whether targeting tumor-intrinsic pathways may be vital to extending the benefits of immunotherapy to a larger patient population, including those who thus far demonstrated immune-refractory cancer. In this study, we demonstrated that NANOG expression in tumor cells provoke the immune-refractory feature in the TME by simultaneously disrupting various steps of the anti-tumor immunity cycle, such as T cell infiltration into tumors and T cell-mediated killing of tumor cells, in an HDAC1-dependent manner. Moreover, the repression of NANOG expression in tumor cells converted the immune-refractory microenvironment into an immune-stimulatory microenvironment that facilitates anti-tumor immunity and overcomes the resistance to anti-PD-1 therapy as shown in Figure 2. Furthermore, repressing NANOG expression may represent a therapeutic strategy for initiating or reinvigorating an anti-tumor response in those patients showing anti-PD-1-therapeutic resistance. Together, our results indicate that NANOG, as a tumor-intrinsic factor, could be a critical determinant that confers resistance to anti-PD-1 therapy by determining the immune feature of the TME.
NANOG also works together with other stemness factors, such as MYC, OCT4, KLF4, SOX2, SOCS2, or ALDH1A1, to control a set of target genes that have important functions in embryonic stem (ES) cells and, plausibly, in tumor cells (59). Interestingly, results from previous studies indicated that these stemness factors including NANOG could induce immune evasion in various cancers by modulating T cell-mediated anti-tumor response (60-62). To test whether other stemness factors may contribute to refractoriness to ICB therapy, we assessed the expression of these stemness factors in transcriptomes of TCGA melanoma patients. Interestingly, not only NANOG but also MYC and SOCS2 were inversely associated with the gene signature that represents T cell infiltration and anti-tumor response in our analysis of the patients (Supplemental Figure 19). These results suggest that, similar to NANOG, elevated MYC or SOCS2 expression in melanoma cells could shape the TME into expressing immune-refractory feature, such as insufficient T cell trafficking to tumors and resistance of tumor cells to T cell-mediated killing. Therefore, it worth understanding the relationship between the stemness factors and the immune-refractory feature of the TME for future studies.

As the NANOG/HDAC1 axis has been implicated as a central channel in the development of resistance to CTL-based immunotherapies, we believe that HDAC1 inhibition may be an effective strategy to control ICB therapy-refractory tumor cells with elevated expressions of NANOG. Indeed, HDAC1 inhibition has received attention for therapeutic purposes in solid tumors and hematologic malignancies, even though HDAC1 inhibitors have shown limited responses as single agents in cancer patients (54, 55). Recently, HDAC1 inhibition in combination with ICB therapy, as a therapeutic strategy aimed at converting non-T cell inflamed into T cell-inflamed tumors, is currently being explored in multiple clinical trials (21, 63-66). Yet, only a minority of patients derives clinical benefit. Thus, it is important to identify the biomarker predicting the responses to HDAC1 inhibitors in combination with ICB therapy. In this study, we found that NANOG is associated with immune-refractory feature of the TME and poor clinical outcomes in cancer patients as well as NANOG expression influences sensitivity of tumor cells to HDAC1 inhibitor. Therefore, these results implicate NANOG can be used as a predictive biomarker that providing a framework for selecting appropriate patients who receive clinical benefit of combinational therapy with HDAC1 inhibitor and ICB therapy.

Altogether, we propose that NANOG+ immune-refractory tumor cells enriched by immune selection drive immune-refractory feature of the TME by simultaneously disrupting multiple steps of the
anti-tumor immunity cycle, which provokes resistance to PD-1 blockade (Figure 8, A and B). In this process, NANOG potentiates resistance to anti-PD-1 therapy via HDAC1-mediated epigenetic reprogramming. Furthermore, we demonstrate that the pharmacological inhibition of the NANOG/HDAC1 axis with FK228 could trigger anti-PD-1 therapy-mediated tumor regression and make immunologically ‘cold’ tumors ‘hot’ by initiating or reinvigorating the anti-tumor immunity cycle. This could help to overcome the local immune-refractory environments as seen in cancer and increase the effectiveness of classical ICB. Therefore, our results provide a strong rationale for the inhibition of HDAC1 as promising strategy, which will be essential to extending the benefit of ICB therapy to larger patient populations, including NANOG+ immune-refractory tumors, particularly in regard to immune-based cancer therapy.
Methods

