Epigenetic silencing of T_{H1}-type chemokines shapes tumour immunity and immunotherapy

Dongjun Peng1, Ilona Kryczek1,2, Nisha Nagarseth1,2, Lili Zhao3, Shuang Wei, Weimin Wang4, Yuqing Sun4, Ende Zhao1, Linda Vatan1, Wojciech Szeliga1, Jan Kotarski1, Rafał Tarkowski1, Yali Dou4, Kathleen Cho4,6, Sharon Hensley-Alford7, Adnan Munkarah7, Rebecca Liu5,8 & Weiping Zou1,2,6,9

Epigenetic silencing including histone modifications and DNA methylation is an important tumorigenic mechanism1. However, its role in cancer immunopathology and immunotherapy is poorly understood. Using human ovarian cancers as our model, here we show that enhancer of zeste homologue 2 (EZH2)-mediated histone H3 lysine 27 trimethylation (H3K27me3) and DNA methyltransferase 1 (DNMT1)-mediated DNA methylation repress the tumour production of T helper 1 (T_{H1})-type chemokines CXCL9 and CXCL10, and subsequently determine effector T-cell trafficking to the tumour microenvironment. Treatment with epigenetic modulators removes the repression and increases effector T-cell tumour infiltration, slows down tumour progression, and improves the therapeutic efficacy of programmed death-ligand 1 (PD-L1; also known as B7–H1) checkpoint blockade2-4 and adoptive T-cell transfusion5 in tumour-bearing mice. Moreover, tumour EZH2 and DNMT1 are negatively associated with tumour-infiltrating CD8^{+} T cells and patient outcome. Thus, epigenetic silencing of T_{H1}-type chemokines is a novel immune-evasion mechanism of tumours. Selective epigenetic reprogramming alters the T-cell landscape6 in cancer and may enhance the clinical efficacy of cancer therapy.

Cancer immunotherapy has demonstrated therapeutic responses4,5,7,8. Yet, the objective responses have been manifested in a fraction of patients. We hypothesized that immune-protective signature genes might be epigenetically silenced in cancer and in turn affect cancer progression and clinical responses to immunotherapy. Cancer epigenetic silencing often includes EZH2-mediated histone modifications and DNMT-mediated DNA methylation. DZNep is an inhibitor of all S-adenosyl-methionine (SAM)-dependent enzymes including EZH2, and EPZ64389 may specifically inhibit EZH2. 5-aza-2'-deoxycytidine (5-aza-dC) is a DNMT inhibitor. Hence, we used these agents to reprogram epigenetic pathways and to test our hypothesis.

In the first setting, we established ID8 ovarian cancer in C57/BL6 mice and treated them with low doses of DZNep and EPZ6438, 5-aza-dC and their combination. Treatment with single agent had minimal effects on tumour volume, whereas the combinatorial treatment caused tumour reduction, increased tumour infiltrating T cells and T_{H1}-type chemokine expression (Extended Data Fig. 1a–e). We observed similar tumour volume (Extended Data Fig. 1f) in ID8-bearing, female NOD-scid Il2r^{null} (NSG) mice11, that received identical treatment. Thus, epigenetic reprograming elicits potent tumour immunity and blocks cancer progression.

We have demonstrated the relevance of the inhibitory B7–H1 (PD-L1) signalling blockade in human cancer2,3. In the second setting, we tested the role of DZNep and 5-aza-dc in anti-tumour immunity elicited by PD-L1 blockade. We observed reduced tumour volume in mice treated with anti-PD-L1 or DZNep plus 5-aza-dC. The combination reduced tumour volume, increased tumour-infiltrating CD8^{+} T cells and T_{H1}-type chemokine expression (Extended Data Fig. 1g–i). Thus, changes in the epigenetic program can augment the therapeutic efficacy of PD-L1 blockade therapy.

In the third setting, we examined the effects of these two epigenetic modulators on adoptive T-cell therapy in our NSG model. We established human ovarian cancer in NSG mice and generated tumour-associated antigen (TAA)-specific CD8^{+} T cells12-14. Then, the NSG mice were treated with DZNep and 5-aza-dC, and/or TAA-specific CD8^{+} T cells. In the absence of TAA-specific T cells, treatment with DZNep and 5-aza-dC had minimal effects on tumour progression. TAA-specific CD8^{+} T cells reduced tumour volume (Extended Data Fig. 1j). Consequently, treatment with DZNep and 5-aza-dC improved the therapeutic efficacy of T-cell therapy, and elevated T_{H1}-type chemokine expression, and tumour effector T-cell infiltration (Extended Data Fig. 1j–l). GS126 is a selective inhibitor of EZH2 methyltransferase activity15. We used GS126 in the human T-cell therapy setting. Treatment with low doses of GS126 and/or 5-aza-dC had no effect on tumour growth in the absence of T-cell transfusion (Extended Data Fig. 1m). Treatment with GS126 and 5-aza-dC synergistically improved the therapeutic efficacy of T-cell therapy (Fig. 1a and Extended Data Fig. 1m), increased tumour CXCL9 and CXCL10 expression (Fig. 1b, c), and CD8^{+} T-cell infiltration (Fig. 1d, e and Extended Data Fig. 1n), and had minimal effects on TNFα and IFNγ expression (Extended Data Fig. 1o–q). Administration of anti-human CXCR3 abrogated the role of GS126 and 5-aza-dC treatment in tumour progression (Fig. 1a), and blocked T-cell tumour trafficking (Fig. 1d, e), and had no effect on T-cell apoptosis (Extended Data Fig. 1r, s). CXCR3^{+} CD8^{+} T cells were observed in the spleen (Extended Data Fig. 1t) and blood (Fig. 1e) with or without anti-CXCR3 treatment. To determine the major source of T_{H1}-type chemokines, we isolated human tumour and immune cells from tumour tissues in the NSG mice (Fig. 1a–e). We found that administration of GS126 and 5-aza-dC increased tumour CXCL10 expression with or without T-cell transfusion (Fig. 1f). Regardless of the treatment, the levels of CXCL10 expression were higher in tumour than immune cells (Fig. 1f). We extended our studies to tumour and immune cells from ovarian cancer patients. Although EZH2 may regulate naive CD4^{+} T-cell interferon-γ (IFNγ) expression16, GS126 and 5-aza-dC treatment increased CXCL10 production by tumour, not by T cells (Fig. 1g), and tumour cells produced higher levels of CXCL10 than immune cells (Fig. 1g). Thus, epigenetic reprograming may predominantly target tumour T_{H1}-type chemokine expression.

