NF-κB-regulated VentX expression mediates tumoricidal effects of chemotherapeutics at noncytotoxic concentrations
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SUMMARY
Limited therapeutic efficacy and severe side effects represent the central hurdles facing cancer chemotherapy. Immune suppression within tumor immune microenvironments (TIME) has been implicated in chemoresistance. In this study, using a TIME-enabling model system (TIME-EMS), we demonstrate that the chemotherapeutic agent doxorubicin has cytocidal effects on tumor cells at high dosage but induces changes in the immune landscape of the TIME at low noncytotoxic concentrations via NF-κB-mediated induction of homeobox protein VentX expression in tumor-associated macrophages (TAMs). We demonstrated that VentX-regulated TAMs drastically promote tumor chemosensitivity >10-fold but exert little effect on chemotoxicity to normal cells through activating cytotoxic T lymphocytes in a tumor-specific manner. Supported by the in vivo synergy of VentX-regulated TAMs and low-dosage noncytotoxic doxorubicin, our data suggest a cell-death-independent immune mechanism for improving the therapeutic index of chemotherapeutic agents.

INTRODUCTION
Cytotoxic chemotherapy is the mainstay therapeutic modality for nonresectable metastatic cancers. Despite the proven efficacy of chemotherapeutic agents, their tumoricidal benefits are accompanied by cytotoxic effects on non-tumor tissues. Clinically, a variety of strategies have been explored over the years to minimize the severe side effects of chemotherapeutics. However, dosage-dependent cytotoxicity remains the major drawback of cancer chemotherapy (Opzoomer et al., 2019; Peters et al., 2005).

Historically, chemotherapeutic agents were developed by targeting cell autonomous mechanisms in two-dimensional cancer cell line cultures (DeVita and Chu, 2008). Recent advances in cancer immunotherapy have led to the appreciation of immunity in mediating the therapeutic effects of chemotherapy (De Palma and Lewis, 2013; Galluzzi et al., 2015). Over the past few decades, stromal cells were found to be a major component of tumors (Powley et al., 2020). Macrophages for example were found to make up more than 50% of tumor mass in a variety of cancer types (Noy and Pollard, 2014). Mechanistic studies suggested that immunogenic cell death (ICD) triggered by the cytotoxicity of chemotherapeutic agents plays an important role in mediating the function of chemotherapy (Kroemer et al., 2013) (Bracci et al., 2014). While the immune-suppressive tumor microenvironment (TME) has been implicated in chemoresistance (De Palma and Lewis, 2013; Galluzzi et al., 2015), without a practical platform to dissect the tumoricidal effect of chemotherapeutics in the context of the TME, there has been little improvement in the therapeutic index of chemotherapeutic agents for more than half a century.

We recently employed en bloc tumor culture models to explore the mechanisms underlying immune suppression within the TME. We found that expression of the homeobox protein VentX, a master regulator of macrophage phagocytosis and plasticity, is downregulated in tumor-associated macrophages (TAMs) (Le et al., 2018, 2020). We demonstrated that VentX controls TAM plasticity and that VentX-regulated TAMs govern immunity in the TME by modulating differentiation and activation of tumor-infiltrating lymphocytes (TILs) (Le et al., 2018, 2020). Using en bloc tumor and autologous TAM co-culture models, we showed that restoration of VentX expression in TAMs converts the immune landscape in the TME from suppression to activation by inhibiting CD4 differentiation into inhibitory Treg cells and by promoting proliferation and activation of cytotoxic CD8 T cells (Le et al., 2018, 2020). Based on our findings, we asked whether...
ex vivo en bloc tumor cultures could be used to evaluate the tumoricidal function of chemotherapeutic agents in the context of the TME.

Using doxorubicin (DOX) as a testing agent, we found that application of DOX to en bloc cultures of colorectal cancer (CRC) causes cytoidal effects on tumor cells at high dosage but induces changes in the immune landscape at low noncytotoxic concentrations via NF-κB-mediated induction of VentX expression in TAMs. We demonstrated that VentX-regulated TAMs drastically promote tumor chemosensitivity >10-fold but exert little chemotoxicity effect on normal cells. Mechanistically, we showed that VentX promotes TAM phagocytosis, which in turn activates cytotoxic T lymphocytes in a tumor-specific manner. A potential role of VentX-modulated TAMs (VentX-TAMs) in promoting the therapeutic efficacy of chemotherapeutic agents was suggested by the findings that VentX-TAMs promote low-dosage noncytotoxic DOX inhibition of tumorigenesis in pre-clinical NSG-PDX models of primary human CRC. Using ex vivo en bloc tumor tissues and TAM co-culture models, we showed that VentX-TAMs promote the tumoricidal function of noncytotoxic DOX on a broad spectrum of cancer types. In summary, our data revealed that a cell-death-independent immune mechanism mediates the tumoricidal effect of chemotherapeutics and suggested a novel venue for improving the therapeutic index of chemotherapy.

RESULTS
Tumoricidal effects of the chemotherapeutic agent DOX in en bloc tissue culture

Cytoidal effects on tumor cells underlie the therapeutic function of chemotherapeutic agents. Historically, chemotherapeutic agents have been developed by targeting cell autonomous mechanisms. Recently, advances in cancer immunotherapy have led to the appreciation of the power of immunity and the tissue microenvironment in chemotherapy for cancers (Bracci et al., 2014). However, in the absence of a practical testing platform, there have been limited mechanistic insights into the cytoidal function of chemotherapeutic agents in the context of the TME (Jo et al., 2018). DOX is a broad-spectrum chemotherapeutic agent and a potent inducer of VentX expression in both cancer cells and macrophages (Gao et al., 2016; Wu et al., 2011b) (Figure S1). Based on our recent employment of an en bloc tumor tissue culture model to study cancer immunotherapy, we asked whether such models could be exploited to study mechanisms underlying the tumoricidal function of DOX in the TME. To this end, en bloc colorectal cancers (CRC) or control non-tumor colon mucosal tissues from the same patients were incubated in RPMI-based media and treated with DOX for 3 days. Based on the results of prior toxicity studies, DOX concentrations of 1 and 10 μM were used as low and high concentrations, respectively (van Oosterwijk et al., 2012). The effects of the treatment on tumor or normal cells were then determined by PI staining and FACS analysis. As shown in Figure 1, we found that DOX treatment elicited dosage-dependent cytoidal effects on tumor cells in the en bloc tumor culture, with little cytotoxic effect at the low 1 μM concentration but significant cytotoxicity at the high 10 μM concentration (Figures 1A and 1C). Consistent with known cancer cell vulnerability to chemotherapeutic agents due to aberration in mechanisms that control adaptive stress response and cell death (Galluzzi et al., 2015; Solimini et al., 2007), we found that DOX treatment exerted less cytotoxic effect on normal epithelial cells in the tissue microenvironment (Figures 1B and 1C). As evidence of the potential involvement of VentX in DOX-induced tumoricidal effects in the TME, we found that DOX induced TAM VentX expression in the en bloc CRC culture in a dosage-dependent manner (Figure 1D).