Mice. Six- to eight-week-old female BALB/c or male C57BL/6 mice were purchased from Central Lab. Animal Inc. (Seoul, KOR). All animal procedures were performed in accordance with recommendations for the proper use and care of laboratory animals.

siRNA constructs. Synthetic small interfering RNAs (siRNAs) were synthesized by Bioneer (Daejeon, KOR): Non-specific GFP (green fluorescent protein), 5’-GCAUCAAGUGAACUCAA-3’ (sense), 5’-UUGAAGUUCACCUCUGAUGG-3’ (antisense); mouse Nanog-#1, 5’-GCCUAGUUCUGGAAAGCAUCGAAU-3’ (sense), 5’-AUUCAUGCUCCUCAGAACUAGGC-3’ (antisense); mouse Nanog-#2, 5’-CCUCAUCUGAACCUGACUUAAGA-3’ (sense), 5’-UUUAGUCAGGGUCAUUGAGG-3’ (antisense); mouse Nanog-#3, 5’-UGAACUGACUUAAGCAGGUAUAA-3’ (sense), 5’-UUUACCUGCUUAUAGCUCAGGUAUCA-3’ (antisense); human NANOG, 5’-GCAACCAGACCUGAACAACG-3’ (sense), 5’-UUUUCAGUGUGCUAGG-3’ (antisense); mouse Hdac1-#1, 5’-GCAUGACUCACAAUUGCUGCUAA-3’ (sense), 5’-UUGAGCGACAAUUGAGUCAUGC-3’ (antisense); mouse Hdac1-#2, 5’-UGUCCGGUGUUUGAUUUGCUUGUCUUAAGGC-3’ (sense), 5’-CAAAAGCCAUCAACCCGACAAA-3’ (antisense); mouse Hdac1-#3, 5’-CCUACCAAGUGACUAACCUUUG-3’ (sense), 5’-AGAAAGCUCACACUUUUGCUUGG-3’ (antisense); human HDAC1, 5’-GAGUUAAACAGAGGAGAUGA-3’ (sense), 5’-UCAUCCUCGUUUUGACUC-3’ (antisense); mouse Mcl1-#1, 5’-GGCAGGAUGACUCUUUUUUC-3’ (sense), 5’-AGAAUUAAGUGACCAACCUUGCC-3’ (antisense); mouse Mcl1-#2, 5’-GGCAGGAUGACUCUUUUUCU-3’ (sense), 5’-AAGAAUUAAGUGACCAAUUGAGACCC-3’ (antisense); and mouse Mcl1-#3, 5’-GCAGGAUGACUCUUUUUUCU-3’ (sense), 5’-AAAGAAUUAAGUGACCAACCUUGGC-3’ (antisense).

Cell lines and reagents. CT26, B16F10, 526mel, H1299, and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were obtained
between 2010 and 2014. YUMM2.1 cell lines were obtained by Marcus W Bosenberg lab. in Yale university (New Haven, CT, USA) at 2021. These cells were tested for mycoplasma using the Mycoplasma Detection Kit (Thermo Fisher Scientific, San Jose, CA, USA). The identities of the cell lines were confirmed by short tandem repeat (STR) profiling by IDEXX Laboratories, Inc., and used within six months. To generate ICB therapeutic-resistant tumor cells, BALB/c or C57BL/6 mice were subcutaneously inoculated with 1 x 10^5 CT26 (CT26 P0) or 1 x 10^6 YUMM2.1 (YUMM2.1 P0) cells per mouse, respectively. Between five and seven days following tumor challenge, the mice were treated anti-PD-1 (200 μg; BioXcell, Lebanon, NH, USA) three times per week. This treatment regimen was repeated for two cycles. The surviving P0 cells were termed P1 cells. This process was repeated for three cycles to derive the P3 line, which was impervious to therapeutic effect by anti-PD-1. As a control, we performed this procedure using IgG antibody in mice inoculated with P0 cells to generate N1, N2, and N3 cells without immune selection. MDA-MB-231 P3 cell lines have been previously described (28).