Next, we investigated how epigenetic modulators regulate tumour T_{H1}-type chemokine expression. We initially examined the effect of DZNep on T_{H1}-type chemokine expression in primary human ovarian...
cancer cells in response to IFNγ treatment. DZNep promoted CXCL9 and CXCL10 mRNA and protein expression (Extended Data Fig. 2a–c), and had no effect on IFNγ receptor (IFNGR2) (Extended Data Fig. 2d). We tested two inhibitors of histone H3 lysine 9 methyltransferase G9a, BIX01294 and UNC0638. The inhibition of G9a had no effect on T H 1-type chemokine expression (Extended Data Fig. 2e). Thus, methylation of H3K27 may mediate T H 1-type chemokine repression in cancer.

EZH2 is the catalytic subunit of the H3K27 methyltransferase complex14,17. DZNep treatment reduced EZH2 protein expression and H3K27 trimethylation (H3K27me3) in primary ovarian cancer cells (Extended Data Fig. 2f). Similarly, EPZ6438 reduced H3K27 trimethylation and increased CXCL9 expression in mouse ID8 cells (Extended Data Fig. 2g, h). We genetically knocked down human EZH2 expression with lentivirus-based short hairpin RNA targeting EZH2 (shEZH2). shEZH2 reduced the expression of EZH2 and H3K27me3 (Extended Data Fig. 2i), and resulted in elevated T H 1-type chemokine mRNA and protein expression (Fig. 2a, b), and no change in IFNGR2 and HLA-B (Extended Data Fig. 2j, k). Thus, EZH2 and its histone methyltransferase activity mediate T H 1-type chemokine repression in primary ovarian cancer cells.

We tested whether EZH2-mediated T H 1-type chemokine repression depends on H3K27me3 changes at the promoter level. Chromatin immunoprecipitation (ChIP) assays revealed that IFNγ treatment reduced H3K27me3 levels on the promoters of CXCL9 and CXCL10 (Extended Data Fig. 2l, m). DZNep (Extended Data Fig. 2l, m) and shEZH2 (Extended Data Fig. 2c) largely removed H3K27me3 on the promoters of CXCL9 and CXCL10, and subsequently increased IFNγ-induced chemokine gene expression (Fig. 2a, b). As a positive control, shEZH2 reduced H3K27me3 occupancy on the promoter of the HOXB1 gene (Extended Data Fig. 2n)18. Thus, H3K27me3 removal results in the abrogation of the T H 1-type chemokine gene silencing in primary ovarian cancer.

GSK126 is a selective inhibitor of EZH2 methyltransferase activity15. GSK126 treatment abolished the global level of H3K27me3 without inhibiting EZH2 (Extended Data Fig. 3a, b), IFNGR2 expression or cell survival (Extended Data Fig. 3c, d). GSK126 treatment led to higher levels of IFNγ-induced T H 1-type chemokine expression in two primary and three established ovarian cancer cell lines (Fig. 2d and Extended Data Fig. 3e–g). JmjC-domain-containing protein (JMJD3) is an H3K27-specific demethylase18. Ectopic expression of JMJD3 reduced H3K27me3 (Extended Data Fig. 3i), increased T H 1-type chemokine expression (Fig. 2e and Extended Data Fig. 3j), and had no effect on HLA-B and IFNGR2 expression (Extended Data Fig. 3k, l). JMJD3 deficiency introduced by a shJMJD3 inhibited CXCL10 (Fig. 2f), but not IFNGR2 expression (Extended Data Fig. 3m).

To define the gene profile altered by EZH2 and H3K27me3 in response to IFNγ, we performed several microarrays in primary ovarian cancer cells transfected with shEZH2, control, or treated with GSK126 and medium. We found that 155 and 124 genes were altered by shEZH2 and GSK126 treatment, respectively, and the expression of 20 genes was increased or decreased by both shEZH2 and GSK126 treatment. CXCL9 and CXCL10 were at the top 1 and 3 positions among the increased genes in the arrays (Fig. 2h). Altogether, the data indicate that H3K27me3 specific methyltransferase and demethylase preferentially and predominantly regulate T H 1-type chemokine repression in primary ovarian cancer cells.

DNA methylation regulates gene expression through DNA methyltransferases (DNMTs). We treated two primary and one established ovarian cancer cell line with 5-AZA-dC. 5-AZA-dC treatment increased CXCL9 and CXCL10 mRNA and protein expression (Fig. 3a–c and Extended Data Fig. 4a–d). IRF1 and IFNGR2 were not affected by 5-AZA-dC treatment (Extended Data Fig. 4e, f). Specific knockdown of DNMT1 (Extended Data Fig. 4g) increased CXCL9 and CXCL10 mRNA and protein expression (Fig. 3d–f), but had no effect on IFNGR2 (Extended Data Fig. 4h). To demonstrate DNA methylation status in the CXCL10 locus, we carried out bisulfite genomic sequencing on the CXCL10 gene locus and analysed the methylation at the CXCL10 gene loci containing the STAT1-binding site (Fig. 3g and
Extended Data Fig. 4i). The genomic location of bisulfite sequencing was 3′ upstream of the CXCL10 promoter (−5 kb to −4.7 kb). 5-AZA-dC treatment reduced DNA methylation of the CXCL10 gene locus (Fig. 3g). Thus, DNA methylation regulates T_{H}1-type chemokine expression.

