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Inhibition of Histone Lysine-specific Demethylase 1 Elicits Breast Tumor Immunity and Enhances Antitumor Efficacy of Immune Checkpoint Blockade

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Abstract

Immunotherapy strategies have been emerging as powerful weapons against cancer. Early clinical trials reveal that overall response to immunotherapy is low in breast cancer patients, suggesting that effective strategies to overcome resistance to immunotherapy are urgently needed. In this study, we investigated whether epigenetic reprograming by modulating histone methylation could enhance effector T lymphocyte trafficking and improve therapeutic efficacy of immune checkpoint blockade in breast cancer with focus on triple negative breast cancer (TNBC) subtype. *In silico* analysis of TCGA data shows that expression of histone lysine specific demethylase 1 (LSD1) is inversely associated with the levels of cytotoxic T cell attracting chemokines (CCL5, CXCL9, CXCL10) and programmed death-ligand 1 (PD-L1) in clinical TNBC specimens. Tiling chromatin immunoprecipitation study showed that re-expression of chemokines by LSD1 inhibition is associated with increased H3K4me2 levels at proximal promoter regions. Rescue experiments using concurrent treatment with siRNA or inhibitor of chemokine receptors blocked LSD1 inhibitor-enhanced CD8+ T cell migration, indicating a critical role of key T cell chemokines in LSD1-mediated CD8+ lymphocyte trafficking to the tumor microenvironment. In mice bearing

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TNBC xenograft tumors, anti PD-1 antibody alone failed to elicit obvious therapeutic effect. However, combining LSD1 inhibitors with PD-1 antibody significantly suppressed tumor growth and pulmonary metastasis, which was associated with reduced Ki-67 level and augmented CD8+ T cell infiltration in xenograft tumors. Overall, these results suggest that LSD1 inhibition may be an effective adjuvant treatment with immunotherapy as a novel management strategy for poorly immunogenic breast tumors.

Keywords

LSD1/KDM1A; breast cancer; PD-1/PD-L1; LSD1 inhibitor; T-cell chemokine; immunotherapy

Introduction

Unlike some other types of tumors, breast cancer was thought to be non-immunogenic and the relevance of the host immune response to breast tumors has long been debated. A growing body of evidence has shown that some breast tumors, particularly the more aggressive TNBC, do elicit host antitumor immune responses, and the robustness of the response correlates with prognosis (1–3). Recent data from phase I trials with immune checkpoint inhibitors in TNBC patients reported an encouraging overall response (OR), up to 20%, with durable clinical responses (4–7). However, the majority of TNBC patients are still refractory to immunotherapy. This raises the question of whether combining immunotherapy with other approaches could augment clinical response rate for this devastating disease.

One of the best predictors of response to immunotherapy is the number and phenotype of tumor-infiltrating CD8+ cytotoxic T lymphocytes that are recruited at the tumor site by the locally secreted chemokines (8). Chemokines are a family of small heparin-binding proteins, which mediates immune cell trafficking and lymphoid tissue development (9). Among these chemokines, elevated levels of the C-C motif chemokine ligand 5 (CCL5) and T helper 1 (Th1)-type chemokines, C-X-C motif chemokine ligand 9 and 10 (CXCL9 & CXCL10), are frequently associated with increased recruitment of CD8+ T lymphocytes to tumor sites (10–12). A large body of evidence exists to show that increased expression of cytotoxic T cell attracting chemokines correlates with decreased levels of cancer metastasis and improved clinical outcome in cancer patients (13, 14). However, it is not well understood about the molecular mechanisms controlling down-regulation of cytotoxic T cell chemokine expression in cancer and how reduced expression of these chemokines subsequently deters effector T cell trafficking to the tumor microenvironment.

Epigenetic alterations are associated with all stages of breast tumor formation and progression (15, 16). The best characterized chromatin dysregulation is epigenetically-mediated transcriptional silencing which is typically associated with increased DNA methylation and histone function abnormalities (17, 18). Recent studies indicate that epigenetic dysregulation plays a critical role in silencing expression of certain effector T cell chemokines which may lead to inefficient recognition and elimination of cancer cells by the host immune system (11, 12). The results from these studies also suggest that aberrant

suppression of effector T cell chemokines could be reversed by epigenetic reprogramming, which may, in turn, improve T cell infiltration and expand the efficacy of immunotherapy. However, the nature of epigenetic silencing in governing cancer immunopathology and immunotherapy remains very elusive.

In this study, we explored the mechanisms of how epigenetic dysregulation of expression and activity of cytotoxic T cell chemokines impedes trafficking of antitumor immune cells and facilitates TNBC progression. We also investigated whether epigenetic agents could augment antitumor immune responses and improve therapeutic efficacy of immune checkpoint blocking antibodies. Our findings indicate that a key epigenetic modifier, Lysine-Specific Demethylase 1 (LSD1), plays an important role in mediating epigenetic reprogramming that alters the T cell landscape in TNBC. We have also put forth preclinical evidence that combined use of LSD1 inhibitor effectively enhances the therapeutic efficacy of anti PD-1 immunotherapy.

Results

Negative correlation between expression of LSD1 and immune regulatory genes in TNBC specimens

The correlation of expression between key epigenetic modifiers (histone deacetylases, histone lysine demethylases, DNA methyltransferases, etc.) and immune signature genes such as CD8+ T cell attracting chemokines (CCL5, CXCL9, CXCL10) and the immune checkpoint molecule PD-L1 was first evaluated in a cohort of 222 TNBC clinical specimens (19). We observed a negative correlation between multiple epigenetic regulators and immune-related genes (Supplementary Table 1). Among these epigenetic modifiers, the FAD-dependent histone demethylase, LSD1, appears to be negatively correlated with the chemokines and PD-L1 with most overall significant p values (Supplementary Table 1; Figure 1a). Further analysis showed that LSD1 gene expression was inversely associated with these immune factors in estrogen receptor negative (ER−), but not in ER+ or HER2+ tumors (Figure 1b–d). Overall, these *in silico* data revealed a negative correlation between expression of LSD1 and cytotoxic T cell attracting chemokines and PD-L1 in aggressive TNBC or ER negative breast tumors. Analysis of TCGA data indicates that LSD1 expression is greatly increased in breast tumor specimens compared with adjacent normal tissues (Figure 1e; Supplementary Figure 1a). The analysis also indicated a significantly elevated level of LSD1 mRNA expression in ER- or basal-like breast cancer in comparison to other subtypes (Figures 1e & f; Supplementary Figure 1b).

Inhibition of LSD1 induces expression of effector T cell attracting chemokines and PD-L1

The dysregulation of LSD1 activity has been implicated in tumorigenesis for various cancers including breast cancer (20–22). To determine whether overexpression of LSD1 aberrantly suppresses expression of immune protective factors, we tested several LSD1 inhibitors for their impact on expression of CD8+ T cell attracting chemokines and PD-L1. Among these LSD1 inhibitors, HCI-2509 and Tranylcypromine (TCP) significantly increased the expression of PD-L1, CCL5, CXCL9, and CXCL10 in human TNBC MDA-MB-231 cells (Figure 2a). TCP is an irreversible LSD1 inhibitor that has been used as a chemical scaffold

to design new generations of LSD1 inhibitors (21) (Supplementary Figure 2a). HCI-2509 is a non-competitive and highly potent reversible LSD1 inhibitor that effectively inhibits LSD1 activity at micromolar levels in MDA-MB-231 cells (Supplementary Figure 2b). HCI-2509 induced mRNA expression of PD-L1 and T cell chemokines in a dose dependent manner in MDA-MB-231 cells, and mouse TNBC cell line models, 4T1 and EMT6 (Figure 2b). In agreement with the effects of the LSD1 inhibitors, depletion of LSD1 by siRNA in MDA-MB-231 or MDA-MB-468 cells significantly increased expression of CCL5, CXCL9 and CXCL10 (Figure 2c; Supplementary Figure 3a), whereas overexpression of LSD1 via transfection of pReceiver-LSD1 plasmids attenuated expression of these genes in both cell lines (Figure 2d; Supplementary Figure 3b). It is noted that either depletion or overexpression of LSD1 exerted negligible effects on expression of other types of chemokines such as CCL2, CCL3 or CCL4 whose activities are known to have pro-tumor roles (23), suggesting that targeting LSD1 may have a favorable impact on promoting antitumor immunity. Similarly, transfection of a second LSD1 siRNA also significantly induced mRNA expression of CCL5, CXCL9 and CXCL10 in both MDA-MB-231 and MDA-MB-468 cells (Supplementary Figures 3c & d). Moreover, stable LSD1 knockdown in 4T1 cells was established through infection with shRNA lentiviral particles. In two LSD1-KD clones showing best knockdown efficacy (Supplementary Figure 4a), stable loss of LSD1 consistently induced mRNA expression of CCL5, CXCL9 and CXCL10 (Supplementary Figure 4b).