VentX mediates DOX effects on the immune landscape of the tumor microenvironment at noncytotoxic concentrations

Though it has long been known that TAMs modulate tumor response to DOX, the underlying mechanisms remain poorly understood (De Palma and Lewis, 2013; Mantovani et al., 1979). VentX is a key regulator of TAM plasticity and immunity at the TME (Le et al., 2018, 2020; Wu et al., 2011a, 2014). Our finding that DOX effectively induces VentX expression in TAMs at the noncytotoxic concentration of 1 μM (Figure 1D) made us wonder whether DOX might also alter the immune landscape of TME at low noncytotoxic concentrations.

To test this hypothesis, en bloc CRC cultures were treated with noncytotoxic 1 μM DOX for 3 days and the effects on TAM phenotypes were determined by FACS analysis. We found that noncytotoxic DOX treatment shifted the population of TAMs from an M2-like phenotype to an M1-like phenotype by promoting the expression of M1 markers and cytokines but inhibiting the expression of M2 markers and cytokines in TAMs (Figures 2A–2D). Consistent with the effects of DOX on TAM plasticity, the effects of the treatment on the immune landscape of the TME were further found to encompass the alteration of TIL differentiation,
Figure 1. Tumoricidal effects of DOX in en bloc CRC culture
(A and B) En bloc CRC (A) or normal colon mucosa (B) were cultured in RPMI media and treated with 1 or 10 μM of DOX for 3 days, and single-cell suspensions were then generated through mechanical disruption. CRC cells and normal epithelial cells were stained with CK20 or EP4, respectively. The effects of the treatment on the apoptosis of CRC cancer cells or normal epithelial cells were determined by PI staining and FACS analysis. The data represent the mean ± SD of 4 independent experiments. "ns" indicates no statistically significant difference. "**" indicates p < 0.01 by one-way ANOVA analysis.

(C) En bloc CRC or normal colon mucosa were treated with DOX at indicated dosages for 3 days. The viability of cancer cells and normal epithelial cells were then determined as described above. The data represent the mean ± SD of 4 independent experiments. "*" indicates p < 0.05 by two-tailed t-test.

(D) qRT-PCR measurement of VentX mRNA expression in TAMs isolated from en bloc CRC treated with DOX at indicated dosages for 3 days and n = 5 independent patient samples. "ns" indicates no statistically significant difference. "**" indicates p < 0.01 by two-tailed t-test vs untreated samples.
including increased CD8 T cell activation but reduced CD4 T cell differentiation into Treg cells (Figures S2A and S2B). Consistent with the key regulatory role of VentX in mediating the effects of noncytotoxic DOX on TAM plasticity and the immune landscape of the TME, we showed that knocking down VentX expression in TAMs attenuated DOX effects on the expression of M1 and M2 markers and inhibited DOX-induced secretion of pro-inflammatory cytokines (Figures 2E–2G). The effects of VentX-modulated TAM in mediating DOX effects on the immune landscape were further revealed by the abolishment of DOX-induced alteration of TIL differentiation by VentX-modified TAMs (Figures S2C and S2D).

NF-κB mediates DOX-induced VentX expression in TAMs
To determine the mechanism underlying DOX-induced VentX expression, we analyzed the VentX promoter with the ECR Browser and identified three potential NF-κB binding sites (Figure 3A). NF-κB is a key regulator of macrophage function in the pathogenesis of inflammation and cancers (Ben-Neriah and Karin, 2011; Biswas and Lewis, 2010). Previous studies suggested that NF-κB plays diverse roles depending on cell type (Arlt et al., 2001; Wang et al., 2002). To determine whether NF-κB plays a role in DOX activation of VentX expression in monocytes, we examined the effects of DOX on NF-κB expression in primary human monocytes and found that it induces NF-κB expression in a dosage-dependent manner (Figure S3). Consistent with the role of NF-κB in mediating DOX induction of VentX expression, we found that VentX expression in TAMs correlates with DOX-induced NF-κB expression and is blocked by NF-κB inhibitors (Figure 3B). Supporting the role of NF-κB as a transcription factor that mediates DOX induction of VentX expression, we showed that DOX promoted the interaction of NF-κB with the VentX promoter, and CHIP analysis showed that enhanced binding between NF-κB and VentX promoter was abolished by NF-κB inhibitors (Figure 3C). The direct interaction between NF-κB and the VentX promoter was further supported by EMSA and ELISA findings that mutations of the potential proximal NF-κB binding site sequence attenuated the interaction between NF-κB and the VentX promoter probe (Figures 3D and 3E).