We examined the relationship between EZH2/H3K27me3 and DNA methylation in the regulation of chemokine expression. We treated primary ovarian cancer cells with 5-AZA-dC or GSK126, and found that 5-AZA-dC reduced DNMT1 expression but had no effect on H3K27me3 or H3K9me2, whereas GSK126 reduced H3K27me3 but had no effect on DNMT1 expression (Fig. 4a). Thus, DNA methylation and EZH2/H3K27me3 histone modification may not reciprocally regulate at the protein level in primary ovarian cancer. We explored whether EZH2/H3K27me3 and DNA methylation could independently mediate T_{H}1-type chemokine expression. We examined the effects of 5-AZA-dC on shEZH2 cells, and observed that 5-AZA-dC enhanced the expression of T_{H}1-type chemokine genes.

**Figure 3** | DNA methylation regulates T_{H}1-type chemokine expression. a–c, Effects of 5-AZA-dC on T_{H}1-type chemokine expression. Primary ovarian cancer cells were treated with 5-AZA-dC and IFNγ for 24 (a, b) or 48 (c) hours. CXCL9 and CXCL10 transcripts (a, b) and protein (c) were quantified by real-time PCR or ELISA (mean ± s.d., n = 7, *P < 0.05, Wilcoxon test). d–f, Effects of shDNMT1 on T_{H}1-type chemokine expression. Primary ovarian cancer cells were transduced with shDNMT1 or non-target shRNA and stimulated with IFNγ. Chemokine transcripts (d, e) and protein (f) were quantified by real-time PCR and ELISA (mean ± s.e.m., n = 5, *P < 0.05, Wilcoxon test). g, Effects of 5-AZA-dC on DNA methylation on the promoter of CXCL10. Schematic diagram of CXCL10 gene locus is shown (top). Primary ovarian cancer cells were treated with 5-AZA-dC. DNA methylation at CpG sites was quantified by bisulfite sequencing. Results are shown as the percentage of methylation. Filled circle, methylated; open circle, unmethylated. The arrows indicate the locations of primers. All gene expressions are represented as relative values normalized to GAPDH mRNA level.
Figure 4 | EZH2/H3K27 and DNMT1 interaction and its impact on clinical outcome. a, Effects of GSK126 and 5-AZA-dC on H3K27me3 and DNMT1. Primary ovarian cancer tissues were treated with GSK126 and/or 5-AZA-dC for 48 h. Histone marks and DNMT1 were detected by western blotting. b–d, Effects of 5-AZA-dC and GSK126 on tumour CXCL10. shEZH2 (b), shDNMT1 (c) expressing cells or primary ovarian cancer cells (d) were treated with 5-AZA-dC, GSK126 and/or DZNep for 1–2 days followed by IFNγ stimulation for an additional 24 h (b, c) or 48 h (d). CXCL10 was quantified by real-time PCR (b, c) and ELISA (d) (mean ± s.e.m., n = 5–6, \( P < 0.05 \), Wilcoxon test). e, f, The association between EZH2 (e), DNMT1 (f) and overall survival; \( P < 0.0001 \). g, Impact of EZH2, DNMT1 and CD8+ T cells on overall survival. The ROC curve analysis was applied to evaluate the predictive accuracy of each marker for overall survival (\( t = 60 \) months). h, Impact of EZH2 and DNMT1 on overall survival. EZH2\textsuperscript{low}DNMT1\textsuperscript{low} (n = 50), EZH2\textsuperscript{high}DNMT1\textsuperscript{high} (n = 57), EZH2\textsuperscript{low}DNMT1\textsuperscript{high} (n = 32), and EZH2\textsuperscript{low}DNMT1\textsuperscript{low} (n = 66) were compared. Log-rank test, \( P < 0.0001 \). i, The Pearson correlation between intratumoural CD8+ T cells and overall survival; \( P < 0.0001 \). j, The association between EZH2 and DNMT1 (n = 168, \( r = 0.24 \), \( P = 0.002 \)) (j) and DNMT1 (n = 170, \( r = 0.28 \), \( P = 0.0003 \)) (k) and the accumulating levels of EZH2 and DNMT1 (n = 168, \( r = -0.32 \), \( P < 0.0001 \)) (l). Gene expression levels are represented as relative values normalized to GAPDH mRNA level.

CXCL10 expression (Fig. 4b). Similarly, GSK126 increased CXCL10 expression in shEZH2 cells (Fig. 4c). Primary ovarian cancer cells released higher levels of CXCL10 in the combinatorial treatment of DZNep and 5-AZA-dC compared to either treatment alone (Fig. 4d). These data suggest that H3K27me3 and DNA methylation can independently repress tumour T\textsubscript{H1}1-type chemokine expression.

We studied the interaction between EZH2 and DNMT1 in the ovarian cancer microenvironment and its clinical relevance (Extended Data Table 1). We quantified the nuclear levels of EZH2 and DNMT1 (Extended Data Fig. 5a, b) via immunohistochemistry in human ovarian cancer tissues by the H-score method\textsuperscript{14} and analysed their impact on patient survival (Extended Data Table 1). Based on the median values of EZH2 and DNMT1 intensity, we divided patients into ‘low’ and ‘high’ groups. Overall survival and disease-free-interval (DFI) (Extended Data Table 1) were shorter in patients with high levels of EZH2 (Fig. 4e and Extended Data Fig. 5c) and DNMT1 (Fig. 4f and Extended Data Fig. 5d), compared to patients with low levels of these markers. After adjusting for the prognostic clinical factors, overall survival and DFI remained shorter in patients with high EZH2 and DNMT1 expression (Extended Data Table 2). Tumour EZH2 and DNMT1 were positive predictors of risk of death in univariate (Extended Data Table 1) and multivariate analyses (Extended Data Table 2). The reduced overall survival and DFI were more pronounced in the combination of high levels of EZH2 and DNMT1 than EZH2 or DNMT1 alone (Extended Data Table 3).

