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DNA methyltransferase inhibition promotes recruitment of myeloid-derived suppressor cells to the tumor microenvironment through induction of tumor cell-intrinsic interleukin-1

Sofie Traynor a, Mikkel Green Terp a, Aaraby Yoheswaran Nielsen a, Per Guldberg a, b, Mie Jakobsen a, Pernille Gejl Pedersen a, Odd Lilleng Gammelgaard a, Christina Bøg Pedersen a, Mathilde Thybo Pedersen a, Sofie Rattenborg a, Henrik Jørn Ditzel b, c, d, Morten Frier Gjerstorff a, c, d, *  

a Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, J. B. Winsløws Vej 25, Odense, Denmark  
b Molecular Diagnostics Group, Danish Cancer Society Research Center, Strandboulevarden 49, DK-2100, Copenhagen, Denmark  
c Department of Oncology, Odense University Hospital, J.B. Winsløws Vej 4, Odense, Denmark  
d Academy of Geriatric Cancer Research (AgeCare), Odense University Hospital, J.B. Winsløws Vej 4, Odense, Denmark

A B S T R A C T

DNA methyltransferase (DNMT) inhibitors are used for treatment of certain hematological malignancies and exert anti-cancer activity through diverse mechanisms, including reexpression of tumor suppressor genes and anti-viral responses triggered by expression of endogenous retroviruses. Despite advances in the pharmacokinetic properties of DNMT inhibitors, the efficacy of these drugs in solid cancers remains low. Here, we show in cell lines and clinical and experimental tumors across multiple cancer types that DNMT inhibition induces the expression of interleukin-1 (IL-1), a cytokine with proinflammatory and protumorigenic properties. Specifically, this tumor-intrinsic IL-1 expression modulates the chemokine landscape of tumors and leads to the recruitment of monocyte-like myeloid-derived suppressor cells to the tumor microenvironment, processes that can be blocked by IL-1 antagonists. Molecular analysis demonstrates complex patterns of IL-1 and interferon activation and crosstalk in response to DNMT inhibition, which depend on the integrity of IRF- and NF-κB-mediated antiviral pathways and may determine the outcome of DNMT-inhibitor treatment. Together, our results show that DNMT inhibitors may negatively affect the microenvironment of a large subset of tumors and suggest that co-treatment with IL-1 antagonists may be a favorable combination for these patients.

1. Introduction

Chemical inhibitors of DNA methylation, also known as hypomethylating agents, such as 5-azacytidine (azacitidine) and 5-aza-2’-deoxycytidine (decitabine), are used for the treatment of myelodysplastic syndrome and myeloid leukemias [1]. These agents are nucleoside analogues that become incorporated into DNA where they covalently bind DNA methyltransferases (DNMTs), the enzymes catalyzing the addition of a methyl group to cytosine within CpG dinucleotides. Inhibition and subsequent depletion of DNMTs, particularly DNMT1, which maintains methylation patterns following DNA replication, leads to a genome-wide reduction in DNA-methylation content.

The anti-cancer activities of DNMT inhibitors are complex and involve multiple biological and physiological processes. When administered at high concentrations, azacitidine and decitabine have cytostatic activities and are toxic for patients, whereas at lower and repeated doses, they induce DNA hypomethylation in cancer cells and are better tolerated. A direct consequence of DNA hypomethylation is reexpression of genes aberrantly silenced by CpG island-associated promotor hypermethylation in cancer cells, including tumor suppressor genes involved in multiple cellular pathways [2]. Another consequence is to create long-lasting effects on anti-tumor immunity [3,4] by reverting DNA methylation-dependent repression of MHC-I genes [5–7] and by inducing the expression of cancer/testis antigens, thereby increasing the availability of T-cell antigens on cancer cells [8]. In addition, DNMT inhibitors can induce a state of viral mimicry in cancer cells by activating the expression of human endogenous retroviruses (HERVs) that reside in the genome and are normally silenced by DNA methylation [9–13]. The transcription of HERV-derived double-stranded RNAs (dsRNAs) elicits cytosolic dsRNA-sensing mechanisms.