VentX-TAMs promote cytotoxic function of DOX in a tumor-specific manner
The effect of immunogenic cell death (ICD) in mediating the therapeutic effects of chemotherapeutic agents has been well appreciated, and its underlying mechanisms have been linked to events triggered by chemo-cytotoxicity (Kroemer et al., 2013). Our finding that DOX induces VentX expression in TAM at noncytotoxic concentrations prompted us to explore whether VentX-regulated TAMs (VentX-TAMs) enhance the therapeutic efficacy of DOX. To this end, en bloc CRC tumor or control colon mucosal tissues were treated with DOX at the low noncytotoxic concentration of 1 μM and then co-cultured with TAMs transfected with VentX or control GFP. After 5 days’ co-culture, effects on the survival of tumor cells or control normal epithelial cells were determined by PI staining and FACS analysis. We found that at the noncytotoxic concentration of 1 μM, DOX alone exerts little cytotoxic effect on tumor cells. However, when VentX-TAMs were added, the efficacy of DOX against tumor cells increased >10-fold (Figures 4A, 4C, and 4D). Strikingly, we found that this additive cytotoxic effect of VentX-TAMs is tumor specific. In comparison with its effect on tumor tissues, VentX-TAMs did not enhance the cytotoxic effects of DOX on normal epithelial cells (Figures 4B and 4E).

To elucidate the mechanisms by which VentX-TAMs promote the cytotoxic effects of DOX against tumor but not normal cells, and on the strength of our prior finding that VentX promotes TAM phagocytosis, we hypothesized that VentX promotes CRC-TAM phagocytosis of CRC tumor cells, which in turn activate cytotoxic CD8 T lymphocytes in a tumor-specific manner. To test this hypothesis, CRC-TAMs were transfected with GFP or GFP-VentX and then incubated with CellTrace Yellow-labeled purified autologous CRC cancer cells or normal epithelial cells for 24 h. The effects of VentX on TAMs phagocytosis were determined by FACS analysis. As shown in Figure 5A, VentX promotes phagocytosis of both cancer and normal epithelial cells. After the phagocytosis step, we incubated VentX-TAMs with autologous CD8 T cells and...
found that phagocytosis of cancer cells but not normal epithelial cells by VentX-TAMs led to significant enhancement of CD8 T cell proliferation and activation (Figures 5B–5D).

**VentX-TAMs promote DOX inhibition of CRC tumorigenesis in NSG-PDX models of primary human CRC**

NSG-PDX models of primary human tumors are becoming powerful tools for evaluating the therapeutic efficacy of chemotherapeutic agents. Using NSG-PDX models of primary human tumors, our recent studies showed that VentX-TAMs exert strong inhibition on the tumorigenesis of primary CRC in a dosage-dependent manner (Le et al., 2018). Our finding that VentX-TAMs promote the tumoricidal effects of DOX ex vivo prompted us to examine the potential synergy between VentX-TAMs and DOX in combination therapy for CRC in vivo. NSG-PDX models of primary human CRC were developed in the laboratory (Le et al., 2018). One week post implantation of primary CRC tumors, mice were tail-vein

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**Figure 3. NF-κB mediates DOX-induced VentX expression**

(A) Location of 3 putative NF-κB binding sites on the VentX promoter as revealed by ECR Browser analysis. (B and C) NF-κB mediates DOX induction of VentX expression. Monocytes in RPMI medium were pre-incubated with NF-κB inhibitor FeTBAP (10 μM) or PBS as a control for 2 h and then treated with 1 μM DOX for 24 h. The effects of the treatment on the expression of VentX mRNA were determined by qRT-PCR. The data represent the mean ± SD of 6 independent experiments. ‘∗’ indicates p < 0.05, and ‘∗∗’ indicates p < 0.01 by two-tailed t-test (B). The effect of the treatment on the interaction between NF-κB p65 and the VentX promoter was determined by CHIP analysis (C). (D and E) (D) Gel shift and ELISA (E) analysis showed the binding of NF-κB to the wild-type (WT) but not the mutated (Mut) VentX promoter probe. ‘∗∗’ indicates p < 0.01 by two-tailed t-test.
Figure 4. Effects of VentX-TAMs on the tumoricidal effects of DOX in en bloc CRC culture

(A and B) En bloc cultures of colon cancer (A) or control non-tumor tissues (B) were treated with 1 μg/mL DOX or PBS as a control and then co-cultured with autologous TAMs transfected with GFP-VentX or control GFP. After 5-day co-culture, single-cell suspensions were obtained through mechanical disruption.
injected with low dosages of VentX-TAMs. Three days later, low dosages of DOX (1.5 mg/kg) were given through tail-vein injection and repeated two weeks later following an established protocol (Podyacheva et al., 2021) (Figure 6A). The growth of the implanted tumors was observed for up to six weeks. As shown in Figure 6, while low dosages of DOX exerted small but discernible inhibition on CRC tumorigenesis in vivo, the inhibitory effects of low-dosage DOX on CRC tumorigenesis were significantly enhanced by the addition of low-dosage VentX-TAMs. The combination regimen of low-dosage DOX and VentX-TAMs was well tolerated, suggesting a potential novel approach to improve the efficacy of chemotherapeutic agents.

**VentX-TAMs promote tumoricidal function of DOX across a broad spectrum of tumor types**

Originating from investigations of the mechanisms of dorsoventral axis formation during early vertebrate embryogenesis, our studies led to the appreciation of a central regulatory role of VentX in governing immunity within the TME (Gao et al., 2007, 2010, 2012; Le et al., 2018, 2020; Wu et al., 2011a, 2011b, 2014, 2017; Zhu and Kirschner, 2002). Besides CRC, we have demonstrated that VentX-TAMs govern immunity within the TME of pancreatic ductal adenocarcinoma (PDAC) and non-small-cell lung cancer (NSCLC) (Le et al., 2020) (unpublished data). As DOX is a broad-spectrum chemotherapeutic agent, we asked whether the addition of VentX-TAMs promotes the tumoricidal effects of DOX more broadly. Using en bloc culture models of PDAC, NSCLC, esophageal, stomach, and ovarian cancers, we found that (similar to CRC) VentX-TAMs promote the tumoricidal function of low-dosage noncytotoxic DOX on all tumor types tested (Figure 7).