To estimate the performance of EZH2 and DNMT1 on predicting survival, we used the time-dependent receiver operating characteristic (ROC) curve analysis\textsuperscript{20}. The area under the ROC curve (AUC) is calculated to evaluate the predictive accuracy of each marker for estimating survival. The analysis revealed similar AUCs for EZH2 and DNMT1 in predicting overall survival and DFI (Fig. 4g, Extended Data Fig. 5e and Extended Data Table 4). The expression of EZH2 and DNMT1 may be equally important in ovarian cancer pathology. Next, we evaluated significance of the two parameters for ovarian cancer survival. Given the negative and independent impact of EZH2 and DNMT1, we reasoned that both parameters are more efficient at predicting survival than individually. Indeed, EZH2\textsuperscript{high}DNMT1\textsuperscript{high} patients experienced a shorter overall survival and DFI (Extended Data Table 3) than EZH2\textsuperscript{low}DNMT1\textsuperscript{low} patients in univariate and multivariate analysis (Fig. 4h). Patients with mixed patterns had similar and moderate survival (Fig. 4i and Extended Data Fig. 5f). Thus, the combination of EZH2 and DNMT1 allows for improved prognostic stratification of ovarian cancer survival as compared to EZH2 or DNMT1 alone. Next, we quantified intratumoural CD8\textsuperscript{+} T cells by immunohistochemistry (Extended Data Fig. 5g), and found that there was a correlation between tumour CD8\textsuperscript{+} T-cell content and survival (Extended Data Tables 1 and 2). We divided the patients into two groups based on the median values of CD8\textsuperscript{+} T-cell number. Patients with more CD8\textsuperscript{+} T cells experienced longer overall survival and DFI scores compared to those with fewer CD8\textsuperscript{+} T cells\textsuperscript{11,22} (Fig. 4i and Extended Data Fig. 5h). ROC curve analysis revealed similar power of CD8\textsuperscript{+} T cells in predicting overall survival (Fig. 4g) and DFI (Extended Data Fig. 5e).

Finally, we observed that the levels of EZH2 (Fig. 4j) and DNMT1 (Fig. 4k) were inversely associated with CD8\textsuperscript{+} T cells. The accumulating
levels of EZH2 and DNMT1 negatively correlated with the number of intratumoural CD8+ T cells (Fig. 4l). Altogether, the results suggest that tumours co-opt certain epigenetic pathways, silence T_{H1}-type chemokine expression and repress T-cell tumour homing, which functions as a novel immune-evasion mechanism.

To maintain cellular identity, through epigenetic mechanisms, key ‘stemness’ genes may be repressed in somatic cells, and key effector genes may be silenced in stem cells.11,24 Tumour cells may gain stem cell properties.11 This may explain why T_{H1}-type chemokines, as effector genes, are repressed in cancer. Epigenetic silencing is an intrinsic tumorigenic mechanism.1 We propose a unifying model of cancer in which epigenetic dysregulation has dual biological and immunological roles in supporting tumour progression. Cancer immunotherapies and classic therapies rely on efficient T-cell tumour trafficking, and may induce and/or expand TAA-specific T cells.29,30 Applicable, our work suggests that epigenetic reprogramming may condition tumours from poor T-cell infiltration to rich T-cell infiltration, and ultimately potentiate cancer therapy (Extended Data Fig. 5i).

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 9 December 2014; accepted 24 August 2015.

Published online 26 October; corrected online 11 November 2015 (see full-text HTML version for details).

Acknowledgements This work is supported (in part) by the NIH grants (CA190176; CA123088; CA099885; CA156685; CA193136; CA152470; CA171306; 5P30CA46592), the Rivkin Ovarian Cancer Center, and the Ovarian Cancer Research Fund. We appreciate discussion with B. Richardson. We thank L. Cabrera, D. Postiff, M. Vinco, R. Craig and J. Barikdar for their technical support. We are grateful for the professional help from W. Wu and C. Johnson in our Microarray Core and Bioinformatics Core. We particularly appreciate the support of B. Leclair and D. Leclair.

Author Contributions D.P. and W.Z. initiated and designed the research. D.P., I.K. and W.Z. wrote the manuscript. D.P., I.K., N.N., S.W., E.Z., L.V., W.W. and S.H.-A., A.M., K.C. and R.L. provided intellectual and technical support, clinical specimens, and clinical and pathological information.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to W.Z. (wzou@med.umich.edu).

© 2015 Macmillan Publishers Limited. All rights reserved
METHODS

Ovarian cancer patients, cancer tissue samples and primary ovarian cancer cells. Patients diagnosed with high-grade serous ovarian carcinomas were recruited for this study. Human subject use in this study was approved by the local Institutional Review Boards and informed consent was obtained from the patients. We collected 186 formalin-fixed, paraffin-embedded ovarian tumour tissue blocks (Extended Data Table 1) and 20 fresh ovarian cancer tissues for this study as described previously11,13,33. After pathological review, a tissue microarray (TMA) was constructed from the most representative area of paraffin-embedded ovarian tumour tissue. For each tumour, a minimum of two representative tumour areas were selected from a haematoxylin and eosin-stained section of a donor block. Core cylinders (1 mm) were punched from each of these areas and deposited into a recipient paraffin block. Consecutive 6-μm-thick TMA sections were cut and placed on charged poly-L-lysine-coated slides for immunohistochemistry analyses. Epithelial cell adhesion molecule-expressing (EpCAM+) primary tumour cells, CD3+CD45+ T cells, and CD45+ immune cells were isolated and sorted from fresh ovarian cancer tissues for functional studies. Primary human ovarian cancer cell lines (OC8 and OC17) were generated from fresh ovarian cancer tissues and/or ascites fluid in our laboratory as previously described11,13,33.

Three commercialized ovarian cancer cell lines A2780, CAOV3 and ES-2 were included in the study. The cell lines were routinely tested for mycoplasma contamination. All the commercially collected cell lines were authenticated by the supplier and used within ten passages. Primary ovarian cancer cells were authenticated in the laboratory.