Next, we investigated whether LSD1 inhibitor-induced expression of chemokines was accompanied by changes in H3K4me2 levels at specific gene promoters. Primers spanning the proximal promoter region for chemokines and PD-L1, from approximately −1,200 to +400 bp relative to the transcriptional start site (TSS), were designed for quantitative tiling ChIP assays (Figure 3a). Treatment with HCI-2509 led to increase of H3K4me2 enrichment at proximal elements or core regions of transcription start site (P4, P5 or P6) at promoters of chemokines and PD-L1. HCI-2509 also enhanced H3K4me2 occupancy at distant region upstream of the TSS site of CCL5 and PD-L1 promoters (Figure 3b). These studies illustrate the effect of LSD1 inhibitor on the enrichment of the active histone mark, H3K4me2, in important promoter regions that are likely responsible for LSD1 inhibitor-induced re-expression of immune regulatory genes.

Next, we used a mouse chemokine array to determine whether upregulation of T cell chemokine expression by LSD1 inhibition would increase chemokine protein synthesis and secretion. Supernatants from 4T1 cells were collected and incubated with the detection antibody, and then added onto the blocked membrane that contains various chemokine capture antibodies (Supplementary Figure 4c). Immunoblotting results indicated that LSD1 RNAi or treatment with HCI-2509 stimulated the protein secretion of CCL5 and CXCL10 in 4T1 culture medium. CXCL9 protein level was elevated by HCI-2509 but was basically not affected by LSD1 RNAi (Figure 3c & d; Supplementary Figures 4d & e).

PD-L1 is a transmembrane glycoprotein that binds to its receptor, PD-1, on T cells, which leads to suppression of immune function (24). PD-L1 expression has been speculated as a critical predictive parameter of sensitivity to therapeutic agents targeting the PD-L1/PD-1 immune checkpoints (25). To test whether LSD1 inhibitor-induced PD-L1 expression results

in increased cell surface expression, FACS analysis was used to measure the PD-L1 level on the cell surface of several TNBC cell lines, with IgG as a negative control. We found that the basal membranous PD-L1 expression is very low in TNBC cells and treatment with HCI-2509 significantly up-regulated surface expression of PD-L1 (Figures 3e & 3f; Supplementary Table 2).

LSD1 inhibits CD8+ T lymphocyte trafficking in TNBC microenvironment

To examine whether LSD1 inhibition-induced chemokine products could enhance CD8+ T cell trafficking and tumor infiltration, we carried out an *ex vivo* chemotaxis assay. Briefly, naïve CD8+ T cells were purified from mouse spleen and activated with Dynabeads containing mouse T-activator CD3/CD28 and recombinant mouse IL-2 (Figure 4a). A pool of activated CD8+ T cells was subsequently collected (Supplementary Figure 5a) and then placed onto top chambers of plates and allowed to migrate for 24 h towards cellular supernates of 4T1 cells that were treated with DMSO or HCI-2509. The FCM result showed that HCI-2509 significantly increased the migration of CD8+ T cells (Figure 4b).

To characterize the role of CD8+ T cell chemokines in LSD1 inhibition-induced T cell recruitment, a rescue study was carried out. 4T1 or EMT6 cellular supernatants were added with TAK-779, a potent antagonist for CCR5 and CXCR3 which are the receptors for CCL5 and CXCL9/10/11 respectively (26). Chemotaxis assays showed that concurrent treatment with TAK-779 hindered HCI-2509-enhanced CD8+ T cell migration in both cell lines (Figure 4c). We validated this result by concurrent transfection of CCL5 or CXCL10 siRNA into EMT6 cells. Transfection with siRNAs effectively knocked down more than 70% of mRNA expression of CCL5 or CXCL10 (Supplementary Figure 5b). Chemotaxis assay demonstrated that simultaneous depletion of either CCL5 or CXCL10 effectively blocked HCI-2509-induced T cell migration (Figure 4d). Collectively, these results point to an important role of T cell chemokines in regulation of CD8+ T cell recruitment to tumor microenvironment.

To evaluate the potential effect of LSD1 inhibitor on normal activities of immune tissues, splenocytes were extracted from BALB/c mice and treated with various concentrations of HCI-2509 for 24 h. qPCR results indicated that HCI-2509 had no noticeable impact on the mRNA expression of examined immune factors (Supplementary Figure 5c). Next, we examined the potential influence of LSD1 inhibition on expression of key T cell exhaustion regulators, PD-1, TNF± and IFN**γ**, and chemokine receptors CCR5 and CXCR3 in activated CD8+ cells. Naïve CD8+ T cells were purified from mouse spleen and stimulated with Dynabeads with mouse T-activator CD3/CD28 and recombinant mouse IL-2. Activated CD8+ cells were then treated with 2.5 μM HCI-2509 for 24 h. Results of quantitative RT-PCR indicated that HCI-2509 significantly decreased mRNA expression of PD-1 and increased level of CCR5 in activated CD8+ cells (Figure 4e). Treatment with LSD1 inhibitor resulted in a small reduction of TNF± expression and the impact on IFN**γ** failed to attain statistical significance (Figure 4e). FACS results showed that decreased PD-1 mRNA expression by HCI-2509 led to reduced cell surface expression of PD-1 (Supplementary Figure 5d).

LSD1 inhibition potentiates *in vivo* response of TNBC tumor xenografts to anti PD-1 immunotherapy

Next, we investigated the *in vivo* effect of LSD1 inhibitors on efficacy of anti-PD-1 therapy in BALB/c mice bearing orthotopic EMT6 tumors. Treatment with PD-1 antibody alone failed to elicit obvious therapeutic effects on EMT6 tumor growth. However, combination therapy with HCI-2509 and PD-1 mAb displayed superior inhibitory effect against tumor progression and resulted in 70% reduction in tumor burden as compared to vehicle control group (Figures 5a & b). At the termination of the experiment, the tumor weight in each mouse was measured. Average tumor weight in the combination group was significantly lower than that of control group (Figure 5c). Statistical analysis of average tumor volumes between each group was shown in Supplementary Table 3. Immunohistochemical analysis showed that HCI-2509 decreased Ki-67 expression, and combination therapy led to a more significant reduction of Ki-67 expression in EMT6 tumors (Figures 5d & e).

Mouse 4T1 cell line is highly metastatic and a widely used breast cancer metastasis model. The effect of combination therapy of LSD1 inhibitors and anti-PD-1 antibody on 4T1 tumor growth and metastasis was evaluated in BALB/c mice bearing 4T1 tumors in the mammary fat pads. Mice were treated with HCI-2509 every two days or Tranylcypromine five days a week. Mice were injected i.p. with anti-PD-1 antibodies once every six days. While PD-1 mAb by itself had no obvious impact on 4T1 tumor growth, combining either HCI-2509 or TCP with PD-1 mAb resulted in nearly 40% reduction in primary tumor volumes as compared with the control or single-agent treatment group (Supplementary Figures 6a & b). At the end of experiments, histological assessment was performed to visualize the microscopic tumor lesions in the lungs. 4T1 tumors spontaneously produced highly metastatic lesions in lung tissues. Treatment with HCI-2509 or PD-1 alone had no significant impact on 4T1 metastasis. However, combination therapy significantly reduced the areas of pulmonary metastatic lesions as compared with the control or single-agent treatment group (Figures 5f & g). Similar results were observed in combination therapy of TCP and PD-1 mAb (Supplementary Figures 7a & b). These results indicate that combining LSD1 inhibitors with PD-1 antibody is more effective than either treatment alone in preventing 4T1 tumor metastasis. Overall toxicity of combination therapy against animals in all these studies was insignificant as demonstrated by no animal weight loss (Supplementary Figure 8).