**DISCUSSION**

Human tumor tissues have been used to study chemotherapy response for more than three decades (Freeman and Hoffman, 1986). However, despite their proven accuracy in predicting clinical outcome in a variety of tumor types (Hayashi et al., 2009; Vescio et al., 1991; Yoshimasu et al., 2007a, 2007b), ex vivo cultures of human tumor tissues have yet to become a mainstream testing model for cancer drug development, in part due to the technical difficulties and the limited understanding of the complex molecular and cellular mechanisms involved. In comparison with two-dimensional cancer cell line culture models, patient-derived tissue culture models offer the advantage of preserving the intact architecture of both tumor cells and surrounding stromal cells, thereby allowing the evaluation of drug effects under physiological and patient-specific condition. On the other hand, the limited availability of patient-derived tissues makes them unsuitable for high-throughput drug screens. Moreover, culturing en bloc human tumor tissue cultures has proven technically challenging (Powley et al., 2020). In our current study, using a simplified culture model of en bloc tumor tissues, we demonstrated their feasibility as a tumor immune microenvironment (TIME) enabling model system (TIME-EMS) to evaluate the function and mechanism of the effects of chemotherapeutic agents on tumor and immune cells in the context of the TME. Using the TIME-EMS, we demonstrated that alteration of the immune status of the TME can drastically enhance the therapeutic efficacy of chemotherapeutics 10-fold, thereby allowing chemotherapeutics to exert their therapeutic function at noncytotoxic concentrations.

Employing the TIME-EMS allowed us to appreciate a novel cell-death-independent mechanism in mediating the therapeutic function of DOX. Previously, immunogenic cell death (ICD), which involves chemotherapeutics-induced destruction of cancer cells, the engulfment of the cancer cell fragments by dendritic cells (DCs), and the activation of cytotoxic T cells by DCs through cross priming, has been suggested as the mechanism underlying the immune-stimulatory function of cytotoxic chemotherapeutics (Bracci et al., 2014; Correale et al., 2005; Ma et al., 2013). Our findings that DOX, even at noncytotoxic concentrations, can transform the immune landscape of CRC through NF-κB-mediated induction of VentX expression in...
Figure 5. VentX-TAMs promotes CD8+ T cell activation in a tumor-specific manner

(A) VentX promotes the TAM phagocytosis of cancer and normal epithelial cells. CRC-TAMs were isolated and transfected with plasmids encoding GFP or GP-VentX. The transfected TAMs were then incubated with 1 μM CellTrack Yellow-labeled cancer or normal epithelial cells at a 1:1 ratio for 24 h. The rate of phagocytosis was then determined by flow cytometry. The data represent the mean ± SD of 3 independent experiments. “ns” stands for no statistically significant difference, and “∗∗∗” indicates p < 0.01 by one-way ANOVA analysis.

(B) Cancer-specific stimulation of CD8+ T cells proliferation by VentX-TAMs. VentX-TAMs were mixed with CRC cancer or normal epithelial cells for 24 h and then co-cultured with CellTrack Yellow-labeled autologous CD8+ TIL at a ratio of 1:10 for 5 days. The effects of the incubation on CD8+ T cell proliferation were determined by FACS analysis. A representative figure was shown. The data represent the mean ± SD of 3 independent experiments, “∗∗∗” indicates p < 0.01 by Student’s t test.

(C and D) Tumor-specific activation of CD8+ T cells by VentX-TAMs. VentX-TAMs were mixed with cancer or normal epithelial cells for 24 h and then co-cultured with autologous CD8+ TIL at a ratio of 1:10 (M:T) for 5 days. The effects of the incubation on CD8+ T cell activation were determined by FACS using APC-conjugated anti-IFNγ (C) or PE-conjugated anti-Granzyme B antibodies (D). The data represent the mean ± SD from 3 independent experiments, and “∗” indicates p < 0.05 while “∗∗∗” indicates p < 0.01 by paired Student’s t test.
CRC-TAMs suggested a mechanism of direct activation of antitumor immunity by chemotherapeutics in a cell-death-independent manner. As both the DOX induction of VentX expression in TAMs and the effects of VentX-TAMs on tumorigenesis are dosage dependent (Figure 1) (Le et al., 2018), the induction of VentX expression in TAMs by DOX at noncytotoxic concentrations underlies the synergy of low dosages DOX and VentX-TAMs against CRC tumorigenesis in vivo (Figure 6). VentX expression is significantly decreased in TAMs (Le et al., 2018, 2020). As such, the potential clinical importance of the study was suggested by our finding that restoration of VentX expression in TAMs leads to recovery of TAM phagocytosis, which in turn reinstates the immune surveillance function through cross-priming and activation of CD8 T lymphocytes in a tumor-specific manner (Figures 5B–5D). Though NF-κB has been regarded as a central regulator of macrophage function in tumors, its function and mechanism in tumorigenesis and treatment remain incompletely understood (Biswas and Lewis, 2010). Our findings that NF-κB mediates DOX activation of VentX at noncytotoxic concentrations provided a novel mechanistic insight into the well-appreciated immunogenic function of DOX (Galluzzi et al., 2015).

Figure 6. Effects of VentX-TAMs on DOX inhibition of CRC tumorigenesis in NSG-PDX models of primary human colorectal cancer

(A) A schematic of the experimental protocol. NSG-PDX models of primary human CRC were generated by subcutaneous implantation of small pieces of primary human CRC into the flanks of NSG mice. One week post implantation, the mice were tail-vein injected with VentX-TAMs. 3 days later 1.5 mg/kg DOX was tail-vein injected. DOX injections were repeated two weeks later.

(B) The growth of the CRC was observed for up to 6 weeks, and the results of the treatment are shown. The data represent the mean of 4 independent experiments. “*” indicates p < 0.05, and “**” indicates p < 0.01 by two-way ANOVA with multiple comparison.

(C) The growth curve of individual cases of CRC in NSG-PDX models after treatment.

(D) Representative images of tumors in each treatment group.
Figure 7. Effects of VentX-TAMs on the tumoricidal effects of DOX in en bloc tissue cultures of different cancer types

(A–E) En bloc cultures of PDAC (A), NSCLC (B), esophageal cancer (C), gastric cancer (D), and ovarian cancer (E) were treated with 1 μM DOX or control PBS and then co-cultured with autologous TAMs transfected with GFP-VentX or control.
Our findings using en bloc tissue and macrophage co-culture models suggest a potential application of the TIME-EMS for defining the functions and underlying mechanisms of chemotherapeutics and for testing strategies of combination therapies. Consistent with what we know about the role of immune-suppressive TMEs in chemoresistance and a central regulatory role of VentX-TAMs of immunity within the TME, our preliminary studies using TIME-EMS models suggest that VentX-TAMs promote the tumoricidal function of broad-spectrum chemotherapeutics. As indicated by the synergistic effects of low-dosage DOX and VentX-TAMs in inhibiting CRC tumorigenesis in vivo, further investigation of the molecular interaction between chemotherapeutics and VentX-modulated immunity may suggest novel approaches to improve the therapeutic index of cancer chemotherapeutics.