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leading to increased expression of type I and/or type III interferons (IFNs) [9,10], which have antineoplastic effects and support anti-tumor immunity [14]. The latter includes stimulation of innate and adaptive cytotoxic lymphocyte populations and negative regulation of cell types known to dampen anti-tumor immune responses, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells [15, 16].

Despite the well-documented antineoplastic properties of DNMT inhibitors and the efficacy of these drugs in myeloid malignancies, clinical trials in solid cancers have so far shown limited clinical potential. This inability to effectively target cancer cells may relate to low chemical stability and poor pharmacokinetic properties of the drugs, but may also be caused by tumor-intrinsic mechanisms not operative in myeloid malignancies. In our search for such mechanisms, we investigated the role of interleukin-1 (IL-1), another proinflammatory cytokine that drives local and systemic inflammation in response to viral infection. In contrast to IFNs, IL-1 is considered to have unfavorable effects on anti-tumor immunity [17], which are largely mediated by the ability of IL-1 to enhance the recruitment of repressive myeloid cells to the tumor anti-tumor immunity [17], which are largely mediated by the ability of IL-1 to enhance the recruitment of repressive myeloid cells to the tumor microenvironment (TME) [17–26]. In this study, using the next-generation DNMT inhibitor, guadecitabine, we show that inhibition of DNA methylation leads to induction of IL-1, which drives an unfavorable cytokine response in tumors and thereby mediates recruitment of myeloid cells that may oppose the positive effects of DNMT-inhibitor therapy.

2. Materials and methods

2.1. Cell culture

All cell lines were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (Sigma Aldrich), and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (Sigma Aldrich, Sarbog, Denmark) and 1% penicillin-streptomycin at 37 °C and in 5% CO₂. All cell lines were tested for mycoplasma (Mycoplasma detection kit, Lonza, Copenhagen, Denmark) and cell identities according to ATCC were verified using DNA fingerprinting by short tandem repeat (STR) analysis (Cell IDTM system). Cells were treated daily with 1 μM guadecitabine (SGI-110; Selleckchem, Berlin, Germany) for the number of days indicated in figure legends. The dose was previously demonstrated to effectively reduce DNA methylation as measured by promoter demethylation of LINE-1 and cancer/testis antigen genes [27–30]. Other treatments included: 2 μg/ml Poly(I:C) (HMW)/LyoVec (InvivoGen, San Diego, CA, USA), 10 μg/ml Poly(I:C) (HMW) (InvivoGen), 2 μg/ml Poly(dA:dT)/LyoVec (InvivoGen), 0.1–100 ng/ml recombinant human IFN-A10 (InvivoGen), 0.1–100 ng/ml recombinant human IFN-L1 (InvivoGen), 0.1 μM NF-κB inhibitor Bengamide B or 100 ng/ml IL-1RA (Kineret, Swedish Orphan Biovitrum, Lyngeby, Denmark) as indicated.

2.2. TNBC PDX xenograft models

Tissue biopsies from TNBC patients undergoing routine treatment at Odense University Hospital or frozen stocks of PDX tumors were implanted with Matrigel (Sigma-Aldrich) into the mammary fat pad of 7–8 week-old female NOG CIEA mice (NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac, Taconic) housed under pathogen-free conditions with ad libitum food and water. NOG CIEA mice lack B and T cells due to the SCID mutation which disrupts VDJ recombination. A loss-of-function mutation in the IL-2Rγ further prevents development of NK cells and impairs the function of macrophages and dendritic cells. In contrast, these mice fully support the development of functional monocytes and neutrophils [31]. PDX tumor samples were stored in DMEM with 10% DMSO in liquid nitrogen and used in low passage (second or third generation). PDX models TNBC-4582, -5160, and -5474 were established from primary tumors of untreated patients, while TNBC-9228 was established from a metastasis.