To generate CT26-NANOG cells or CT26-NANOG-CXCL10 cells, pMSCV-NANOG or pMSCV-CXCL10-GFP plasmids were first transfected along with viral packaging plasmids (VSVG and Gag-pol) into HEK293FT cells. Three days after transfection, the viral supernatant was filtered through a 0.45 μm filter and used to infect into CT26 or CT26-NANOG cells. The infected cells were then selected with 1 μg/mL puromycin (CT26-NANOG) or GFP+ cells sorting using flow cytometry (CT26-NANOG-CXCL10). All cells were grown at 37 °C in a 5% CO2 incubator/humidified chamber. Cisplatin, FK228, MS-275, and MGCD0103 were purchased from Selleckchem (Houston, TX, USA).

DNA constructs. The pMSCV-NANOG WT and MT plasmids have been described previously (9, 10). Briefly, to generate pMSCV/Nanog, DNA fragments encoding Nanog were amplified from pSIN-EF2-Nanog-Pur-expressing cells (Addgene, Watertown, MA, USA) using the primer set: 5’-GCCTCGAGATGAGTGTGGATCCAGCTTG-3’ and 5’-GCGAATTCTCACACGTCTTGAGGTTG-3’.

Amplified DNA was cloned into the XhoII/EcoRI site of the pMSCV retroviral vector (Clontech, Mountain View, CA, USA). To create mutations in the Nanog gene, the QuikChange XL Site-Directed Mutagenesis Kit was used (Stratagene. San Diego, CA, USA). Plasmid integrity was verified by DNA sequencing. pMSCV-CXCL10 WT-GFP and MT-GFP plasmids were purchased from Cosmogenetech (Seoul, KOR).
**Real-time quantitative RT-PCR.** The experimental procedure has been described previously (67). Briefly, total RNA was extracted with the RNeasy Mini Kit (QIAGEN, Hilden, DEU) and treated with DNase (Thermo Fisher Scientific, San Jose, CA, USA). Quantitative reverse transcription PCR (qRT-PCR) mixtures were assembled with 1 μl cDNA template, iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and primers for Cxcl9 or Cxcl10. qPCR primers were purchased from Bioneer (Daejeon, KOR): Cxcl9, 5’-CGAGGCACGATCCACTACAA-3’ (forward), 5’-AGGCAGGGTTGATCTCCGT-3’ (reverse); and Cxcl10, 5’-ATGACGGGCCAGTGAGAATG-3’ (forward), 5’-TCAACAGTGGCAGGATAG-3’ (reverse). PCR was carried out for 40 cycles with the following thermal cycling conditions: 95 °C for 10 seconds (denaturation) and 61 °C for 60 seconds (annealing). All data were normalized to Actb mRNA expression levels.

**Western blot analysis.** Lysate extracted from a total of 1 x 10⁵ cells was used to perform Western blots. Primary antibodies against NANOG (A300-379Am Bethyl Laboratories, Montgomery, TX), FLAG (M185-3L, MBL, Nagoya, Japan), HDAC1 (5356S, Cell Signaling Technology, Danvers, MA, USA), AcH3K14 (#4318P, Cell Signaling Technology), AcH3K27 (#4353P, Cell Signaling Technology), MCL1 (sc-819, Santa Cruz Biotechnology, CA, USA), CXCL10 (551215, BD Biosciences, CA, USA), and β-actin (M177-3, BML, MA, USA) were used. Western blotting was followed by incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase. The immunoreactive bands were developed with the chemiluminescence ECL Detection system (GE Healthcare, Chicago, IL, USA), and signals were detected using a luminescent image analyzer (LAS-4000 Mini, Fujifilm, Japan).

**ChIP and quantitative ChIP (qChIP) assays.** The ChIP kit (Millipore, Burlington, MA, USA) was employed according to the manufacturer’s instructions. Briefly, cells (1 x 10⁷ per assay) were bathed in 1% formaldehyde at 25°C for 10 min to crosslink proteins and DNA and then lysed in sodium dodecyl sulfate buffer containing protease inhibitors. DNA was sheared by sonication using a Sonic Dismembrator Model 500 (Fisher Scientific, Pittsburgh, PA, USA). Immunoprecipitation was carried out by incubation with 1 μg of anti-HDAC1 (5356S, Cell Signaling Technology) antibodies or rabbit IgG (Millipore) for 16 hr. For the qChIP assay, immunoprecipitated DNA was quantified by real-time qPCR.
using the following primer sets: 5’- CACTGTCACCTCTATGCGAGAT-3’ (forward) and 5’- CACTCTGCACAGCACCCAAG-3’ (reverse). Each sample was assayed in triplicate, and the amount of precipitated DNA was calculated as the percentage of the input sample.