Limitations of the study
We are only beginning to understand the complex interaction between cancer chemotherapy and immunotherapy. Though our findings revealed a novel NF-κB-VentX axis in mediating DOX induction of antitumor immunity, our understanding of the molecular mechanisms underlying chemo-induced antitumor immunity remains limited. Our current study suggests the role of VentX-regulated phagocytosis in promoting chemosensitivity through tumor-specific activation of cytotoxic lymphocytes; however, the underlying mechanisms remain to be defined. Potential applications of the current findings await further exploration in clinical studies.

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AUTHOR CONTRIBUTIONS

Y.L., H.G., and Z.Z. designed the research and analyzed the data. Y.L., H.G., A.Z., and K.F. performed the experiments and prepared figures. R.B. and S.R. provided tumor samples and clinical information and analysis. Z.Z. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-CD4 (OKT-4), FITC | Thermo Fisher Scientific | Cat# 11-0048-42, RRID:AB_1633390 |
| Mouse monoclonal anti-CD4 (OKT-4), PE | Thermo Fisher Scientific | Cat# 12-0048-42, RRID:AB_2016675 |
| Mouse monoclonal anti-CD8 (SK1), FITC | BioLegend | Cat# 344704, RRID:AB_1877178 |
| Mouse monoclonal anti-CD8 (SK1), PE | BioLegend | Cat# 344706, RRID:AB_1953244 |
| Mouse monoclonal anti-CD14 (61D3), FITC | Thermo Fisher Scientific | Cat# 11-0149-42, RRID:AB_10597597 |
| Mouse monoclonal anti-CD25 (BC96), PE | Thermo Fisher Scientific | Cat# 12-0259-42, RRID:AB_16596682 |
| Mouse monoclonal anti-CD40 (5C3), PE | Thermo Fisher Scientific | Cat# 12-0409-42, RRID:AB_1963582 |
| Mouse monoclonal anti-CD68 (Y1/82A), FITC | Thermo Fisher Scientific | Cat# 11-0689-42, RRID:AB_11149303 |
| Mouse monoclonal anti-CD80 (2D10.4), PE | Thermo Fisher Scientific | Cat# 12-0809-42, RRID:AB_1311209 |
| Mouse monoclonal anti-CD163 (GHI/61), PE | Thermo Fisher Scientific | Cat# 12-1639-42, RRID:AB_1963570 |
| Mouse monoclonal anti-FOXP3 (236/A/E7), APC | Thermo Fisher Scientific | Cat# 17-4777-42, RRID:AB_10804651 |
| Mouse monoclonal anti-IFNγ (4S.B3), FITC | Thermo Fisher Scientific | Cat# 11-7319-41, RRID:AB_11043263 |
| Mouse monoclonal anti-Granzyme B (GB11), PE | Thermo Fisher Scientific | Cat# 12-8899-41, RRID:AB_1659718 |
| Mouse monoclonal anti-Cytokeratin 7 (SD12) | Thermo Fisher Scientific | Cat# MA5-15604, RRID:AB_10981428 |
| Mouse monoclonal anti-Cytokeratin 19 (A53-B/A2) | Thermo Fisher Scientific | Cat# MA5-18158, RRID:AB_2539332 |
| Mouse monoclonal anti-Cytokeratin 20 (Ks20.8) | Agilend | Cat# M0709, RRID:AB_2133718 |
| Mouse monoclonal anti-Epithelial Antigen (Per-EP4) | Agilend | Cat# M0804, RRID:AB_2335685 |
| Goat anti-mouse IgG (H+L) secondary antibody, FITC | Thermo Fisher Scientific | Cat# 62-6511, RRID:AB_2533946 |
| Rabbit monoclonal anti-NF-κB p65 (D14E12) | Cell Signaling Technology | Cat# 8242, RRID:AB_10859369 |
| Anti-CD8 monoclonal antibody for multiplexed IF (1:200) | Leica Biosystems | Cat# NCL-L-CD8 RRID:AB_563637 |
| Rabbit anti-PD-L1 (E1L3N) for multiplexed IF (1:300) | Cell Signaling Technology | Cat# 13684, RRID:AB_2687655 |
| Rabbit anti-FoxP3 (D6C8) for multiplexed IF (1:100) | Cell Signaling Technology | Cat# 12653, RRID:AB_2797979 |
| Rabbit anti-PD1 (EPR4877-2) for multiplexed IF (1:300) | Abcam | Cat# ab137132, RRID:AB_28948676 |
| Mouse anti-Cytokeratin (AE1/AE3) for multiplexed IF (1:100) | Agilent | Cat# M351501-2, RRID:AB_2631307 |
| Mouse anti-CD68 for multiplexed IF (1:100) | Agilent | Cat# M0876, RRID:AB_2074844 |
| **Biological samples** |        |            |
| Human cancers and their adjacent tissues | Brigham and Women’s Hospital | N/A |
| The blood samples | Brigham and Women’s Hospital | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Ficoll-Paque plus | Cell Signaling Tech. | Cat# 5872 |
| Collagenase D | Roche | Cat# 11088858001 |
| Trizol reagent | Life technologies | Cat# 15596026 |
| Propidium iodide staining solution | Thermo Fisher Scientific | Cat# 00-6990-50 |
| Doxorubicin | Sigma-Aldrich | D1515, CAS: 25316-40-9 |
| S-FU | Sigma-Aldrich | F6627, CAS: 51-21-8 |
| Retinoic acid | Sigma-Aldrich | R2625, CAS: 302-79-4 |
| Cisplatin | Sigma-Aldrich | P4394, CAS: 15663-27-1 |
| Gemcitabine hydrochloride | Sigma-Aldrich | G6423, CAS:122111-03-9 |
| Methotrexate | Sigma-Aldrich | A6770, CAS: 133073-73-1 |
| Imatinib mesylate | Sigma-Aldrich | SML1027, CAS: 220127-57-1 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources, reagents should be directed to and will be fulfilled by the lead contact, Zhenglun Zhu (zzhu@bwh.harvard.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study does not report the original code.