For DNMTi treatment, NOG CIEA mice were grafted with second or third generation PDX tumor samples. When tumors were palpable (approximately 2–3 mm), mice were treated with subcutaneous injections of 24.4 mg/kg guadecitabine or PBS control every 5 days for 20 days. This dosing schedule was based on data on the farmacokinetic properties and effect on methylation and expression of LINE-1 and cancer/testis antigen genes in various xenograft models [27,28,30]. The guadecitabine treatment was supplemented with daily injections of 100 mg/kg Kineret (IL-1RA) or PBS (vehicle) where indicated. Mice were euthanized by cervical dislocation 3 days after last treatment and tumors and organs were processed for RNA-sequencing (Supplementary Tables 1 and 2) and/or immunohistochemical staining. The experiments were approved by the Danish Animal Experiments Inspectorate.

2.3. Cytokine ELISA

Cytokines in media from cultured cells were quantified using the following ELISA kits: IL-1 beta human uncoated ELISA kit (Thermo Fisher, Waltham, MA, USA), LumiKine Xpress hIFN-β 2.0 (InvivoGen) and IL-29 (IFN-L1) human uncoated ELISA kit (Thermo Fisher).

2.4. RNA sequencing

RNA was purified from cells using RiboZol (VWR, Sorbo, Denmark) or TRI Reagent (Sigma-Aldrich). For tissues, this step included homogenization using 2.8 mm Zirconium oxide beads (Precellus, Rockville, MD, USA) and a Precellus 24 homogenizer (3 x 15 s, 6500 rpm). Purified RNA was prepared for sequencing on the Illumina NovaSeq 6000 Sequencing Platform using the NENext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA; E7490L) and the NENext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, E7465L) with unique dual indexes according to manufacturer’s instructions. Trimmed and filtered sequencing reads were aligned to the human genome (hg38) or mouse genome (GRCm38) using Spliced Transcripts Alignment to a Reference (STAR) [32] and analyzed using the Quicore analysis platform (Quicore, Lund, Sweden). For the analysis of HERV expression, a list of 519 059 human ERVs was downloaded from http://herv.img.cas.cz [33]. Transcription of the 519 059 HERVs was quantified using the feature Counts function in the Rsubread package [34] and differential HERV expression was analyzed using DeSeq2 [35].

2.5. Gene expression data from public repositories

RNA sequencing gene expression data from lung adenocarcinoma, breast cancer and melanoma were downloaded from The Cancer Genome Atlas (TCGA) repository, while RNA sequencing data from 5-aza-2-deoxycytidine-treated melanoma cell lines (GSE32492) and guadecitabine-treated ovarian cancer patients (GSE102118; NCT01696032) were downloaded from the Gene Expression Omnibus (GEO). All data were processed and normalized using Quicore. Statistical analysis of data was performed with Prism 9.

2.6. Quantitative RT-PCR

RNA was purified from cells using RiboZol (VWR) followed by cDNA synthesis performed with the RevertAid Premium Reverse Transcriptase kit from Fermentas. Quantitative real-time PCR was performed using SYBR green-based expression analysis (Applied Biosystems, Nærum, Denmark) in combination with the following QuantiTect primers (Qiagen, Copenhagen, Denmark): IFNA1 (QTT0021964), IFNB1 (QTT00203763), IFN1J (QTT01035564), IFN1L (QTT01672356), IL1b (QTT0148355), CCL20 (QTT0012971), CSF3 (QTT0001414), CXCL1 (QTT00199752), CXCL8 (QTT0000322) or primers designed in house: IL1A, 5′-TGCT GAA GGA GAT GCC TGA GAT-3′ and 5′-ACA AGT TTG
GAT GGG CAA CTGA-3; IL1B, 5'-CTT CGA GCC ACA AGG CAC AA-3', 5'-TGG CTG CTT CAG ACA CTT GAG-3'; TNF, 5'-TAG CCC ATG TTG TAG CAA ACC C-3', 5'-TCT CTC AGC TTC ACG CCA TT-3'.