LSD1 inhibitor synergizes with PD-1 mAb to enhance *in vivo* breast tumor immunogenicity

LSD1 inhibitor in combination with PD-1 mAb significantly increased the mRNA expression of PD-L1, CCL-5, CXCL9, CXCL10 and CCR5 (receptor of CCL-5) in EMT6 tumors (Figure 6a). The immunohistochemistry assay indicated that recruitment of CD8+ T lymphocyte to EMT6 tumors was increased by HCI-2509 treatment which was further induced in tumors receiving combination therapy (Figures 6b & c). Many clinical studies suggest that lymphatic network facilitates systemic breast tumor metastasis via providing a portal for tumor cell spreading (27, 28). Subtypes of T lymphocytes were sorted and quantified by flow cytometry to determine the effect of combination therapy on status of T cell infiltration in peripheral lymph nodes adjacent to mammary glands with tumor implantation. The combination therapy significantly increased the population of CD3+CD8+

T cells in lymph nodes adjacent to EMT6 tumors (Figure 6d). The ratio of CD4+ to CD8+ T cells in lymph nodes was significantly reduced by combination therapy (Figure 6e). An attenuated CD4+/CD8+ ratio of tumor-infiltrating lymphocytes clearly indicates an enhanced capacity of antitumor immunogenicity (29). The similar results were observed in lymph nodes adjacent to 4T1 xenograft tumors (Supplementary Figure 9). Taken together, our novel findings demonstrate that LSD1 inhibition triggers cytotoxic CD8+ T cell infiltration that in turn enhances the *in vivo* antitumor efficacy of immune checkpoint blockade antibody. A model is proposed to summarize the role of LSD1 regulation on chemokine silencing, effector T cell trafficking, breast tumor immunity, and response to immunotherapy (Figure 7).

Discussion

Accumulating evidence indicates that abnormal epigenetic modifications play important roles in silencing expression of effector T cell chemokines in cancer (11, 12). Analysis of a cohort of TCGA invasive breast cancer datasets revealed that LSD1 expression is negatively correlated with expression of certain CD8+ T cell attracting chemokines and PD-L1. LSD1 is the first identified histone demethylase that has shown great potential as a target in cancer therapy in preclinical models (17, 18, 30–33). In line with *in silico* results, we demonstrated that suppression of LSD1 expression by RNAi or small molecule inhibitors induced expression and activity of anti-tumor chemokines but exerted marginal effect on the expression of those chemokines with pro-tumor activity. The molecular details underlying LSD1 regulation on chemokine transcription are not completely understood. LSD1 has been typically found to be associated with multiple transcription repressors, such as HDAC1, HDAC2 and CoREST, to assemble a transcriptional repressor complex (32, 34, 35). Recent studies showed that knockdown of LSD1 cofactors HDAC1 and HDAC2 failed to affect expression of chemokines and PD-L1 (12, 36). We recently reported that HDAC5, which is a key member of class II histone deacetylase, physically interacts and stabilizes LSD1 protein through up-regulating the expression of LSD1 deubiquitinase in breast cancer cells (37). Similar to data from TCGA TNBC patients for LSD1 mRNA expression, HDAC5 mRNA expression is also negatively associated with CD8+ T cell chemokines and PD-L1 in TNBC patients (Supplementary Table 1). Additional studies would be necessary to address the potential role of HDAC5 in LSD1-mediated repression of T cell chemokine expression. Moreover, the regulation of expression of chemokines by other H3K4me-targeting histone demethylases has been recently reported. For example, the H3K4me3 histone demethylase, Fbxl10, has been found to be associated with promoter of chemokine, CCL7, and mediate its transcription activity (38). Li *et al* has demonstrated that CCL14, an epithelial derived chemokine, is an important regulator of the JARID1B/LSD1/NuRD complex in regulation of angiogenesis and metastasis in breast cancer (39). These findings indicate that multiple histone modifications are involved in regulation of chemokine expression. Continuous studies are needed to clarify the mechanism of how coordinated interaction between LSD1 and other epigenetic modifiers governs chemokines expression.

Immune checkpoint inhibitors (ICIs) have been hailed as a major breakthrough for treatment of malignant diseases, including a subset of breast cancers (40, 41). However, clinical benefit of ICIs remains limited to a fraction of breast cancer patients. The intrinsic

mechanisms of resistance to ICI therapy are multifaceted, dynamic and interdependent, which may be due to due to lack of effective antigen presentation, impaired formation of T cell memory, modified immune checkpoint pathways, changed cellular signaling pathways and tumor microenvironment, etc. However, the precise mechanisms of innate and acquired resistance to ICI therapy in breast cancer patients are still unclear. Our studies have linked the resistance to anti-PD-1 immunotherapy to LSD1-mediated epigenetic silencing of effector T cell chemokines. This notion is supported by the following experimental evidence obtained from our studies: 1) siRNA depletion of specific T cell chemokines, CCL5 or CXCL10, is sufficient to reverse LSD1 inhibitor-induced CD8+ T cell trafficking; 2) PD-1 mAb alone exerts no obvious effect on CD8+ T lymphocyte infiltration and combination therapy leads to significantly increased presence of CD8+ lymphocytes in tumors. Based on these novel findings, we tested a conceptually new strategy to combinatorically target LSD1 and defective immune system to correct the aberrant T cell landscape in low immunogenic triple negative breast cancer, which is an important research area that has been understudied. Our *in vivo* results show that combination therapy significantly enhanced the response to PD-1 immunotherapy in TNBC. Importantly, in addition to enhancing chemokine expression and infiltration of effector CD8+ T cells in tumors, our combination approach also increased the ratio of CD8+/CD4+ T cells in lymph tissues adjacent to mouse mammary glands, which is considered as an important marker of immunological defense against tumor spreading.

Although PD-L1 is generally regarded as an immunosuppressive molecule, several clinical trials have shown the positive association of PD-L1 expression with higher overall response rates to anti-PD-1/PD-L1 therapy (40, 42). Thus, PD-L1 has been considered as a predictive parameter of sensitivity to therapeutic agents targeting the PD-L1/PD-1 pathway in cancer patients (43, 44). Elevated PD-L1 expression has been reported to correlate with increased infiltrating lymphocytes, which in turn leads to stronger cytotoxic immune response and improved survival in breast tumors (45, 46). By using large-scale genomic data sets of solid tissue tumor biopsies, Rooney *et al.* reported that amplification of PD-L1 was positively associated with high local immune cytolytic activity (47). Our recent bisulfite sequencing study depicted that CpG islands at PD-L1 promoter are mostly unmethylated in MDA-MB-231 cells and treatment with DNMT inhibitor exerted insignificant effect on PD-L1 expression (data not shown). We speculate that dysregulated histone functions such as LSD1-mediated H3K4 demethylation at key elements of PD-L1 promoter could be a critical epigenetic mechanism contributing to PD-L1 silencing.

It is still unclear about the mechanism of how LSD1 inhibition potentiates anti-PD-1 therapy in breast cancer. One major function of CD8+ T cell destruction of targeted cancer cells is via Fas/FasL interaction. Anti-PD-1/PD-L1 therapy renders tumor cells sensitive to CD8+ T cell and FasL-mediated lysis (48, 49). Moreover, multiple lines of evidence showed that adaptive resistance to anti-PD1 therapy is mediated by the PI3K/Akt pathway in cancer (50, 51). Our recent microarray has shown that inhibition of LSD1 increases the expression of Fas and down-regulates of PI3K/Akt signaling (52). Future work is needed to determine whether inhibition of LSD1 enhances therapeutic efficacy of immune checkpoint blockade through regulation of activities of Fas and PI3K/Akt signaling in TNBC.

In conclusion, we have demonstrated that inhibition of LSD1 reactivates key immune checkpoint regulator and cytotoxic T cell attracting chemokines which in turn augments sensitivity of TNBC to immune checkpoint blocking antibodies (Figure 7). Our studies identify a new strategy to target crosstalk between epigenetic modulators and immune compartments as a novel therapeutic strategy for breast cancer patients with poor immune response. The development of novel LSD1 inhibitors is progressing rapidly and several clinical trials of LSD1 inhibitors are ongoing in cancer patients. We strongly believe that our new combination strategy, using these potent LSD1 inhibitors with immune modulators, would carry high innovation and translational potential.