**CTL-mediated apoptosis assay.** Tumor cells were labeled with CFSE (10 μM, Molecular Probes, Eugene, OR, USA) in RPMI supplemented with 0.1% fetal bovine serum (FBS). The CFSE-labeled CT26 cells were mixed with cognate AH1-specific CD8+ CTLs at a 1:1 ratio and incubated for 4 h at 37 °C. The cells were stained for active caspase-3 as an index of apoptosis and examined by flow cytometry.

**Granzyme B-mediated apoptosis assay.** Recombinant human granzyme B (Enzo Life Sciences, New York, NY, USA) was mixed with BioPorter Reagent (Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 5 min. The tumor cells were mixed with BioPorter-granzyme B complexes for 4 h at 37 °C. The cells were stained for active caspase-3 as an index of apoptosis and examined by flow cytometry.

**Trypan blue exclusion assay.** To determine cell viability, the trypan blue exclusion assay was performed. Briefly, cells were seeded at 1 x 10^5 cells per well in 12-well plates one days prior to the assay. The treatments were added at the concentrations indicated in the figures. After 24 h, the cells were detached and stained with 0.4% trypan blue. The unstained cells were counted using a hemocytometer. The data are expressed as the percentages of unstained cells compared to the control cells not exposed to the chemical reagents.

**TCGA data collection and analysis.** Gene expression data for more than 10,000 cancer samples profiled by TCGA were collected from the FireHose data repository (https://gdac.broadinstitute.org/). Clinical data were also retrieved from the same source. The T cell infiltration or T cell-mediated anti-tumor response gene expression signature were previously defined (29-32). We used the single-sample Gene Set Enrichment analysis algorithm implemented in R package GSVA, to calculate the T cell
infiltration or anti-tumor response signature scores for each sample. The default parameters from the GSVA package were used. Spearman’s correlation was used to quantify the association between NANOG signature, T cell infiltration, and anti-tumor response scores individually for each tumor type. The association between NANOG signature expression and survival was evaluated by Cox regression and Kaplan-Meier analyses. For the latter, the samples were stratified into three groups according to their NANOG signature expression (low, intermediate, high). The 20th and 80th percentiles were used as cutoff thresholds.

**Tumor treatment experiments.** BALB/c mice were inoculated subcutaneously with 1 x 10^5 CT26 P3 cells per mouse. Seven days following. Seven days following tumor challenge, FK228 (0.05 mg/kg) or PBS was treated via intraperitoneal route. Following day after FK228 treatment, mice administered anti-PD-1 (BioXcell, Lebanon, NH, USA) or isotype antibody control that was administrated every 3 days at a dose of 200 μg per mice in accordance with the schedule described in Supplemental Figure 16. This treatment regimen was repeated for three cycles. Mice were monitored for tumor burden and survival for 20 and 50 days after challenge, respectively.

**Tumor digestion, cell isolation, and flow cytometric analysis.** Treated mice were euthanized on day 18 following tumor inoculation and the tumors were harvested. The tumors were dissected into fragments by cutting and digested by collagenase (0.5 mg/mL, Sigma-Aldrich) and DNase (1 μg/mL, Sigma-Aldrich) at 37°C for 45 min. The digested samples were then filtered through a 70 μm cell strainer and washed with phosphate-buffered saline (PBS) buffer. The cell pellets were then incubated with red blood cell (RBC) lysis buffer to lyse the RBCs. The cell suspensions were stained for the intracellular and extracellular protein markers of interest. The stained samples were assessed on a flow cytometer (BD biosciences) along with CellQuest Pro software. The staining antibodies used were anti-CD3, anti-CD4, anti-CD19, anti-Foxp3, anti-CD8, anti-granzyme B, anti-active caspase 3, and anti-IFN-γ. All antibodies were purchased from BD Biosciences.
Statistical analysis. All data are representative of at least three separate experiments. Statistical differences were calculated by either Student’s t-test (two-tailed, unpaired), one-way ANOVA, or two-way ANOVA using GraphPad Prism software. Spearman’s rank correlation analysis was used to evaluate the association between the indicators. Survival curves were calculated using the Kaplan-Meier method and the differences between the survival curves were calculated by the long-rank test. A Cox proportional hazards model was created to identify the independent predictors of survival. Results with two-tailed p-values of < 0.05 were considered statistically significant.