2.7. Immunohistochemistry

Formalin-fixed paraffin-embedded sections (4 μm) of tumors and mouse tissues were cut with a microtome, mounted on ChemMateTM Capillary Gap Slides (Dako, Santa Clara, CA, USA), dried at 60 °C, deparaffinized and hydrated. Hydrogen peroxide (1.5%) in TBS buffer (pH 7.4) was used for blocking endogenous peroxidase and antigen-retrieval was achieved by microwave boiling in TEG buffer (Dako) for 15 min. Sections were incubated with the primary antibody: anti-mouse CD11b (ab133357, Abcam, Cambridge, UK, 1:1000) at room temperature for 32 min before detection with the OptiView DAB IHC detection kit (760–700, Vienna Medical systems). For fluorescence MPO staining of PDX tumors, tissue sections were stained with goat anti-MPO (AF3667, Rndsystems, Minneapolis, USA, 1:100), washed in PBS and stained with donkey anti-rabbit Alexa Fluor 488. Stained tissue sections were mounted under cover slides with ProLong Gold Antifade with DAPI (Life Technologies). Imaging was performed with an Olympus IX73 microscope fitted with a PlanApo N 60X/1.42 oil objective.

2.8. Quantification of CD11b immunohistochemical staining

Immunohistochemical staining of whole tumor sections or partial sections of livers and spleens were analyzed using Image J. Pictures of CD11b-stained tissues were analyzed by adjusting the color threshold to mark all stained cells or all cells in total and the selected areas were measured. The relative levels of CD11b-positive cells in tissues are expressed as arbitrary units (area CD11b staining/area total cells).

2.9. Immunocytochemistry

Cells were grown on glass slides and treated with ice-cold methanol for 10 min. Next, cells were incubated with 3% BSA, PBS for 30 min and stained with anti-P-IRF3 (Ser386) (E7J8G, Cell Signaling, Herlev, Denmark, 1:500) in 1% BSA, PBS at room temperature for 90 min. Finally, cells were incubated with goat anti-rabbit IgG (H + L) cross-adsorbed Alexa Fluor 488 (ThermoFisher Scientific) at room temperature for 60 min and mounted under cover slides with ProLong Gold Antifade with DAPI (Life Technologies). Imaging was performed with an Olympus IX73 microscope fitted with a PlanApo N 60X/1.42 oil objective.

2.10. Statistical analysis

Statistical testing was performed in GraphPad Prism v8 (GraphPad Software, Inc.) using either two-tailed Student’s t-Test or one-way analysis of variance (ANOVA) as indicated in figure legends.

3. Results

3.1. DNMT inhibition induces IL-1 expression in tumors across cancer types

To investigate the potential role of IL-1 in the tumor cell response to DNMT inhibition, we first established patient-derived xenograft (PDX) mouse models of triple-negative breast cancer (TNBC) and treated the mice with guadecitabine administered as subcutaneous injections at 5-day intervals. Consistent with previous studies [10,36], DNMT inhibition led to activation of HERV expression in all four models (Fig. S1). In three of these models, expression of IL-1 genes (i.e. IL1A and IL1B) was