Materials and methods

Cells and reagents

MDA-MB-231, MDA-MB-468 and EMT6 cell lines were from ATCC. 4T1 cell line was provided by Dr. Adrian Lee (University of Pittsburgh). HCI-2509 was purchased from Xcessbio Biosciences Inc. (San Diego, CA). GSK-LSD1 and TAK-779 were obtained from Sigma (St. Louis, MO).

LSD1 siRNA transfection and shRNA infection

Pre-designed LSD1 siRNA #1 (Santa Cruz Biotechnology, Dallas, TX) and siRNA #2 (Thermo Fisher Scientific, Waltham, MA) were transfected into cells following the manufacturer's protocol. Scramble control and LSD1-specific shRNA lentiviral particles (Santa Cruz Biotechnology) were infected into 4T1 cells, according to the manufacturer's protocol. Cells were harvested 72-h post-infection and seeded into dishes with 2 μg/ml puromycin. Individual colonies were picked and analyzed for LSD1 expression.

Quantitative PCR

Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) as described previously (53, 54). Quantitative real-time PCR was performed on the StepOne real-time PCR system (Life Technologies). TaqMan® Gene Expression Assays were predesigned and obtained from Life Technologies.

Immunoblotting

Western blotting was performed as previously described (55, 56). Antibodies used in this study are shown in Supplementary Table 4. Nitrocellulose membranes were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Proteome antibody arrays

Mouse chemokine array kit ARY020 (R&D Systems, Minneapolis, MN) was used to detect 25 mouse chemokines. Briefly, cell culture supernatant was collected and incubated with the detection antibody cocktail. The sample/antibody mixture was then added onto the blocked membrane, containing 25 different capture antibodies. After washing, membrane was incubated with diluted Streptavidin-HRP and Chemi Reagent Mix was added, and the membrane was then exposed to X-ray film.

Flow cytometry analysis

One million cells were collected and stained with a variety of antibodies (Supplementary Table 4) or isotype control antibody. Stained cells and fixed cells were suspended in FACS buffer and analyzed on the LSR-II flow cytometer (BD Biosciences, San Jose, CA). Data was processed with FACSDIVA™ software (BD Biosciences).

Chromatin immunoprecipitation (ChIP)

ChIP was performed using methods as reported previously (37). Cells treated with vehicle or LSD1 inhibitor were exposed to 1% formaldehyde to cross-link proteins, and two million cells were used for each ChIP assay and performed as previously described (31, 56). Quantitative ChIP confirmed changes in H3K4me2 at the promoters of examined genes using qPCR with primer sets indicated in Supplementary Table 5.

Activation of effector CD8$^+$ T cells

Naïve CD8$^+$ T cells were purified from mouse spleen using EasySep mouse CD8$^+$ T cell isolation kit (StemCell Technologies, Cambridge, MA). Isolated naïve CD8$^+$ T cells were stimulated with Dynabeads, mouse T-activator CD3/CD28 (Thermo Fisher Scientific) and recombinant mouse IL-2 (R&D Systems) for 10 days. After activation, effector CD8$^+$ T cells were harvested using magnetic plate and counted for further analysis.

Chemotaxis assay

After treatment, cell culture medium was replaced with drug free medium for 24 hours. Cell-free supernatant was then transferred to 24-well plates with membranes of 5 μm pore size (Corning, Corning, NY). 2×10^5 effector CD8+ T cells were loaded onto top chambers and allowed to migrate for 24 h towards cell supernatants. The migrated cells were harvested and re-suspended in 4% paraformaldehyde solution to fix. The number of CD8+ T cells was quantified by fixed 30 s runs on LSR-II flow cytometer.

Immunohistochemistry (IHC)

Tumors were fixed in Bouin's solution (Sigma) and processed for paraffin embedding. Antigen retrieval was performed using X buffer and stained with primary antibodies overnight at 4 °C. Secondary antibody (1:200, eBioscience) was used followed by 3,3'-diaminobenzidine (DAB, 34002, Thermo Fisher Scientific) and then counterstaining with hematoxylin (Sigma). The percentage of CD8+ cells was analyzed using Image-pro Plus software (Media Cybernetics, Rockville, MD) and the staining of Ki-67 was quantitated by ImageJ as previously described (37) through blinded evaluation.

Animal studies

All animal studies were conducted in accordance with protocol approved by IACUC of University of Pittsburgh. In EMT6 xenografts, 0.5×10^6 cells were injected into mammary fat pad of six to eight-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). When tumor volume reached 50–100 mm^3, mice were randomized into experimental groups. HCI-2509 was injected intraperitoneally (i.p.) (50 mg/kg) every day and anti-PD-1 antibodies (10 mg/kg, i.p.) were injected every 3 days. Vehicle and isotype control

antibodies were injected in control mice. Tumor volumes were assessed every four days. In 4T1 xenograft model, HCI-2509 was injected (30 mg/kg, i.p.) every two days. Tranylcypromine (TCP) was injected (10 mg/kg, i.p.), five days a week. Anti-PD-1 antibodies were injected (10 mg/kg, i.p.) every 6 days. At the end of study, animal lung tissues were processed into paraffin sections, and then subjected to hematoxylin-eosin (H&E) staining at the histology and microimaging core facility at MWRI. The blinded evaluation of metastasis was conducted by a pathologist (Y.F.) and the areas were calculated by SZX-16 microscope and CellSens Dimension software (Olympus, Shinjuku, Tokyo, Japan).

Statistical analysis

Two-tailed Student's t-test was used to determine the quantitative variables. Gene expression profiles in 222 TNBC patient specimens were analyzed using Affymetrix Human Genome U133A Plus 2.0 microarray data files from 21 breast cancer data sets (19). Data files were then processed using the affy and bioconductor packages in the R statistical programming language. The data files were combined and then background corrected, log2 transformed, normalized, and summarized using Robust Multi-chip Average (RMA) algorithm to generate the expression data. For those genes containing multiple corresponding probe sets in a microarray, the expression measure of the probe set with the maximum interquartile expression range value was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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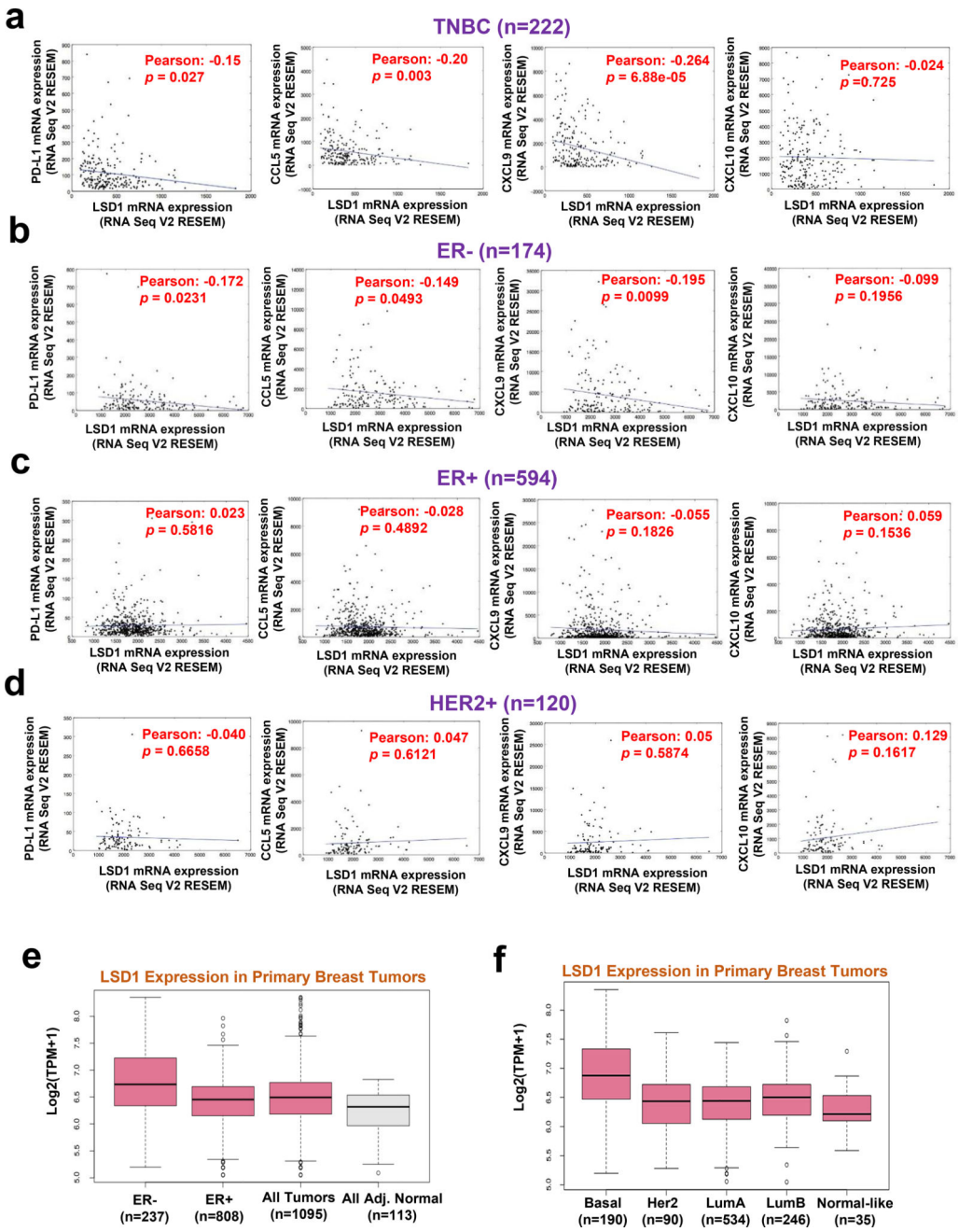