Plasmids, shRNAs and antibodies. pCMV-HA-MJD3 plasmid was obtained from Addgene (#24167). Lentiviral shRNAs (Extended Data Table 5) were provided by the Vector Core at the University of Michigan or provided by A. Chinnayyan (University of Michigan). Antibodies including monoclonal anti-EZH2 (1:2000, BD Biosciences, 612667), anti-H3K27me3 (1:1000, Millipore, 07-449), H3K9me2 (1:2000, ab1220, Abcam), H3K9me3 (1:500, ab8898, Abcam), H3K4me1 (ab8895, Abcam), H3K4me2 (ab194678, Abcam), H3K4me3 (ab1012, Abcam), anti-Histone H3 (1:2000, Cell Signalling, 9715), anti-HA (1:200, Santa Cruz Biotechnology, sc-805), anti-DNMT1 (1:250, Abcam, ab13537) were used for western blotting. Anti-human CXCRC3 (1G6) blocking antibody was prepared from mouse hybridoma (ATCC, #HB-12330) by Hybrion Core at the University of Michigan.

Quantitative Real-time RT–PCR and microarray. RNA extracted with TRIzol (Invitrogen) was used for cDNA synthesis with high capacity-cDNA reverse transcript kit (Applied Biosystems, 4374966). Real-time RT–PCR was performed on the Eppendorf Realplex real-time PCR system or StepOne Plus real-time PCR system (Life Technologies). The mRNA was quantified and normalized to GAPDH. The specific primers used for Real-time PCR are listed in the Extended Data Table 5.

Gene expression microarray was carried out at the University of Michigan Microarray Core Facility using Affymetrix Human Gene ST 2.1 Chip according to the standard protocol. Data was analysed by the University of Michigan Bioinformatics Core Facility. Two biological replicates of each sample were prepared independently from primary human ovarian cancer cells for gene expression profiling. Genes with >1.5-fold changes were selected for further analysis.

Chromatin immunoprecipitation (ChIP). ChIP assay was performed as described previously11,13. Sonication was performed with the Misonix 4000 bath sonication unit at 15% amplitude for 10 min. ChIP-enriched chromatin was analysed by real-time PCR with SYBR Green Master Mix, and normalized to the internal control GAPDH. These experiments were started before T-cell transfection by intraperitoneal administration three times per week. In some cases, mice received CD8+ T cells which were pre- incubated with anti-CXCR3 for 1 h before in vivo transfection, followed by intraperitoneal administration of 300 μg anti-CXCR3 three times per week. Tumour growth was monitored and recorded. T cells and tumour infiltrating immune cells were isolated and studied by FACS, real-time PCR and/or immunohistochemistry. All animal protocols were approved by the University of Michigan Committee on Use and Care of Animals (UCUA).

Statistical analysis. Wilcoxon rank-sum tests were used to compare two independent groups, and for paired groups, Wilcoxon signed rank tests were used for the comparison. Correlation coefficients (Spearman correlation, denoted by ρ, for ordinal data and Pearson correlation, denoted by r, for continuous data), together with a P value (null hypothesis is that r/p is zero), were computed to measure the degree of association between biomarkers. log-rank test was used to compare time to tumour initiation between two groups. Overall patient survival was defined as the time from date of diagnosis to disease-related death. Survival functions were estimated using Kaplan–Meier methods. Cox’s proportional hazards regression was performed to model survival as a function of EZH2, DNMT1 and CD8+ T cells. The data were analysed as continuous or categorized values and classified as low and high based on the median values, or the combination of EZH2 and DNMT1 (classified as EZH2highDNMT1high and EZH2lowDNMT1low), after adjusting for age and stage. We assessed the adequacy of the Cox regression model. Graphical and numerical methods were described42.

We used ROC analysis to evaluate the predictive accuracy of the levels of EZH2 and/or the combination of EZH2 and DNMT1 (classified as EZH2highDNMT1high and EZH2lowDNMT1low), and outcome assessment unless state differently.

31. Zou, W. et al. Stromal-derived factor-1 in human tumors recruits and alters the function of circulating T cells. Cancer Res. 67, 1339–1346 (2001).
32. Kryczek, I. et al. IL-22CD4 T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase DNMT1. Int. J. Cancer 130, 29–39 (2012).
33. Roby, K. F. et al. Development of a syngeneic mouse model for events related to ovarian cancer. Cancer Genes 21, 585–591 (2020).
34. Lin, D. Y., Wei, L. J. & Ying, Z. Checking the Cox model with cumulative sums of martingale-based residuals. Biometrika 80, S57–S72 (1993).
Extended Data Figure 1 | Epigenetic reprogramming alters immunotherapy. a–c, Effects of DZNep and 5-AZA-dC on ID8 mouse ovarian cancer progression. The ID8 tumour-bearing-bearing mice (C57BL/6) were treated with DZNep and 5-AZA-dC. a, Tumour growth was recorded by bioluminescence imaging and quantified by calculating the total flux (photons per second). The representative images and tumour volume at day 22 are shown. Day 0, tumour inoculation. Mean ± s.e.m. per high-power field. b, Tumour growth was recorded and quantified by bioluminescence imaging. The representative bioluminescence images on day 15 (days after tumour inoculation) are shown. Mean ± s.e.m. per high-power field. c, Tumour growth was recorded and quantified by bioluminescence imaging. The representative bioluminescence images on day 24 are shown. Day 0, tumour inoculation. Mean ± s.e.m. per high-power field.

d–f, Effects of EPZ6438 and 5-AZA-dC on anti-PD-L1 immunotherapy. Anti-PD-L1 (10 mg/kg) was given to ID8 tumour-bearing mice (C57BL/6) with or without DZNep and 5-AZA-dC (5-Aza). Tumour growth was recorded. The representative images and tumour volume at day 18 are shown. The mice were treated with DZNep or 5-AZA-dC. Mean ± s.e.m.; n = 5 per group; *P < 0.05; one-way ANOVA.

g–i, Effects of GSK126 and 5-AZA-dC on T-cell immunotherapy. Ovarian-cancer-bearing NSG mice were treated with or without GSK126 and 5-AZA-dC, and received autologous human ovarian-cancer-specific CD8+ T cells. Circles represent each mouse from the group. Mean ± s.e.m.; n = 5 per group; *P < 0.05 Mann–Whitney U-test.