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Fig. 1. IL-1 expression is frequently induced by DNMT inhibitors across cancer types (A) NOG CIEA mice challenged with TNBC PDX tumors (i.e., TNBC-4582, 5160 -5474 and 9228), were treated with guadecitabine (Gua) or vehicle (Veh) and the expression of IL1A and IL1B was investigated with RNA sequencing analysis. Data represent the mean ± SD for four biological replicates. (B) Expression levels of IL1A and IL1B in TNBC cell lines: BT20, MDA-MB-231 (MB231), MDA-MB-157 (MB157), MDA-MB-468 (MB468) were treated with guadecitabine or vehicle for one, three or six days and measured by quantitative PCR. Guadecitabine induced pronounced cell death in MDA-MB-468 cells and therefore the day six timepoint could not be analyzed. Data represent the mean ± SD for four biological replicates. (C) The effect of the DNMT inhibitor 5-aza-2′-deoxycytidine (AZA) on the expression of IL-1A and IL-1B in melanoma cell lines was investigated using data from a previously published study (GSE32492) [54] (top). The expression levels of IL-1A and IL-1B in tumor biopsies from ovarian cancer patients treated with two cycles of guadecitabine were investigated using previously published RNA-seq data (GSE102118) [55] (bottom). − and + indicate biopsies collected before and after treatment, respectively. Values are the mean of two biopsies collected from the same tumor. (D) IL-1B levels were measured by ELISA in culture medium from TNBC, ER-positive breast cancer (ER + BC), melanoma and non-small cell lung cancer (NSCLC) cells treated with guadecitabine or vehicle for six days. Data represent the mean ± SD of three biological replicates. Statistical differences between vehicle- and guadecitabine-treated samples were analyzed by t-tests. ns = non-significant. ***P < 0.0001; ****P < 0.0001; **P < 0.001; *P < 0.01.
significantly increased, with the highest levels in TNBC-4582 (Fig. 1A).
To examine whether IL-1 expression was tumor-cell intrinsic, we treated
four TNBC cell lines (BT-20, MDA-MB-157, MDA-MB-231 and
MDA-MB-157) with guadecitabine. These cell lines exhibited potent
depletion of DNMT1 upon guadecitabine treatment (Fig. S2). Three
responded to DNMT inhibition by increased expression of IL-1 (Fig. 1B).
A similar effect was observed with decitabine, confirming that the
induction of IL-1 expression is a genuine effect of DNMT1 (Fig. S3). We
next analyzed gene expression data sets from melanoma cell lines
treated with decitabine and tumor biopsies from ovarian cancer patients
treated with two cycles of guadecitabine. Increased expression of IL-1
was found in nine of twelve melanoma cell lines (75%) and two of
eight ovarian cancers (25%; Fig. 1C). Since bioactive IL-1 is generated
from the proteolytic processing of pro-IL-1A/B, we analyzed the protein
levels of IL-1 in culture medium from TNBC, estrogen receptor (ER)-positive breast cancer, melanoma and non-small cell lung cancer (NSCLC) cell lines treated with guadecitabine. This analysis confirmed that DNMT inhibition induces IL-1 expression in TNBC and melanoma cells, whereas no expression was observed in ER-positive breast cancer and NSCLC cells (Fig. 1D). In contrast to the activation of IL-1 expression by DNMT inhibition in human cancer models, guadecitabine treatment did not induce IL-1 in murine breast cancer and melanoma models (Fig. S4). It remains to be determined if this reflects a genuine mouse–human difference or a variation between cancer cell models.

3.2. Differential activation and negative crosstalk between IL-1- and IFN-mediated antiviral signaling in response to DNMT inhibition

Having demonstrated IL-1 expression activated by DNMT inhibition in a subset of tumors, we investigated the relationship between IL-1- and IFN-mediated antiviral responses. The frequency of IFN induction in response to guadecitabine was similar to that of IL-1 induction, with increased expression of type I IFN (i.e. IFN-A and -B) and type III IFN (i.e. IFN-L1 and L3) in three of eight and six of eight TNBC models, respectively (Fig. 2A–C). In addition, the expression of interferon induced genes (ISGs) followed that of IFN genes in TNBC cell lines (Fig. S5). The patterns of IL-1, type I and type III IFN expression across models were complex with no obvious correlations (Fig. 2C), suggesting that these antiviral responses are activated by different signals in response to DNMT inhibition.