Figure 1. LSD1 expression and its correlation with immune-related factors in breast cancer TCGA database

(a–d) The Pearson correlation between immune regulatory factors and LSD1 across breast cancer subtypes: TNBC **(a)**, ER negative **(b)**, ER positive **(c)** or HER2 amplified **(d)** breast cancer. (**e**) LSD1 mRNA level in ER positive vs. ER negative breast cancer specimens and all tumors vs. adjacent normal tissues (downloaded from TCGA database: https://www.ncbi.nlm.nih.gov/pubmed/26209429). **(f)** LSD1 mRNA level in PAM50 intrinsic breast cancer subtypes in TCGA data.

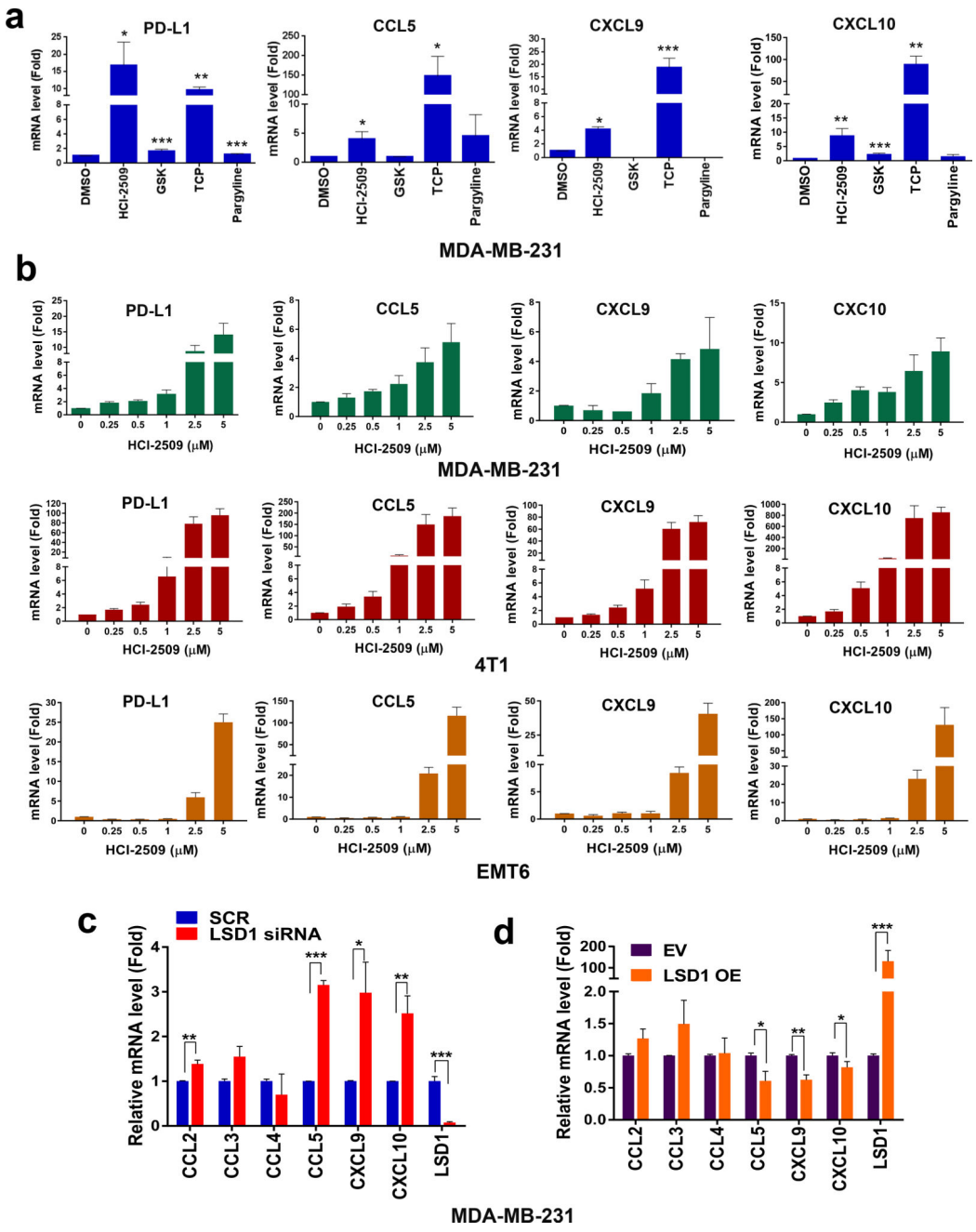

Figure 2. LSD1 inhibition induces expression of CD8+ T cell attracting chemokines and PD-L1

Real-time RT-PCR analysis was performed to analyze relative mRNA expression level of indicated genes. β-actin was included as an internal control. **(a)** MDA-MB-231 cells were treated with LSD1 inhibitors (2.5 μM HCL-2509, 100 μM GSK-LSD1, 2.5 mM Tranylcypromine or 2.5 mM Pargyline) for 24 h. Relative mRNA expression of indicated immune regulatory factors is shown. **(b)** MDA-MB-231, 4T1 and EMT6 cells were exposed to increasing concentrations of HCI-2509 for 24h. Relative mRNA expression of PD-L1, CCL5, CXCL9 and CXCL10 compared to vehicle (set to fold change = 1) is shown. **(c)** MDA-MB-231 cells were transiently transfected with scramble or LSD1 siRNA for 48 h.

Effect of LSD1 knockdown on mRNA expression of indicated chemokines was examined by real-time RT-PCR. **(d)** MDA-MB-231 cells were transiently transfected with control or pReceiver-FLAG-LSD1 plasmids for 48 h and analyzed by real-time RT-PCR for expression of indicated chemokines. Histograms represent the mean fold change in mRNA expression compared to control group (set to fold change = 1) for three independent determinations ± s.d. Bars marked with asterisks indicate a statistical difference by Student's t-test. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***. EV = empty vector, OE = overexpression.