Data reported in this paper will be shared by the lead contact upon request.

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Bleomycin sulfate   | Sigma-Aldrich | 1076308, CAS: 9041-93-4 |
| Paclitaxel          | Sigma-Aldrich | T7191, CAS: 33069-62-4 |
| Hydroxyurea         | Sigma-Aldrich | H8627, CAS: 127-07-1 |
| Asparaginase        | Sigma-Aldrich | A3809; CAS: 9015-68-3 |
| Dexamethasone       | Sigma-Aldrich | D1756, CAS: 50-02-2 |

Critical commercial assays

| CellTrace yellow cell proliferation kit | Thermo Fisher Scientific | Cat# C34567 |
| Human macrophage nucleofector kit     | Lanzo                     | Cat# F12796 |
| Griess reagent kit                    | Thermo Fisher Scientific  | Cat# G7921 |
| EasySep human monocyte/macrophage kit | Stemcell Technologies     | Cat# 19359 |
| EasySep human CD8 T cell kit          | Stemcell Technologies     | Cat# 17953 |
| Human IL-1 beta ELISA kit             | Thermo Fisher Scientific  | Cat# 88-7261 |
| Human IL-12 p70 ELISA kit             | Thermo Fisher Scientific  | Cat# 88-7126 |
| Human TNF alpha ELISA kit             | Thermo Fisher Scientific  | Cat# 88-7346 |
| Fixation/Permeabilization buffer set  | Thermo Fisher Scientific  | Cat# 88-8823-88 |
| Superscript III reverse transcript kit | Thermo Fisher Scientific  | Cat# 18080-051 |
| TransAM NF-κB p65 activation assays   | Active Motif              | Cat# 40097 |
| TNT quick coupled transcription/translation system | Promega | Cat# L1170 |
| SimpleChip Plus Enzymatic Chromatin IP kit | Cell Signaling Technology | Cat# 9004 |

Experimental models: Organisms/strains

| NSG mouse: NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ | Jackson Laboratory | Cat# 005557 |

Oligonucleotides

| The primers for RT-PCR are listed in Table S1 | Thermo Fisher Scientific | N/A |
| Morpholino: VentX-MO; (TACTCAACCCTGACATAGAGGGTAA) | Gene Tools               | N/A |
| Wild type VentX probe for NF-κB p65 binding (CCGGGTCTCTTTCCTGGGGAAGGCCTCCGTGGGCTTGC) | Thermo Fisher Scientific | N/A |
| Mutant VentX probe for NF-κB p65 binding (CCGGGTCTCTTCCCTGGGTTCCGTGGGCTTGC) | Thermo Fisher Scientific | N/A |

Software and algorithms

| GraphPad prism v9.2 | Dotmatics | N/A |
| ImageJ             | NIH Image | https://imagej.nih.gov/ij/ |
| FlowJo v10.7       | Flowjo LLC | N/A |
| CaseViewer         | 3DHISTECH Ltd. | N/A |
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**NSG-PDX model of primary human colorectal cancers**

Animal models of primary human colon cancers were developed in the laboratory (Le et al., 2018; Mittal et al., 2015). Both male and female mice were used. All animal experiments were approved by the Institutional Animal Care and Use Committee at Brigham and Women’s Hospital. Briefly, 8-week-old NOD.Cg-Ptkdscid Il2rgrtm1Wjl/SzJ mice (commonly known as NOD scid gamma, or NSG mice) were purchased from Jackson Laboratory and maintained under specific pathogen-free conditions. Tumors were cut into around 0.5 cm pieces and then surgically implanted into subcutaneous spaces in the flanks of NSG mice. One week after implantation, 0.25 x 10^6 TAMs transfected with GFP-VentX or control GFP were injected into the mice through the tail vein. After three days, 1.5 mg/Kg of DOX or PBS control were tail-vein injected, which was repeated two weeks later. Tumor growth was monitored twice weekly and measured using a caliper for 6 weeks. Tumor volumes were calculated according to the formula ½ (length x width^2).

**Study approval**

All animal experiments were approved by the Institutional Animal Care and Use Committee at Brigham and Women’s Hospital; Animal Approval Number 2016N000353.

**Collection of human tissue samples**

All samples were collected from patients who were scheduled for surgical resection at Brigham and Women’s Hospital and consented to have a portion of resected tissues and blood collected for research purposes. Human materials from 75 individuals, including 45 male and 30 females, were used for the study. All patients signed an informed consent document that was approved by the Institutional Review Board of Brigham and Women’s Hospital. Around 5-10 grams of tissue were collected from tumor mass or normal tissues. Tumor samples and control tissues were verified by board-certified pathologists at the institution.

**Study approval**

The study using discarded human material was approved by the Institutional Review Board of Brigham and Women’s Hospital, Boston, MA. Tumor samples and control tissues were verified by board-certified pathologists at the institution. The IRB study Number is 2006P1354.

**METHOD DETAILS**

**Preparation of lymphocytes and macrophages from normal and tumor tissues**

Lymphocytes were isolated essentially as described (Kamada et al., 2008; Le et al., 2018; Rogler et al., 1998). Briefly, dissected fresh normal tissues and tumors were rinsed in a 10-cm Petri dish with Ca^{2+}-free and Mg^{2+}-free Hanks’ balanced salt solution (HBSS) (Life Technologies) containing 2% fetal bovine serum (FBS) and 2 mM Dithiothreitol (DTT) (Sigma-Aldrich). The normal and tumor tissues were then cut into approximately 0.1 cm pieces with a razor blade and incubated in 5 mL HBSS containing 5 mM EDTA (Sigma-Aldrich) at 37°C for 1 hour. The tissues were then passed through a gray-mesh (100 micron) filter. The flow-throughs containing lymphocytes and epithelial cells were then analyzed using flow cytometry.