Fig. 3. DNMT inhibitor-induced IL-1 expression mediates tumor-intrinsic expression of chemokines for myeloid cell recruitment (A) NOG CIEA mice challenged with TNBC PDX tumors (i.e., TNBC-4582, -5160, -5474 and -9228) were treated with guadecitabine or vehicle, and the expression of selected cytokines was investigated with RNA sequencing analysis. Data represent the mean ± SD of four biological replicates. (B) The expression levels of IL-1A and IL-1B and selected chemokine genes were investigated in previously published RNA-seq data generated from tumor biopsies of ovarian cancer patients treated with two cycles of guadecitabine (Gua). → and + indicate biopsies collected before and after treatment, respectively (bottom). Values are the mean of two biopsies collected from the same tumor (GSE102118) [55]. Red boxes indicate tumors with coordinated activation of IL-1 and cytokine genes. (C) The correlation between expression of IL-1 and selected chemokines in melanoma, lung and breast cancer tumors was investigated using RNA-sequencing data from the TCGA repository. (D) MDA-MB-231 TNBC cells were treated with vehicle, guadecitabine or guadecitabine in combination with the IL-1 receptor antagonist IL-1RA for four days and subjected to RNA-sequencing analysis. Shown in the relative induction of cytokine genes by guadecitabine/IL-1RA treatment compared to vehicle treatment. Data represent the mean ± SD of three biological replicates. (E) A2058 and FM3 melanoma cells were treated with vehicle, guadecitabine or guadecitabine in combination with IL-1RA for four days and subjected to qPCR analysis to quantify expression of selected cytokines. Data represent the mean ± SD of three biological replicates. Statistical differences between samples were analyzed by One-way ANOVA followed by Tukey’s multiple comparison test. ns = non-significant. ***P < 0.0001; **P < 0.001; *P < 0.01.
DNMT inhibition and that the integrity of these signals differs among tumors. This is consistent with previous studies demonstrating that IL-1, type I IFN and type III IFN mediate distinct types of inflammatory responses to viral infection and are activated to different extents, depending on stimuli and cellular setting [37,38]. The observed differences in IL-1 and IFN expression may reflect different modes of activation. dsRNA activates IFN expression through the cytosolic detectors RIG1 and MDAS and downstream signaling mediated by MAVS and phosphorylation-mediated activation of IRF transcription factors (e.g. IRF3 and IRF7) [39], a mechanism also shown to be the main route of IFN activation in cancer cells in response to DNMT inhibition [10,36]. Consistent with these findings, induction of type I IFN by guadecitabine in TNBC cells correlated with the activity of IRF phosphorylation (Fig. S6), whereas the activation of type III IFN may involve additional factors. IL-1 expression, on the other hand, is driven by NF-kB signaling in response to dsRNA [40]. Indeed, inhibition of 1xNR, an essential component of the NF-kB pathway, significantly reduced IL-1B production in response to guadecitabine (Fig. 2D). Thus, the relative activity of IRF- and NF-kB-mediated antiviral pathways in tumors may account for the production of IFN and IL-1 in response to DNMT inhibition.

Previous studies suggested negative crosstalk between IL-1 and IFN antiviral pathways in immune cells [41–44] that could potentially contribute to the complex patterns of IL-1 and IFN expression in cancer cells treated with DNMT inhibitors. Indeed, the addition of IFN-A10 (a type I IFN) during guadecitabine treatment repressed production of IL-1B protein (Fig. 2E and S7). This effect was only partially caused by repression of IL-1 gene expression (Fig. 2F), suggesting that type I IFN may also repress post-translational processing of IL-1. Like IFN-A10, IFN-L1 (a type III IFN) repressed IL-1 production, albeit to a lesser extent (Fig. 2E and S8). In contrast, IL-1 did not affect DNMT inhibitor-induced activation of IFN expression (Fig. 2G).

3.3. DNMT inhibitor-induced IL-1 modulates the cytokine landscape of tumors

Gene expression profiling of guadecitabine-treated tumors from TNBC PDX mice and ovarian cancer patients showed that induction of IL-1 expression correlated with the expression of a broad panel of cytokines (Fig. 3A–B), including multiple members of the CXC- and CCL-chemokine families and other cytokines (e.g. CSF3, TNF and IL-6). Using RNA-sequencing data from the TCGA repository, we found a significant correlation between the expression of IL-1 and cytokines in tumors from patients with melanoma, breast cancer and lung cancer (Fig. 3C). In comparison, neither type I nor type III IFNs correlated with the expression of these cytokines in untreated (Fig. S8) or DNMT inhibitor-treated tumors (Fig. S9). Functionally blocking the IL-1 receptor using the IL-1-receptor antagonist IL-1RA prevented guadecitabine-mediated activation of numerous cytokine/chemokines in both TNBC and melanoma cells (Fig. 3D–E).