Data availability. Transcriptome data from melanoma patients classified as responders (R) or non-responders (NR) to anti-PD-1 treatment were deposited into the Gene Expression Omnibus (GEO) under the accession code GSE91061 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE91061] (Riaz et al., cohort), the European Nucleotide Archive (ENA) under accession number PRJEB23709 [https://www.ebi.ac.uk/ena/browser/view/PRJEB23709] (Gide et al., cohort), and dbGaP under the accession code phs000452.v3.p1 [https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000452.v3.p1] (Liu et al., cohort). Transcriptome data from TCGA were deposited into FireHose data repository portal (https://gdac.broadinstitute.org/). The gene ontology (GO) analysis which supported the findings of this study is publicly available online at [https://amp.pharm.mssm.edu/Enrichr/]. The raw images for the immunoblots are provided in the Supplemental Information. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

Study approval. All mice were maintained and handled under the protocol approved by the Korea University Institutional Animal Care and Use Committee (KOREA-2017-0141, Seoul, KOR).

Author contributions
SJO, HJL, KHS, and TWK designed the study. SJO, HJL, KHS, SYK, and EHC conducted the experiments. SJO, HJL, and KHS acquired and analyzed data. MWB provided experimental materials. SJO, JYL and TWK wrote the manuscript. All authors read and approved the final manuscript.
Acknowledgements

This work was funded by the National Research Foundation of Korea (NRF-2021R1C1C2008422 to S.J. Oh, NRF-2021R1C1C2013395 to H.J. Lee, NRF-2019R1A4A1029000 to T.W. Kim, and NRF-2020R1A2B5B03095410 to T.W. Kim) and the Korea Drug Development Fund (HN21C0486 to T.W. Kim). Multiplex immunohistochemistry (mIHC) with computational image processing workflows was supported by the PrismCDX (Hwaseong, KOR).
References


34. Gide TN, Quek C, Menzies AM, Tasker AT, Shang P, Holst J, Madore J, Lim SY, Velickovic R,


64. Setiadi AF, Omilusik K, David MD, Seipp RP, Hartikainen J, Gopaul R, Choi KB, and Jefferies WA. Epigenetic enhancement of antigen processing and presentation promotes immune


Figures and Figure Legends

**Figure 1.** NANOG is inversely associated with anti-tumor immune state of the TME in cancer patients. (A) Expression of top 600 differentially expressed genes in NANOG\textsuperscript{high} versus NANOG\textsuperscript{low} melanoma patients. (B) Gene ontology term enrichment analysis for the top five biological processes controlled by differentially expressed genes among patients with high NANOG signature expression. (C and D) Comparisons of expression levels of T cell infiltration, anti-tumor response, and anti-tumor immune state signatures in NANOG\textsuperscript{low} (n=315) and NANOG\textsuperscript{high} (n=158). (E) Kaplan-Meier analysis of overall survival (calculated as months to death or months to last follow-up) and median cutoffs values for the expression level of NANOG signature (NANOG\textsuperscript{high} > median; NANOG\textsuperscript{low} < median, p = 0.0391). (F) Comparisons of the expression levels of anti-tumor immune state signature in non-responders with
low levels (low, n = 8) and high levels (high, n = 9) of NANOG signature. The 25th and 75th percentiles were used as cutoff thresholds. (G) Pearson’s correlation between expression level of NANOG signature and anti-tumor immune states in non-responders. (H) Correlation plot of NANOG and anti-tumor immune state signature in pan-tumor types. Correlation and two-tailed p-values were assessed using the Pearson’s correlation coefficient and the unpaired t-test. (C, D, and F) The p-values were determined by unpaired, two-tailed Student’s t-test. In the box plots, the top and bottom edges of boxes indicate the first and third quartiles, respectively; the center lines indicate the medians; and the ends of the whiskers indicate the maximum and minimum values, respectively. Source data are provided as a Source Data file. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. NS, not significant)
Figure 2. CT26 P3 cells display the immune-refractory feature of the TME. (A to I) CT26 P0, P3, or N3 tumor-bearing mice treated with IgG or PD-1 antibody. (A) Tumor growth curves and (B) changes in tumor volume 17 days after challenge compared to baseline. (C) FFPE sections of CT26 P0 or P3 tumors treated with IgG or PD-1 antibody were stained with the indicated markers by pseudo-coloring. The indicated markers are shown on the right. (D) The frequency of tumor-infiltrating CD8+ T cells. (E)
The frequency of apoptotic cells in the tumors. (F) Flow cytometry profiles of the tumor-infiltrating CD3+ CD8+ T cells. (G) The ratio of granzyme B+ to tumor-infiltrating CD3+ CD8+ T cells. (H) The frequency of apoptotic cells in the tumors. (I) Quantification of antigen-specific CTLs in spleens derived from the tumor-bearing mice. Ten mice from each group were used for in vivo experiments. The graphs represent three independent experiments performed in triplicate. (D to I) The p-values by one-way ANOVA are indicated. The data represent the mean ± SD. Source data are provided as a Source Data file. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. NS, not significant)
Figure 3. NANOG repression enhances response to anti-PD-1 therapy by inducing immune-stimulatory feature in the TME. (A) Top, quantification of NANOG expression in tumor cells at different stages of immune selection (P0 to P3). Parallel stages without selection are labeled as N1 to N3. Bottom, representative Western blot images. (B) Top, representative images of flow cytometry analysis of NANOG+ tumor cells. Bottom, quantification of the frequency of NANOG+ tumor cells. (C to H) CT26 P3
tumor-bearing mice administered siGFP or siNanog with or without treatment with PD-1 antibody. 