j–l, Effects of anti-CXCR3 on T-cell immunotherapy. Autologous tumour-specific human CD8+ T cells were transfused into ovarian-cancer-bearing NSG mice with or without DZNep and 5-AZA-dC treatment. The mice were treated with anti-CXCR3. j, Tumour volume was monitored. k, Tumor type chemokine expression was quantified by real-time PCR. i, Tumour-infiltrating T cells were determined by FACS. Mean ± s.e.m.; n = 5 per group; *P < 0.05 Mann–Whitney U-test. Total tumour-infiltrating CD8+ T cells were normalized to the tumour volume (absolute number of CD8+ T cells per mm3 of the tumour). T-cell cytokine profile was determined by FACS via gating on human CD45+ CD8+ cells in tumour tissues. The percentages of TNFα+ and IFNγ+ cells are shown in CD8+ cells. Circles represent each mouse from the group. One of two experiments is shown. r–t, Effects of anti-α-CXCR3 on T-cell survival. Tumour-specific human CD8+ T cells were transfused into NSG mice. The mice were treated with anti-α-CXCR3. Peripheral blood annexin V+ CD8+ human T cells (mean ± s.e.m.; n = 5 per group) (r, s) and spleen CXCR3+ CD8+ human T cells (t) were determined by FACS.

© 2015 Macmillan Publishers Limited. All rights reserved
Extended Data Figure 2 | EZH2 controls T H 1-type chemokines. a, Effect of DZNep on CXCL10 transcript expression. Primary ovarian cancer cells were treated with DZNep in the presence of IFNγ for different time (hours). CXCL10 gene expression was quantified by real-time PCR. One of three experiments is shown. b, Effects of DZNep on ovarian cancer T H 1-type chemokine expression. Human primary ovarian cancer cells were treated for 24 (b) or 48 (c) hours with DZNep in the presence of IFNγ. CXCL9 and CXCL10 expression was quantified by real-time PCR (b) or detected by ELISA (c). (mean ± s.e.m.; n = 5, *P < 0.05, Wilcoxon test).

e, Effects of histone methyltransferase G9a/GLP inhibitors on T H 1-type chemokine expression. Primary ovarian cancer cells were treated with BIX01294 or UNC0638 in the presence of IFNγ for 24h. IFNGR2 expression was quantified by western blotting. One of three experiments is shown. f, Effects of EZH2 knockdown in primary ovarian cancer cells mediated by EZH2 shRNA. Primary ovarian cancer cells were stably transduced with a lentiviral shRNA expressing vector (non-target shRNA (Ctl) or EZH2 shRNA, shEZH2). The levels of EZH2 and H3K27me3 were detected by western blotting. g, h, Effects of EPZ6438 on histone marks (g) and CXCL9 mRNA expression (h). Mouse ID8 ovarian cancer cells were treated with EPZ6438 in the presence or absence of IFNγ for 48h. H3K27me3 and H3K9me2 were detected by western blotting. CXCL9 transcripts were quantified by real-time PCR. (Mean ± s.e.m.; four repeats, *P < 0.05, Wilcoxon test). i, EZH2 knockdown in primary ovarian cancer cells mediated by EZH2 shRNA. Primary ovarian cancer cells were stably transduced with a lentiviral shRNA expressing vector (non-target shRNA (Ctl) or EZH2 shRNA, shEZH2). The levels of EZH2 and H3K27me3 were detected by western blotting. j, k, Effects of EZH2 knockdown on IFNGR2 and HLA-B gene expression. Primary ovarian cancer cells were stably transduced with non-target shRNA (Ctl) or EZH2 shRNA (shEZH2). IFNGR2 (j) and HLA-B (k) gene expression was quantified by real-time PCR. (mean ± s.e.m.; n = 4).

l, m, Effect of DZNep on H3K27me3 occupancy at T H 1-type chemokine promoters. H3K27me3 ChIP assay was performed in primary ovarian cancer cells treated with DZNep with or without IFNγ. H3K27me3 levels at the gene promoter of CXCL9 and CXCL10 were normalized to the input (mean ± s.e.m.; n = 5, *P < 0.05, Wilcoxon test).
Extended Data Figure 3 | H3K27-specific methyltransferase and demethylase regulates T\(\text{H}1\)-type chemokine expression.  

**a**, Effects of GSK126 treatment on H3K27me3. Primary ovarian cancer cells were treated with GSK126 (0, 0.05, 0.2, 0.5, 2 or 10 \(\mu\)M) for 48 h. H3K27me3 was detected by western blotting. One of three experiments is shown. 

**b**, c, Effects of GSK126 on EZH2 and IFNGR2 transcript expression. Primary ovarian cancer cells were pre-treated with GSK126 for 48 h and stimulated with IFNγ for an additional 24 h. EZH2 (b) and IFNGR2 (c) transcripts were quantified by real-time PCR. Results are expressed as the mean ± s.e.m. Results from three experiments with duplicates are shown. 

**d**, Effects of GSK126 on cell apoptosis. Primary ovarian cancer cells were treated with GSK126 and stained with propidium iodide and annexin V. The percentage of apoptotic cells (annexin V-positive) was quantified. Mean ± s.e.m. from three experiments with duplicates. 

**e–h**, Effects of histone methyltransferase inhibitors on ovarian cancer T\(\text{H}1\)-type chemokine expression. Human primary ovarian cancer cells (OC17) (e), or ovarian cancer cell lines (ES2, CAOV3 and A2780) (f–h) were treated with GSK126 with or without IFNγ. CXCL9 and CXCL10 expression was measured by ELISA (e) or real-time PCR (f–h) (mean ± s.e.m., \(n = 5\), \(\star P < 0.05\), Wilcoxon test). I, Effects of ectopic JMJD3 on histone marks. Primary ovarian cancer cells were transiently transfected with plasmid encoding HA–JMJD3. Histone markers H3K9me2, H3K9me3 and H3K27me3 were detected by western blotting. One of three experiments is shown. 

**j**, Effect of ectopic expression of JMJD3 on CXCL10 transcript expression. Primary ovarian cancer cells were transiently transfected with vector or different amounts of HA–JMJD3 plasmids and stimulated with IFNγ. CXCL10 transcripts were quantified by real-time PCR. Results are expressed as the mean values ± s.e.m. Data represent three independent experiments. (\(n = 5\), \(\star P < 0.05\), Wilcoxon test). 