3.4. DNMT inhibitor-induced IL-1 expression is associated with recruitment of monocyctic MDSCs to tumors

Some of the chemokines/ cytokines induced by IL-1 in response to DNMT inhibition (Fig. 3) are known to be important for mobilizing and recruiting myeloid cells, including MDSCs, to the tumor microenvironment. We investigated this possibility in CIEA NOG mice, which support the growth of clinically-relevant PDX tumors. These mice lack B, T and NK cells, and have impaired functions of macrophages and dendritic cells, while fully sustaining the development of neutrophils and monocytes [31]. Furthermore, monocytes and neutrophils are recruited to PDX tumors in these mice dependent on tumor intrinsic factors such as expression of chemokines (manuscript submitted). Thus, CIEA NOG mice are highly suitable for studying mobilization and recruitment of tumor promoting MDSCs of the monocytic and granulocytic lineage. We first quantified the levels of myeloid-cell infiltration in guadecitabine-treated TNBC PDX tumors using CD11b as a marker. Guadecitabine-treated TNBC-4582 tumors, which exhibited strong induction of IL-1 (Fig. 1A) and IL-1-regulated chemokines (Fig. 3A), showed a major increase in myeloid-cell infiltration, while PDX models with lower or no IL-1 induction showed no change or a statistically insignificant tendency towards a reduced number of myeloid cells (Fig. 4A). Microscopic evaluation of the myeloid-cell component in PDX tumors revealed the presence of two cell types morphologically consistent with polymorphonuclear (PMN)-MDSCs and monocytic (M)-MDSCs, respectively. Guadecitabine enhanced infiltration of M-MDSCs in TNBC-4582 tumors (Fig. 4B), which was supported by enhanced expression of the monocyte markers Cd14 and F4/80 (encoded by Adgre1) [45] and the monocyte receptors Marco and Clec4e [46] (Fig. 4C). Expression of Ly6c (encoded by Ly6c1) was not enhanced suggesting that the infiltrating monocytes were of a non-classical subtype [45]. Recruitment of M-MDSCs to TNBC-4582 tumors was also associated with increased expression of Arg1 and iNOS/Nos2, which are important suppressors of anti-tumor immune responses [47,48] (Fig. 4C). In contrast, tumors from untreated TNBC-5474 PDX mice were infiltrated with high numbers of PMN-MDSCs, which were reduced upon guadecitabine treatment (Fig. 4B). This pattern was validated by immunostaining for the PMN-MDSC marker myeloperoxidase (MPO) (Fig. 4D) and consistent with a reduction in the expression of the PMN-MDSC chemokine receptor Cxcr2 in tumors (Fig. 4C).

3.5. Blocking IL-1 signaling inhibits DNMT inhibitor-mediated recruitment of M-MDSCs without affecting reexpression of genes silenced by DNA methylation

To validate the role of IL-1 in DNMT inhibitor-mediated recruitment of M-MDSCs to the TME, we treated TNBC-4582 PDX mice with a combination of guadecitabine and IL-1RA (Fig. 5A), a naturally-occurring IL-1 receptor (IL-1R1) antagonist used for treatment of inflammation-driven chronic diseases such as rheumatoid arthritis. In this model, the accumulation of M-MDSCs in response to treatment with guadecitabine could be rescued by co-treatment with IL-1RA (Fig. 5B). To examine whether blocking IL-1 signaling affects the hypomethylating activity of DNMT inhibitors, we measured the expression of cancer/testis antigens, which are normally silenced by DNA methylation. As shown in Fig. 5C, guadecitabine induced expression of these genes in TNBC cells, and this induction was not reduced by co-treatment with IL-1RA.

4. Discussion

The clinical use of DNMT inhibitors as mono or combination therapies is currently restricted to myeloid malignancies. Clinical trials of patients with solid cancers suggest that DNMT inhibitors can improve patient response to chemotherapy or immunotherapy [2], but further investigations and developments are needed to increase efficacy. While differences in response to DNMT inhibitors between myeloid and solid cancers may partly be due to tumor-specific differences in DNA-methylation patterns, factors related to the TME of solid tumors are likely to be major determinants. Our present data based on clinical samples, and clinically-relevant PDX models suggest that DNMT inhibitors can lead to the accumulation of tumor-infiltrating M-MDSCs through the induction of tumor-intrinsic IL-1 expression. These findings may have clinical implications given the importance of M-MDSCs in the repression of anti-tumor T-cell responses [49].