To isolate the macrophages, normal and tumor tissues were rinsed with HBSS, cut into approximately 0.1 cm pieces using a razor blade and then incubated in HBSS (with Ca^{2+} and Mg^{2+}), containing 2% FBS, 1.5 mg/mL Collagenase D (Roche), 0.1 mg/mL Dnase I at 37°C for 1 hour. The digested tissues were then passed through a gray-mesh (70 micron) filter. The flowthroughs were collected, washed, and resuspended in RPMI 1640 medium. Normal tissue macrophages and TAMs were further purified using EasySep™ Human Monocyte/Macrophage Enrichment kit without CD16 depletion (StemCell Technologies, Cat# 19085) according to the manufacturer’s instructions. The isolation process does not lead to activation of macrophages, and the purity of isolated macrophages was above 95% (Kamada et al., 2008; Rogler et al., 1998; Wu et al., 2011a). More than 98% of cells isolated by the techniques were viable according to propidium iodide (PI) staining tests.
**FACS analysis**

Phenotypic analysis of macrophages and lymphocytes was performed using flow cytometry after immuno-labeling of cells with fluorescence dye–conjugated antibodies. Extracellular staining was performed for 30 minutes at 4°C. Intracellular staining was performed using fixation/permeabilization solution (Fisher Scientific) following the manufacturer’s protocol. Isotope control labeling was performed in parallel. Antibodies were diluted as recommended by the supplier. Labeled cells were acquired using the BD LSRFortessa at the Flow Cytometry Core of the Dana Farber Cancer Institute with the FACS Diva software (BD Biosciences) and analyzed using the FlowJo 10.7 software (Treestar). Typically, 20,000 cells were analyzed per sample according to the standard FACS analysis procedure. Compensation was performed with two or more fluorescence of antibody staining and the instrument was calibrated daily using CS&T beads. Gating was performed on live single cells. Results are expressed as the percentage of positive cells.

**En bloc tissue culture and drug treatment assays**

Tumor tissues were washed with 1x PBS buffer plus antibiotics and then cut into 0.5 cm pieces. Tissues were cultured in 2 mL of RPMI 1640 medium, supplemented with 5% FBS (Sigma) and 1% antibiotic-antimyotic solution (Gibco) in 24-well plates (Corning). The cultures were incubated at 37°C, 5% CO2 and treated with chemo-reagents at indicated concentrations or PBS control. For en bloc and macrophage co-culture experiments, autologous TAMs or macrophages were transfected with plasmid encoding GFP-VentX or the control GFP and then cultured in RPMI media (Le et al., 2018, 2020). 48 hours post transfection, 0.25 x 10⁶ GFP-VentX or GFP-transfected macrophages were added to the wells containing en bloc cultures of tumor or control non-tumor tissues. After gentle shaking, the plate was incubated at 37°C, 5% CO2 for 3-5 days. The tissues were then subjected to cell isolation. Single-cell suspensions were generated by mechanical disruption of the tissues, followed by filtering of cell suspensions through 70 um nylon mesh, staining with fluorescent conjugated antibodies, and analysis by flow cytometry or immunohistochemistry (Le et al., 2018, 2020).

**Cell viability assay**

Single-cell suspensions generated from en bloc tissue culture were washed with FACS staining solution (PBS plus 2% FBS), fixed and permeabilized (Invitrogen), and then stained with Ber-EP4 antibodies for normal epithelial cells and CK7, CK19, and CK20 for cancer cells for 30 minutes on ice. After washes with FACS staining solution, cells were stained with FITC-conjugated secondary antibodies for 30 minutes on ice. The cells were then washed again with FACS staining solution and fixed in 2% paraformaldehyde in PBS for 30 min or overnight. The cells were washed and resuspended in 200 ul of FACS staining solution, and 5 ul of PI staining solution (eBioscience/Fisher Scientific) was added to the solution for 15 minutes. Cell viability was then analyzed using flow cytometry.

**Quantitative RT-PCR**

Total RNA was isolated using the TRIzol reagent (Life Technologies), and RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Equal amount of RNA was used for first-strand cDNA synthesis with the SuperScript III First-Strand Synthesis System (Life Technologies) according to the manufacturer’s protocol. The AccuPrime Taq DNA polymerase system (Life Technologies) was used to amplify VentX cDNA with conventional PCR. Quantitative measurements of VentX and other cDNA were carried out with SYBR Green, using a Mastercycler ep Gradient S (Eppendorf). GAPDH was used as a housekeeping gene to normalize mRNA expression. The primers used are listed in Table S1. Relative expression profiles of mRNAs were calculated using the comparative Ct method (DDCT method).

**Transfection assays**

Plasmids encoding GFP-VentX and control GFP were generated in the laboratory (Gao et al., 2010). Transfection of GFP-VentX and GFP into macrophages was performed using the Human Macrophage Nucleofector Kit (Catalog #: WPA-1008, Lonza, Walkersville, MD). Briefly, 2x10⁶ cells were suspended into 100 µl nucleofector solution with 5 µg of plasmid DNA for 20 minutes on ice. Transfections were performed using a Amxara Nucleofector II Device (Lonza). After transfection, cells were placed on ice immediately for 1 minute and then cultured in pre-warmed RPMI 1640 complete medium, containing 10% FBS and 1% antibiotic-antimycotic solution (Gibco, Cat# 15240062) for 48 hours before cells were used for experiments.
ChIP assay

Human monocytes were treated with 1 μM DOX or PBS control for 24 h and harvested for ChIP assay (Upstate Biotechnology) following the manufacturer’s instructions. The NF-κB p65 antibody (Cell Signaling Technology, Cat# 8242) was used for immunoprecipitation. The Rabbit IgG antibody was served as a negative control. The VentX promoter region containing a putative NF-κB binding site was amplified with specific primers 5’-CGCGGAAAGACCGTCTTA-3’ and 5’- TGGGAGCAGGCTCCTCGGGGT-3’. All PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Gel shift assay