(C) Tumor growth curves and (D) changes in tumor volume 17 days after challenge compared to baseline. 

(E) Flow cytometry profiles of tumor-infiltrating CD3+ CD8+ T cells. (F) The ratio of granzyme B+ to tumor-infiltrating CD3+ CD8+ T cells. (G) The frequency of apoptotic cells in the tumors. (H) Quantification of antigen-specific CTLs in spleens derived from the tumor-bearing mice. Ten mice from each group were used for in vivo experiments. The graphs represent three independent experiments performed in triplicate. The p-values by two-tailed Student’s t-test (A and B) or one-way ANOVA (E to H) are indicated. The data represent the mean ± SD. Source data are provided as a Source Data file. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. NS, not significant)
Figure 4. NANOG blocks CD8+ T cell infiltration through HDAC1-mediated epigenetic repression of CXCL10. (A) Correlation plot of NANOG and T cell infiltration signature in pan-tumor types. Correlation and two-tailed p-values were assessed using the Pearson’s correlation coefficient and unpaired t-test. (B) Pearson’s correlation of NANOG signature expression with indicated transcripts of T cell-recruiting chemokines. RSEM, relative s.e.m (C) Cxcl9 or Cxcl10 mRNA expression was analyzed.
by qRT-PCR. (D) Western blot analysis of the expression of CXCL10. (E to G) CT26-no insert or CT26-Nanog cells were transfected with siGFP or siHdac1. (E) Western blot analysis of the expression of CXCL10. (F) Cxcl10 mRNA expression was analyzed by qRT-PCR. (G) Relative occupancy of AcH3K14, AcH3K27, and HDAC1 in the Cxcl10 promoters was assessed by qChIP analysis. The ChIP data values represent relative ratios to the input. (H) Transwell T cell migration assay in CT26-Nanog-siGFP or CT26-Nanog-siHdac1 cells were treated with IgG or anti-CXCL10. (I) Western blot analysis of the expression of GFP (CXCL10) in CT26-no insert or CT26-Nanog WT were transduced with Cxcl10 WT or Cxcl10 MT. (J to L) CT26-no insert, CT26-Nanog-Cxcl10 WT, or CT26-Nanog-Cxcl10 MT tumor-bearing mice treated with PD-1 antibody. (J) Tumor growth curves. (K) Flow cytometry profiles of tumor-infiltrating CD3+ CD8+ T cells. (L) The ratio of Granzyme B+ to tumor-infiltrating CD3+ CD8+ T cells. Five mice from each group were used for in vivo experiments. (D, E and I) β-actin was used as an internal loading control. The graphs represent three independent experiments performed in triplicate. The p-values by one-way ANOVA (C, F, G, H, K, and L) or two-way ANOVA (J) are indicated. The data represent the mean ± SD. Source data are provided as a Source Data file. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. NS, not significant)
Figure 5. Resistance to CTL-mediated killing mediated by the NANOG axis is one of the key steps driving the immune-refractory feature of the TME. (A and B) CT26-no insert or CT26-Nanog cells were transfected with siGFP or siHdac1. (A) Western blot analysis of the expression of MCL1. \(\beta\)-actin was used as an internal loading control. (B) The frequency of apoptotic cells. (C and D) CT26-Nanog cells were transfected with siGFP or siMcl1. (C) Western blot analysis of the expression of MCL1. \(\beta\)-actin was used as an internal loading control. (D) The frequency of apoptotic cells. (E to H) CT26-Nanog tumor-bearing mice administered siGFP or siMcl1 with or without PD-1 antibody treatment. (E) Tumor growth curves and (F) changes in tumor volume 17 days after challenge compared to baseline. (G) The frequency of apoptotic cells in the tumors. (H) Quantification of antigen-specific CTLs in spleens derived from the tumor-bearing mice. Ten mice from each group were used for in vivo experiments. The graphs represent three independent experiments performed in triplicate. The p-values by one-way ANOVA (B, G, and H) or two-tailed Student’s t-test (D) are indicated. The data represent the mean ±
SD. Source data are provided as a Source Data file. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. NS, not significant)
Figure 6. The NANOG/HDAC1 axis is conserved across multiple types of NANOG\textsuperscript{high} tumor cells.