Our study confirms and extends earlier findings that DNMT inhibitors can affect tumor growth via the activity of antiviral response pathways triggered by induced expression of HERVs [9,10]. The involvement of IL-1 in these responses adds another layer of complexity given the opposing effects of IFN and IL-1 on the immune response to tumors, with type I and III IFNs being essential components of effective...
anti-tumor immunity [14], whereas IL-1 is a key factor in tumor-promoting inflammation [50]. Tumors with a clear IL-1 response to DNMT inhibitor treatment had chemokine profiles favoring the recruitment of MDSCs, consistent with preclinical studies implicating IL-1 in the recruitment of repressive species of myeloid cells and promotion of tumor immune escape [18–20,22–24]. In contrast, IFN-driven
tumor responses to DNMT inhibitor were less potent in inducing chemokine expression and did not promote MDSC recruitment. The latter is supported by findings demonstrating that IFN generally represses recruitment of MDSCs to the TME [15,51]. Together, these findings suggest that the tumor response to DNMT inhibitors may be determined by the relative involvement of IFN and IL-1 signaling and the recruitment of MDSCs to the TME. While the expression of IL-1 and IFN in response to DNMT inhibitors depends on IRF and NF-κB signaling, respectively, the reason for the differential activation of these two antiviral pathways in tumors remains elusive. Future studies, should focus on identifying deficiencies in IRF and NF-κB signaling that determine the relative expression of IL-1 and IFN in the response to DNMTi.

Studies in syngeneic mouse tumor models have shown that epigenetic therapy with DNMT inhibitors can potentially enhance the anti-tumor responses with respect to both primary tumors and metastases [52]. This effect has been attributed to a reduction of immune suppressive MDSCs in primary tumors and metastatic niches caused by a DNMT inhibitor-mediated phenotype switch of MDSCs [52] or repression of tumor-induced production of MDSCs in the bone marrow [53]. These results focused on PMN-MDSCs and are supported by our study demonstrating that DNMT inhibitor treatment reduces tumor infiltration of PMN-MDSCs. The TNBC-5474 PDX model used in this study, exhibiting massive infiltration of PMN-MDSCs prior to DNMT inhibition, which were completely lost upon treatment. In contrast to this effect on PMN-MDSCs, we found an IL-1-dependent enhanced recruitment of M-MDSCs with a non-classical, immune suppressive phenotype to PDX tumors exhibiting high chemokine expression as the result of DNMT inhibitor treatment (i.e. TNBC-4584 PDX tumors). Similar results have not been reported by studies using mouse tumor models, which may reflect differences in tumor responses between mouse and human models. Although the relative involvement of IFN and IL-1 anti-viral response pathways in the DNMT-inhibitor response of syngeneic mouse tumors was not addressed in previous studies, we found that mouse cancer cell lines, in contrast to human cell lines and PDX models, did not express IL-1 in response to DNMT inhibition. This suggests that the use of human models provides a more detailed view on the response to DNMT inhibitors, better reflecting the diversity of the response of patient tumors.

In conclusion, our data suggest that treatment with DNMT inhibitors may impair anti-tumor responses in subsets of patients due to IL-1-mediated recruitment of M-MDSCs to the TME. These findings may pave the way for development of molecular biomarkers predicting response to DNMT inhibitors. Furthermore, our study shows that accumulation of M-MDSCs can be prevented by IL-1 receptor antagonists, suggesting that the combination of DNMT inhibitors and therapies antagonizing IL-1 signaling, such as IL1RA or IL-1R-blocking antibodies, may be favorable in patients with tumors mounting an IL-1 response to DNMT inhibitors. This combination maintains the effect of DNMT inhibitors on tumor cell-specific induction of tumor suppressor genes as well as cancer/testis antigens, potentially enhancing anti-tumor T-cell activity.

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Data availability
Data supporting the study can be found in the supplementary information file, and the corresponding author will make any further data and materials available upon request. Previously published datasets analyzed in this study includes: GSE32492 and GSE102118, and data from the TCGA repository.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data
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