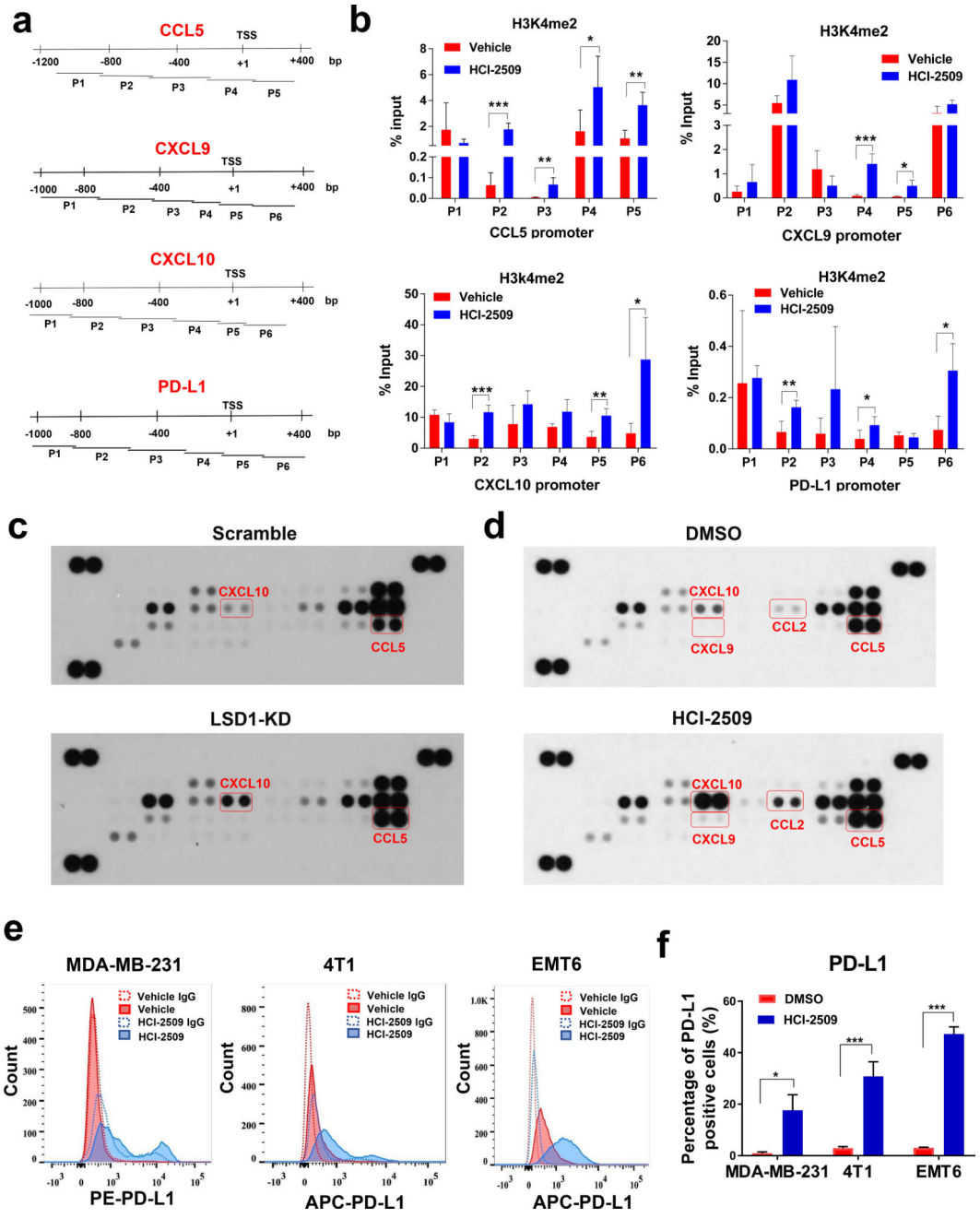

Figure 3. Inhibition of LSD1 promotes protein production and secretion of chemokines and PD-L1

(a) Tiling ChIP primers were designed spanning from −1200 to +400 bp around the transcription start sites (TSS) of indicated genes. **(b)** MDA-MB-231 cells were treated with 2.5 μM HCI-2509 for 24 h. Quantitative ChIP studies were conducted to characterize the enrichment of H3K4me2 at promoters of indicated genes. **(c–d)** Mouse chemokine antibody array was performed following the manufacturer's protocol to detect secreted chemokines from 4T1 cells that were **(c)** infected with scramble or LSD1 shRNA lentivirus, or **(d)** exposed to DMSO or 2.5 μM HCI-2509 for 24 h. Red boxes designate the chemokines

whose expression was altered by LSD1 RNAi or inhibitor. **(e)** Flow cytometry analysis was carried out to detect the percentage of PD-L1 positive MDA-MB-231, 4T1 or EMT6 cells after treatment with DMSO or 2.5 μM HCI-2509 for 24 h. IgG was used as a negative control to normalize the expression levels of PD-L1 on the surface of the tumor cells. Shown are representative FACS images. **(f)** Histogram shows the quantified percentage of PD-L1 positive cells after treatment with vehicle or HCI-2509. All the experiments were performed three times. Student's *t*-test was performed to assess significance. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***.

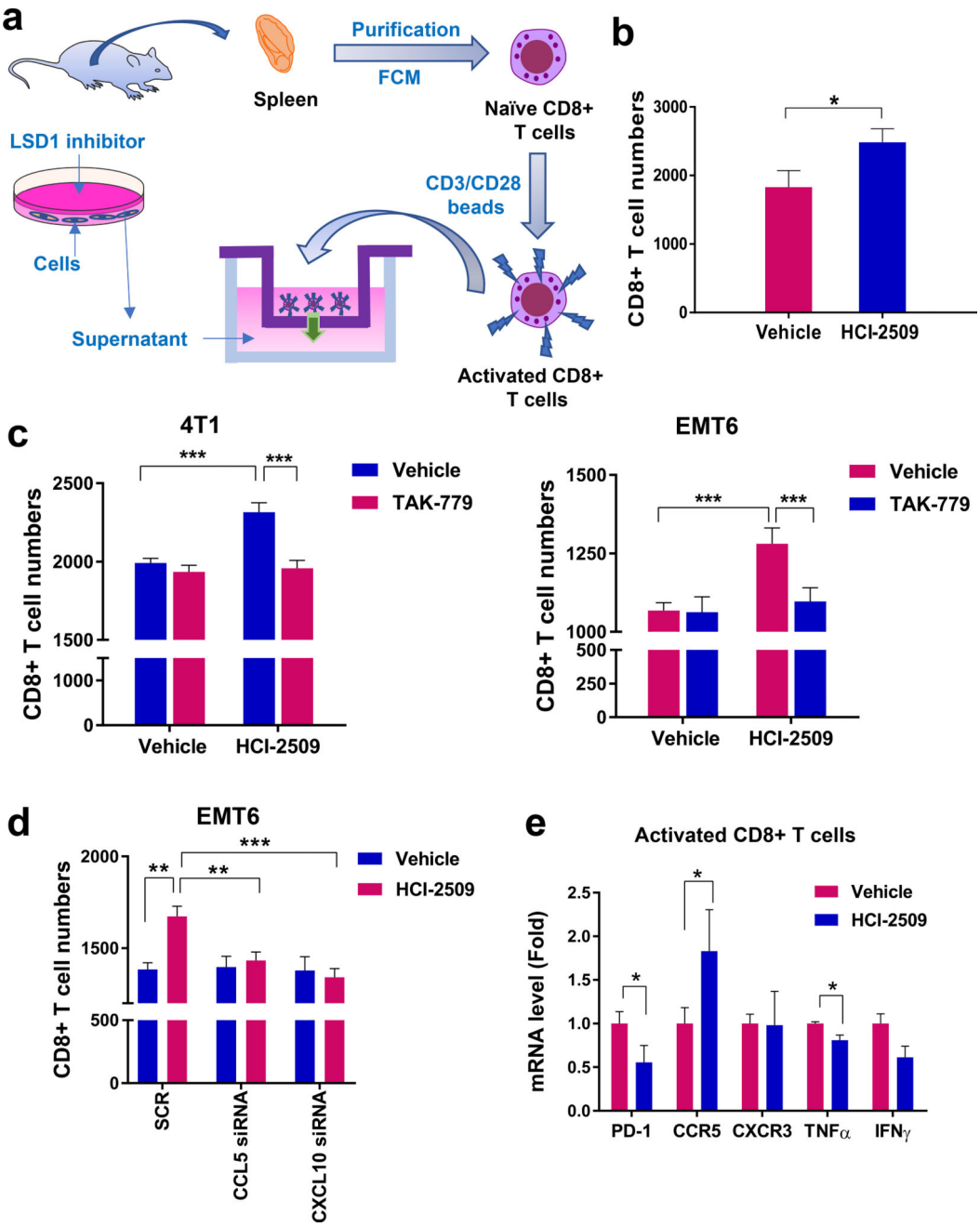

Figure 4. LSD1 inhibition induces effector T cell migration and tumor infiltration