**k**, l, Effects of ectopic JMJD3 on IFNGR2 (k) and HLA-B (l) gene expression. Primary ovarian cancer cells were transiently transfected with plasmid encoding HA–JMJD3 and stimulated with IFNγ. IFNGR2 and HLA-B transcripts were quantified by real-time PCR. Results are expressed as the mean ± s.e.m. Data represent three independent experiments. 

**m**, Effect of JMJD3 knockdown on IFNGR2 gene expression. Primary ovarian cancer cells were stably transduced with a lentiviral shRNA specific for JMJD3 (shJMJD3) or non-target shRNA (Ctl), and stimulated with IFNγ. IFNGR2 transcripts were quantified by real-time PCR. Results are expressed as the mean ± s.e.m. Data represent three independent experiments. 

**n**, Effects of GSK-J4 on histone marks. Primary ovarian cancer cells were treated with GSK-J4 (10 \(\mu\)M) for 48 h. H3K27me3, H3K4me1, H3K4me2 and H3K4me3 were detected by western blotting. One of three experiments is shown. 

**o**, Effects of GSK-J4 on IFNGR2 gene expression. Primary ovarian cancer cells were pre-treated with GSK-J4 (10 \(\mu\)M) for 48 h and stimulated with IFNγ for an additional 24 h. IFNGR2 transcripts were quantified by real-time PCR. Results are expressed as the mean ± s.e.m. Data represent three independent experiments.
Extended Data Figure 4 | DNA methylation controls T helper 1-type chemokine expression. a, b, Effects of 5-AZA-dC on T helper 1-type chemokine expression. Human ovarian cancer cell line (A2780) or primary ovarian cancer cells (OC17) were treated with 5-AZA-dC and IFNγ. CXCL9 and CXCL10 expression were quantified by real-time PCR (a) or ELISA (b) (mean ± s.e.m., n = 6, *P < 0.05, Wilcoxon test). c–f, Effects of 5-AZA-dC on IFNγ associated gene expression. Primary ovarian cancer cells (OC8) were treated with 5-AZA-dC and IFNγ for 24 h. CXCL9 (c), CXCL10 (d), IRF1 (e) and IFNGR2 (f) transcripts were quantified by real-time PCR. Results are expressed as the mean ± s.e.m. One of three independent experiments is shown. g, DNMT1 knockdown via DNMT1 shRNA. Primary ovarian cancer cells (OC8) were transduced with a lentiviral shRNA specific for DNMT1 (shDNMT1) or non-target shRNA (Ctl). DNMT1 was detected by western blotting. h, Effect of DNMT1 knockdown on IFNGR2 expression. Primary ovarian cancer cells (OC8) were transduced with a lentiviral shRNA specific for DNMT1 (shDNMT1) or non-target shRNA (Ctl) and stimulated with IFNγ for 24 h. IFNGR2 transcripts were quantified by real-time PCR. Results are expressed as the mean ± s.e.m. Data represent three independent experiments. i, STAT1-binding site at CXCL10 gene promoter. STAT1 ChIP-seq data set from ENCODE/SYDH (top panel). K562 cells were treated with IFNγ for 30 min or 6 h. Non-treated GM12878 cells were used as control (no IFNγ). Gene Expression Omnibus accession numbers: GSM935487, GSM935488 and GSM935612. STAT1 occupancy at CXCL10 promoter (−5,143 to −4,699 base pairs) is shown as the peaks. Middle panel, schematic diagram of CXCL10 gene locus. STAT1-binding site (TTCCCGGAA) was predicted by TFSEARCH, score = 100. STAT1 ChIP-seq peaks overlap with the predicted STAT1-binding site (indicated as vertical lines). Bottom, homologous STAT1-binding site at CXCL10 gene promoter (Ensemble Genomic alignment).
Extended Data Figure 5 | EZH2/H3K27 and DNMT1 interaction affects clinical outcome. 

**Extended Data Table 1**

| Parameter | Value |
|-----------|-------|
| EZH2 low | DNMT1 low | DFS 1 year |
| EZH2 high | DNMT1 high | DFS 1 year |

© 2015 Macmillan Publishers Limited. All rights reserved
Extended Data Table 1  |  Patient characteristics and risk factors for overall survival and DFS

|                              | N   | P       | Hazard Ratio | 95% Hazard Ratio Confidence Limits |
|------------------------------|-----|---------|--------------|-----------------------------------|
| **Overall survival**         |     |         |              |                                   |
| Age: mean (min-max)          | 59  | (19-87) | 186          | 0.004                             |
| I, II                        | 30  | (16%)   |              | 1.020                             |
| III, IV                      | 152 | (82%)   | 0.0004       | 1.006                             |
| ND                           | 4   | (2%)    | 2.955        | 1.627                             |
| EZH2 ·                       |     |         |              |                                   |
| High                         | 103 |        | < 0.0001     | 3.019                             |
| Low                          | 83  |        |              | 2.083                             |
| DNMT1 ·                      |     |         |              |                                   |
| High                         | 90  |        | < 0.0001     | 3.49                              |
| Low                          | 83  |        |              | 2.383                             |
| CD8 ·                        |     |         |              |                                   |
| High                         | 97  |        | < 0.0001     | 0.263                             |
| Low                          | 89  |        |              | 0.181                             |

| **Disease free survival**    |     |         |              |                                   |
| Age: mean (min-max)          | 59  | (19-87) | 178          | 0.01                              |
| I, II                        | 28  | (16%)   |              | 1.016                             |
| III, IV                      | 146 | (82%)   | 0.0004       | 1.004                             |
| ND                           | 4   | (2%)    | 2.739        | 1.572                             |
| EZH2 ·                       |     |         |              |                                   |
| High                         | 99  |        | < 0.0001     | 2.112                             |
| Low                          | 79  |        |              | 1.487                             |
| DNMT1 ·                      |     |         |              |                                   |
| High                         | 86  |        | < 0.0001     | 2.08                              |
| Low                          | 80  |        |              | 1.46                              |
| CD8 ·                        |     |         |              |                                   |
| High                         | 90  |        | < 0.0001     | 0.422                             |
| Low                          | 88  |        |              | 0.298                             |