(A to C) Various mouse or human cancer cell lines were transfected with \textit{siGFP}, \textit{siNanog}, \textit{siNANOG},
siHdac1, or siHDAC1. (A) Western blot analysis of NANOG, HDAC1, CXCL10, and MCL1 expression. β-actin was used as an internal loading control. Graph depicts the experimental quantitation based on at least three independent experiments. (B) Transwell T cell infiltration assay. (C) The frequency of apoptotic cells. The graphs represent three independent experiments performed in triplicate. (A to C) The p-values by one-way ANOVA are indicated. The data represent the mean ± SD. Source data are provided as a Source Data file. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. NS, not significant)
Figure 7. HDAC1 inhibition renders tumors susceptible to anti-PD-1-mediated anti-tumor immune response. (A) CT26 P0 and CT26 P3 cells were transfected with siGFP or siNANOG. After 16 hours, cells were treated with the indicated concentrations of FK228, MS-275, SAHA or cisplatin for 48 hours. Cell viability was measured by live cell counting using trypan blue. (B) Western blot analysis of expression of NANOG, HDAC1, CXCL10, MCL1, AcH3-K14 and AcH3-K27 in CT26 P3 cells were
treated with dimethyl sulfoxide (DMSO) or FK228. β-actin was used as an internal loading control. (C to I) CT26 P3 tumor-bearing mice administered vehicle or FK228, with or without PD-1 antibody treatment. (C) Tumor growth curves and (D) changes in tumor volume compared to baseline at 17 days after challenge. (E) Survival of mice inoculated with CT26 P3 treated with the indicated reagents. (F) Flow cytometry profiles of tumor-infiltrating CD3+ CD8+ T cells. (G) The ratio of granzyme B+ to tumor-infiltrating CD3+ CD8+ T cells. (H) The frequency of apoptotic cells in the tumors. (I) Quantification of antigen-specific CTLs in spleen derived from the tumor-bearing mice. Ten mice from each group were used for in vivo experiments. The graphs represent three independent experiments performed in triplicate. The p-values by two-way ANOVA (A) or one-way ANOVA (F to I) are indicated. The data represent the mean ± SD. Source data are provided as a Source Data file. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. NS, not significant)
Figure 8. Model depicting the role of the NANO\textsubscript{G}/HDAC\textsubscript{1} axis in resistance to anti-PD-1 therapy. 

(A) NANO\textsubscript{G} drives immune-refractoriness against anti-PD-1 therapy by blocking the anti-tumor immunity cycle. (B) The molecular pathway through which the NANO\textsubscript{G}/HDAC\textsubscript{1} axis represses T cell infiltration of tumors and tumor cell death by CTL-mediated killing.