(a) Schematic diagram of *ex vivo* chemotaxis assay of CD8+ T cell activation and migration. (**b**) Treatment with HCI-2509 induces CD8+ T cell migration. Bar graph shows mean total number of CD8+ T cells ± s.d. (**c**) 4T1 cells were treated with DMSO or 2.5 μM HCI-2509 for one hour. Cellular supernatants were then added with vehicle or 5 nM TAK-779. Chemotaxis assay was subsequently performed to determine the effect of TAK-779 on HCI-2509-induced CD8+ T cell migration. (**d**) EMT6 cells were transiently transfected with CCL5 or CXCL10 siRNA followed by treatment with DMSO or 2.5 μM HCI-2509 for 24 h. Chemotaxis assay was performed to assess the impact of chemokine siRNA on HCI-2509-

induced CD8+ T cell migration and tumor infiltration. (**e**) Activated CD8+ cells were treated with 2.5 μM HCI-2509 for 24 h. mRNA expression of indicated genes was examined by real-time RT-PCR. Histograms represent mean fold change ± s.d. for three independent experiments. Student's *t*-test was performed to assess significance. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***.

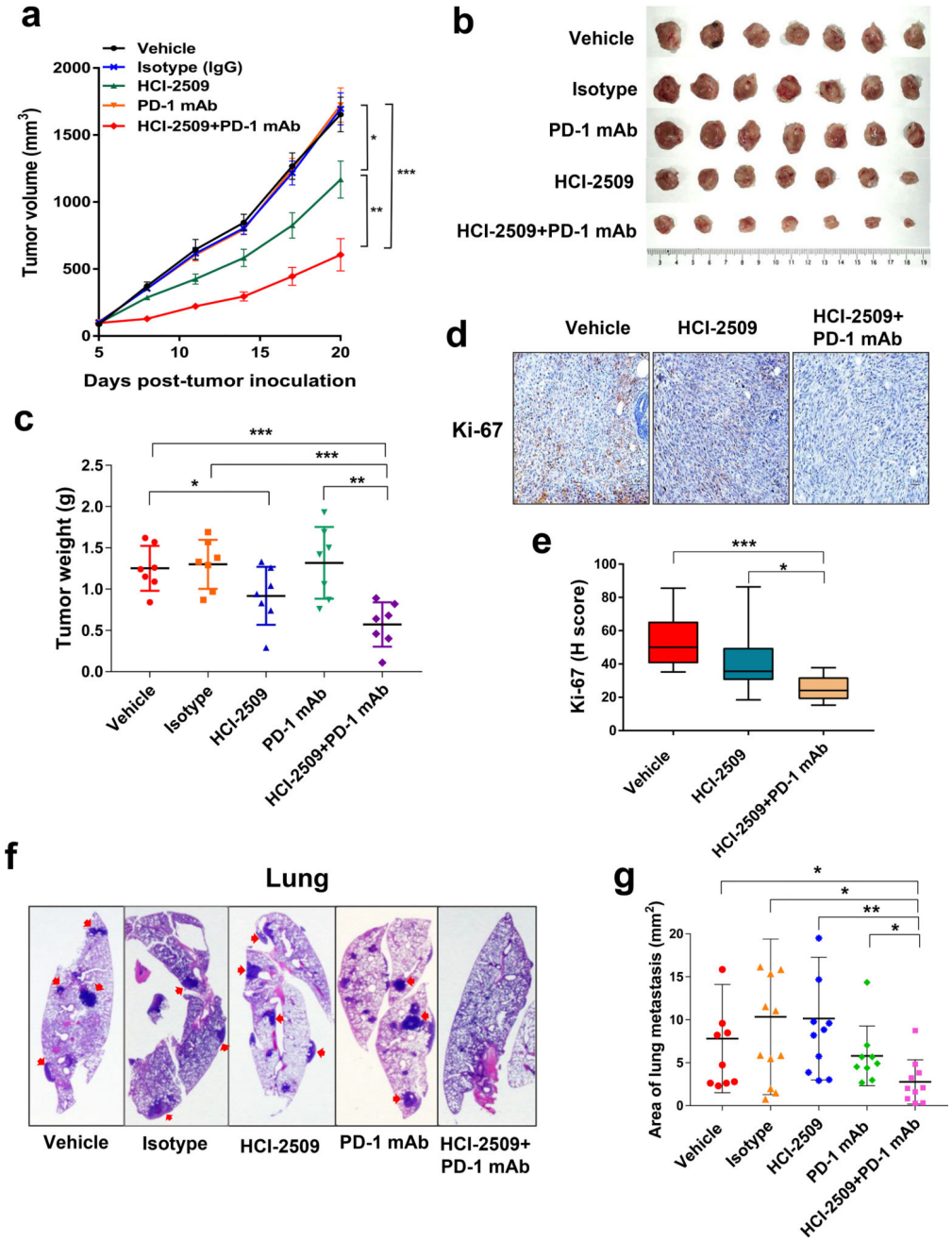

Figure 5. LSD1 inhibitor potentiates antitumor efficacy of PD-1 antibody in mice bearing TNBC tumors

(a) Mouse EMT6 cells were engrafted into the mammary fat pad of BALB/c mice. When established tumors were palpable, mice were treated with vehicle (DMSO, n=7), isotype (IgG, n=7), HCI-2509 (50 mg/kg, 7 days/week, n=7), PD-1 mAb (10 m/kg, once every 3 days, n=7), or combination (n=7) via i.p. injection. Tumors were measured with calipers, and values were plotted. The vertical bars indicate mean tumor size (mm^3) ± s.e. (**b**) EMT6 tumors in each group were harvested and photographed at the end of the experiment. Shown are photographs of the xenograft tumors. (**c**) Tumor weights were measured for each

treatment group at autopsy. Values are mean ± s.d. (**d**) Representative immunohistochemistry staining of Ki-67 in EMT6 xenograft tumors treated with vehicle, HCI-2509, or HCI-2509+PD-1 mAb. (**e**) H-scores represent average staining intensity of Ki-67 in EMT6 xenograft tumors which were treated with vehicle, HCI-2509, or HCI-2509+PD-1 mAb (n=9). **(f)** 4T1 cells were implanted into the mammary gland of BALB/c mice. Vehicle (DMSO, n=10), isotype (IgG, n=12), HCI-2509 (30 mg/kg, every two days, n=11), PD-1 mAb (10 mg/kg, once every 6 days, n=11), and combination (HCI-2509 30 mg/kg+PD-1 mAb 10 mg/kg, n=10) were delivered via i.p. injection. Lung specimens were stained with hematoxylin and eosin. Arrows indicate large metastatic lesions. **(g)** Areas of 4T1 metastasis in each histological section were calculated by microscope and CellSens Dimension software. vehicle, n=10; isotype, n=12; HCI-2509, n=11; PD-1 mAb, n=11; combination, n=10. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, Student's t-test was used to analyze the significance of the results.