Note: univariate analysis.
* Categorized values of EZH2, DNMT1 and CD8 are based on the median.
Extended Data Table 2  |  Risk factors for overall survival and DFS

| Risk factors | P-value | Hazard ratio (HR) | 95% HR Confidence Limits |
|--------------|---------|------------------|--------------------------|
| Overall survival |         |                  |                          |
| EZH2*        | < 0.0001| 2.657            | 1.817 3.884              |
| Age          | 0.0435  | 1.014            | 1 1.029                  |
| Stage        | 0.0236  | 2.038            | 1.1 3.776                |
| DNMT1*       | < 0.0001| 3.217            | 2.182 4.743              |
| Age          | 0.1096  | 1.011            | 0.998 1.025              |
| Stage        | 0.0038  | 2.551            | 1.352 4.814              |
| CD8*         | < 0.0001| 0.31             | 0.211 0.455              |
| Age          | 0.117   | 1.011            | 0.997 1.024              |
| Stage        | 0.0215  | 2.134            | 1.118 4.074              |

Disease free survival

| Risk factors | P-value | Hazard ratio (HR) | 95% HR Confidence Limits |
|--------------|---------|------------------|--------------------------|
| EZH2*        | 0.0004  | 1.916            | 1.34 2.738               |
| Age          | 0.1321  | 1.01             | 0.997 1.024              |
| Stage        | 0.0126  | 2.096            | 1.172 3.749              |
| DNMT1*       | 0.0004  | 1.929            | 1.344 2.77               |
| Age          | 0.0912  | 1.011            | 0.998 1.025              |
| Stage        | 0.0116  | 2.104            | 1.181 3.748              |
| CD8*         | <.0001  | 0.471            | 0.329 0.673              |
| Age          | 0.1653  | 1.009            | 0.996 1.022              |
| Stage        | 0.0134  | 2.084            | 1.164 3.731              |

Note: multivariate analysis.

*Categorized values of EZH2, DNMT1 and CD8 are based on the median.
Extended Data Table 3 | The nested multivariate models of overall survival and DFS

| Overall survival models                  |   | likelihood rate statistics |
|-----------------------------------------|---|---------------------------|
| DNMT1 and EZH2 vs. DNMT1 alone*         | < 0.0001 | 35.5 |
| DNMT1 and EZH2 vs. EZH2 alone*          | < 0.0001 | 47.2 |

| Disease free survival                   |   | likelihood rate statistics |
|-----------------------------------------|---|---------------------------|
| DNMT1 and EZH2 vs. DNMT1 alone*         | < 0.0001 | 16.6 |
| DNMT1 and EZH2 vs. EZH2 alone*          | < 0.0001 | 17  |

*Categorized values of EZH2, DNMT1 are based on the median.
|                        | **DNMT1**       | **EZH2**       | **CD8**        |
|------------------------|-----------------|----------------|----------------|
|                        | *AUC ± s.e.*     | *AUC ± s.e.*   | *AUC ± s.e.*   |
| Overall survival       | 82.66 ± 3.34    | 83.63 ± 3.53   | 75.13 ± 4.18   |
| Disease free survival  | 81.06 ± 3.91    | 71.98 ± 6.28   | 76.53 ± 5.86   |

Extended Data Table 4 | The AUC values of ROCs (t = 60) for DNMT1, EZH2 and CD8
### Primers for quantitative Real-Time PCR

| Gene          | Sequence (5’-3’)         | Sequence (5’-3’)         |
|---------------|--------------------------|--------------------------|
| Human CXCL10  | CTCCAGTCTCAGCACCATGA     | GCTCCCCTCTGGTTTTAAGG     |
| Human CXCL9   | GTGGTGTTTTTTTCTCTTTGG   | ACAGCGACCCTTTCTCACTAC    |
| Human IFNGR2  | TCTCACTGCCGCCAGTCCCTCA   | GGGAGCCTTTCTCCTGGGTCA    |
| human HLA-B   | TCCTAGCAGTTGTGGTCATG     | TCAAGCTGTGAGAGACACAT     |
| Human EZH2    | TTCTAGCAACACCCACACCTT    | GGTGGGGTCTTTATCCGCTC     |
| Human IRF1    | GAGACCCTGGCTAGAGATGC     | CATGGCACAGCAGAAGATTGG    |
| Mouse CXCL10  | AATGAGGAGGCTAGTAGGAAGC   | AGCCATCCACTGGGTAAAGG     |
| Mouse CXCL9   | GAGCAGTGTGGAGTTCGAGG     | TCCGGATCTAGGCAAGGTTTG    |

### Primers for ChIP assay

| Gene  | Sequence (5’-3’)         | Sequence (5’-3’)         |
|-------|--------------------------|--------------------------|
| CXCL10| GGAATGTCTCAGAAAAACGTGGGGC| ACCTTCGAGTCTGCAACATGGGA  |
| CXCL9 | AGGGTTTTCCCCCAGCACAATCA  | ACCAGCAAGATGATGGCCCAAGAGG|
| HOXB1 | GGGTTGGGAGGGAAGGAAAG     | CCCATCCATCTGGAGAGCGAC    |

### Lentiviral shRNA

| shRNA   | Vector                  | Source                                      |
|---------|-------------------------|---------------------------------------------|
| shEZH2  | pGreen-shEZH2-1         | Kindly provided by Dr. Arul Chinnaiyan       |
|         | pGreen-shEZH2-2         | Kindly Provided by Dr. Arul Chinnaiyan      |
| shJMJD3 | pGIPz-shJMJD3 (V3LHS_301324) | Vector Core (University of Michigan)        |
|         | pGIPz-shJMJD3 (V3LHS_310328) | Vector Core (University of Michigan)        |
| shDNMT1 | pGIPZ-DNMT1_V2LHS_113503 | Vector Core (University of Michigan)        |
|         | pGIPZ-DNMT1_V2LHS_113505 | Vector Core (University of Michigan)        |