NF-κB p65 protein was generated with an in vitro translation kit (TNT Coupled Reticulocyte Lysate Systems, Promega). The NF-κB p65 plasmid with T7 promoter was used as a template and the translated NF-κB p65 protein was verified by western blot analysis. Gel shift assay was performed using a LightShift Chemiluminescent EMSA kit from Pierce Biotechnology, following manufacturer’s instructions. Double-stranded oligomers were generated by annealing the pairs of biotin-labelled synthetic oligonucleotides: 5’-CCCGGTCTCTTTCCC GGAAAGCC TCCCTCGGTTCCTGC-3’ (Wild type VentX probe, putative NF-κB p65 binding sequence is in bold and underlined); 5’-CCCGGTCTCTTTCCCTTTAAATCC TCCCTCGGTTCCTGC-3’ (Mutant VentX probe). The binding of NF-κB p65 to VentX promoter DNA was visualized with Odyssey imaging system (LI-COR Biosciences, USA).

NF-κB activation assay

Human monocytes were treated with DOX at indicated concentrations or PBS control. After 24 hours’ treatment, nuclear extracts were prepared using a nuclear extract kit (Active Motif, Cat. 40010). The DNA-binding activity of NF-κB p65 was determined using TransAm assays (Active Motif, Cat. 40097) according to the manufacturer’s instructions. Briefly, 2.5 μg nuclear extracts of each sample were incubated with immobilized NF-κB-specific oligonucleotides for 1 h. The p65 protein bound to DNA was then visualized by incubation with p65-specific antibody, HRP-conjugated secondary antibody and developing solution, and measured with a microplate reader with the absorbance at 450 nm.

NF-κB promoter binding assay

The binding of NF-κB to VentX promoter was quantified using an ELISA kit from eBioscience, according to the manufacturer’s instructions with modifications. Briefly, an anti-NF-κB p65 rabbit mAb (clone D14E12, Cell Signaling Tech) was immobilized on an ELISA plate. NF-κB p65 protein generated by the TNT kit and the biotin-labelled synthetic oligonucleotides of putative NF-κB binding sites of VentX promoter were added and incubated at RT for 60 minutes. The biotin-labelled DNA bound to the NF-κB p65 protein was then visualized by incubation with Avidin-HRP and developing solution. The absorbance at 450 nm was measured with a microplate reader. Triplicate wells were plated for each condition.

Immunohistochemistry

Immunohistochemistry was performed following established protocol (Wong and Chu, 2012). Briefly, colon tumors or normal tissues were fixed in formalin (Fisher Scientific Company, Kalamazoo, MI) for at least 48 hours. The tissues were then embedded in paraffin and sectioned. Haematoxylin/eosin (H&E) staining were performed at the Specialized Histopathology Core at Dana-Farber/Harvard Cancer Center. The images of whole slides were scanned by Pannoramic MIDI II digital slide scanner and analyzed with Caseviewer and Quant center software (3DHistech).

Multiplex immunofluorescence

Multiplex immunofluorescence (MIF) was performed with BOND RX fully automated stainers (Leica Biosystems). Tissue sections of 5-μm thick formalin-fixed, paraffin-embedded (FFPE) tissue sections were baked for 3 hours at 60°C before being loaded into the BOND RX. Tissue sections were deparaffinized (BOND DeWax Solution, Leica Biosystems, Cat. AR9590) and rehydrated with a series of graded ethanol to deionized water. Antigen retrieval was performed in BOND Epitope Retrieval Solution 1 (pH 6) or 2 (pH 9), as shown below (ER1, ER2, Leica Biosystems, Cat. AR9961, AR9640) at 95°C. Deparaffinization, rehydration, and antigen retrieval were all pre-programmed and executed by the BOND RX. Next, slides were serially stained with primary antibodies, such as anti-CD8 (clone 4B11; Leica, dilution 1:200). Incubation time per primary antibody was 30 minutes. Subsequently, anti-mouse plus anti-rabbit Opal Polymer Hors eradish Peroxidase (Opal Polymer HRP Ms + Rb, Akoya Biosciences, Cat. ARH1001EA) was applied as a
secondary label with an incubation time of 10 minutes. Signal for antibody complexes was labeled and visualized using their corresponding Opal Fluorophore Reagents (Akoya) by incubating the slides for 10 minutes. Slides were incubated in Spectral DAPI solution (Akoya) for 10 minutes, air dried, and mounted with Prolong Diamond Anti-fade mounting medium (Life Technologies, Cat. P36965) and stored in a light-proof box at 4°C prior to imaging. The target antigens, antibody clones, dilutions for markers are listed in the key resources table. Image acquisition was performed using the Vectra Polaris multispectral imaging platform (Vectra Polaris, Akoya Biosciences, Marlborough, MA). Representative regions of interest were chosen by the pathologist, and 3-5 fields of view (FOVs) were acquired at 20x resolution as multispectral images. Cell identification was performed (Carey et al., 2017). In short, after image capture, the FOVs were spectrally unmixed and then analyzed using supervised machine learning algorithms within Inform 2.4 (Akoya). This image analysis software assigns phenotypes to all cells in the image, based on a combination of immunofluorescence characteristics associated with segmented nuclei (DAPI signal). Each cell-phenotype specific algorithm is based upon an iterative training/test process, whereby a small number of cells (training phase, typically 15-20 cells) are manually selected as being most representative of each phenotype of interest and the algorithm then predicts the phenotype for all remaining cells (testing phase). The pathologist can over-rule the decisions made by the software to improve accuracy, until phenotyping is optimized. Thresholds for “positive” staining and the accuracy of phenotypic algorithms were optimized and confirmed by the pathologist for each case.

QUANTIFICATION AND STATISTICAL ANALYSIS

Student’s test or one-way ANOVA were used for statistical analysis in Prism version 9 (GraphPad, La Jolla, CA). Data are presented as mean ± standard deviation (SD). Tumor growth curves were analyzed by repeated measurement two-way ANOVA using Sidak’s multiple comparison test. The level of significance was indicated by the p value. In all figures, levels of statistical significance were indicated as: *p < 0.05, **p < 0.01.