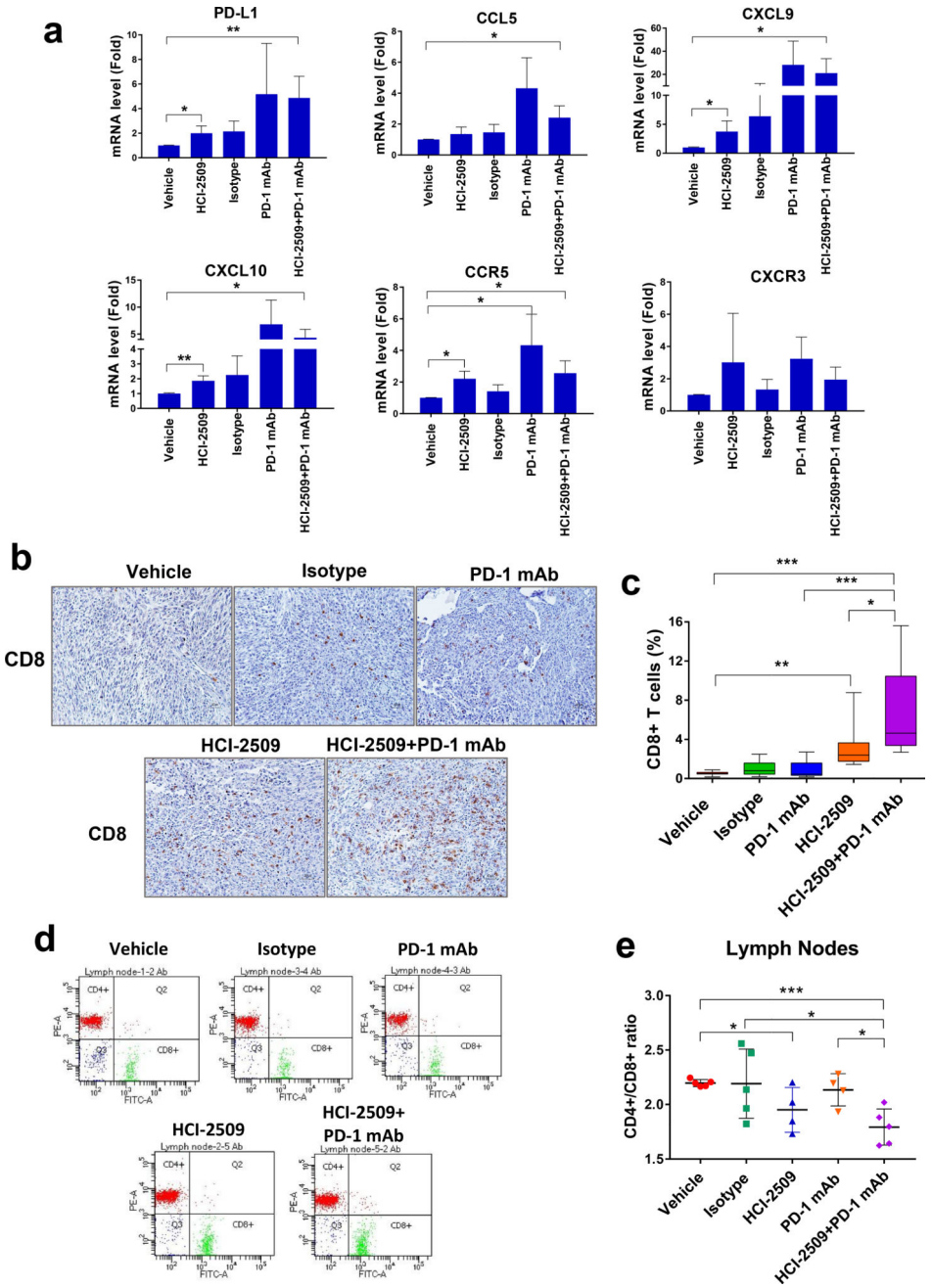

Figure 6. LSD1 inhibitor promotes tumor immunogenicity *in vivo*

(a) Real-time RT-PCR was performed to detect mRNA expression of indicated immune factors in EMT6 xenograft tumors. (**b**) Histological analysis of immune infiltration of CD8+ T cells after therapy. Showed are representative immunohistochemistry staining of CD8+ T lymphocytes in EMT6 tumors treated with vehicle, isotype, PD-1 mAb, HCI-2509, or combination. **(c)** The percentages of CD8+ T lymphocytes in EMT6 tumors were measured and analyzed by software Image-pro Plus. **(d)** The lymph node tissue adjacent to the fourth (inguinal) mammary glands were collected, and subtypes of T lymphocytes were quantified by flow cytometry assay. (**e**) The ratios of CD4+ to CD8+ T cells in lymph nodes of each

group were quantified by FCM. Student's *t*-test was performed to assess significance. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***.

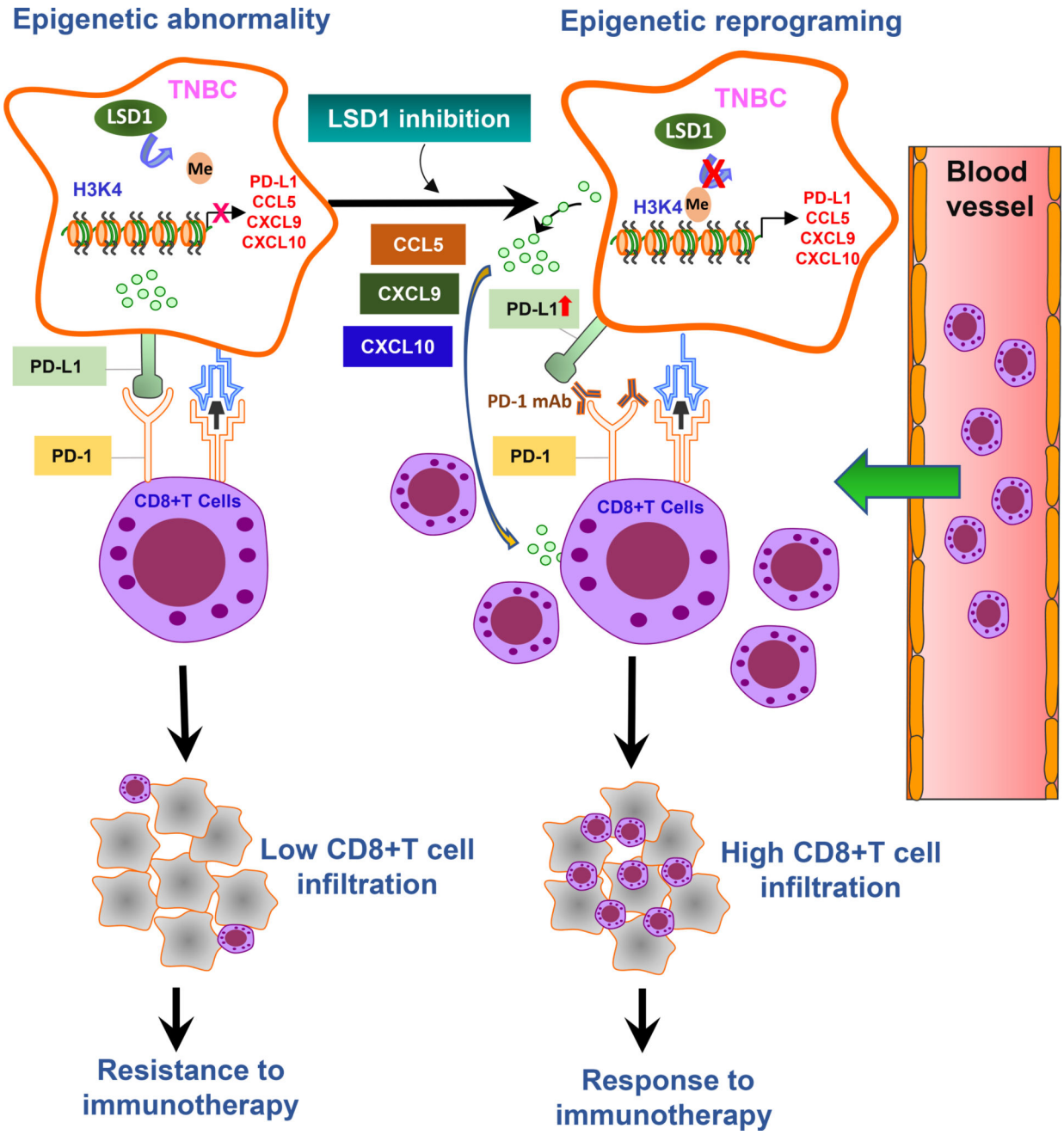

Figure 7. A proposed model of the role of LSD1 in regulation of breast tumor immunogenicity, response to immunotherapy and potential clinical outcome

Our work demonstrated that inhibition of histone lysine specific demethylase 1 (LSD1) increases the expression of key immune checkpoint regulators and effector T cell attracting chemokines which in turn increases CD8+ T cell tumor infiltration and improves the efficacy of immunotherapy. These findings provide supportive evidence to suggest that modulation of breast tumor immunogenicity by drugs that target epigenetic abnormalities may represent a promising area for translational research and clinical intervention for breast cancer therapy.