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27-Hydroxycholesterol acts on myeloid immune cells to induce T cell dysfunction, promoting breast cancer progression.

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Abstract

Breast cancer remains one of the leading causes of cancer mortality in the US. Elevated cholesterol is a major risk factor for breast cancer onset and recurrence, while cholesterol-lowering drugs, such as statins, are associated with a good prognosis. Previous work in murine models showed that cholesterol increases breast cancer metastasis, and the pro-metastatic effects of cholesterol were due to its primary metabolite, 27-hydroxycholesterol (27HC). In our prior work, myeloid cells were found to be required for the pro-metastatic effects of 27HC, but their precise contribution remains unclear. Here we report that 27HC impairs T cell expansion and cytotoxic function through its actions on myeloid cells, including macrophages, in a Liver × receptor (LXR) dependent manner. Many oxysterols and LXR ligands had similar effects on T cell expansion.

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LM, SGN and ERN conceived and designed experiments. LM, LW, ATN, CH, SH, MH, KM, JJC, AEB, AV, SHS and ERN performed experiments. SP and DJS helped develop and provided reagents. LM and ERN analyzed and interpreted the data. LM and ERN wrote the manuscript with input from all authors

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Moreover, their ability to induce the LXR target gene ABCA1 was associated with their effectiveness in impairing T cell expansion. Induction of T cell apoptosis was likely one mediator of this impairment. Interestingly, the enzyme responsible for the synthesis of 27HC, CYP27A1, is highly expressed in myeloid cells, suggesting that 27HC may have important autocrine or paracrine functions in these cells, a hypothesis supported by our finding that breast cancer metastasis was reduced in mice with a myeloid specific knockout of CYP27A1. Importantly, pharmacologic inhibition of CYP27A1 reduced metastatic growth and improved the efficacy of checkpoint inhibitor, anti-PD-L1. Taken together, our work suggests that targeting the CYP27A1 axis in myeloid cells may present therapeutic benefits and improve the response rate to immune therapies in breast cancer.

Keywords

27-hydroxycholesterol; cholesterol; breast cancer; myeloid cell; macrophage; T cell; immune suppression

Introduction

Among women in the United States, breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related death [1]. Worldwide, over 500,000 female lives were lost to breast cancer and 1.7 million incidences were recorded in 2016 [2]. While the mortality rate of breast cancer has significantly declined since 1989, mainly due to screen-detection and improved therapeutics, the overall 5-year survival rate of stage IV metastatic breast cancer remains low [3, 4]. Therefore, there is a continued need to identify targetable drivers of breast cancer, especially for late stage and therapy-refractory disease. Although immune checkpoint blockade (anti-programmed death-ligand 1; αPD-L1) in combination with nab-paclitaxel has recently been approved for the treatment of patients with advanced triple-negative breast cancer (TNBC) staining positive for PD-L1, a majority of these patients do not respond in a clinically meaningful way [5, 6]. Furthermore, immune interventions for the treatment of the other breast cancer subtypes have shown very limited success [7]. Although undoubtedly multifactorial, one major obstacle to immune-therapy is the highly immunosuppressive microenvironment of breast tumors – a phenomenon that is strongly maintained by myeloid immune cells, including macrophages [8, 9]. However, myeloid cells are also critical for a robust antitumor response [10]. Thus, strategies to ‘re-educate’ myeloid cells away from being pro-tumorigenic are warranted and starting to be explored, at least preclinically [11–14].

Cholesterol and control of its metabolism have emerged as potential therapeutic targets. An elevated level of circulating cholesterol has been associated with breast cancer recurrence both pre-clinically and clinically [15–17]. Conversely, several studies have found that patients taking cholesterol-lowering drugs, such as statins, had improved prognosis, as demonstrated by decreased breast cancer recurrence and increased progression-free survival [18–21]. Thus, clinical observations strongly indicate that cholesterol is important for the progression of breast cancer.

Previously, work has shown that a primary metabolite of cholesterol, 27-hydroxycholesterol (27HC), has the capacity to activate the estrogen receptors (ERs) and can directly promote the growth of ERα-positive tumors [22]. Notably, we also found that 27HC can promote the metastatic colonization and progression of breast cancer, regardless of the expression of ER or breast cancer subtype [23]. Furthermore, ER-negative models of ovarian cancer, 27HC was essential for growth [24]. In the lung metastatic site of breast cancer, it was found that neutrophils and γδ T cells were required for the full pro-metastatic effects of 27HC, while in ovarian cancer monocytic myeloid-derived suppressor cells (M-MDSCs, with the same surface markers as inflammatory monocytes) were required [23, 24]. These observations suggest that 27HC is a critical factor in shaping myeloid cell function, directly or indirectly, and that the type of myeloid cell required for its pro-tumorigenic actions is dependent on the metastatic site and type of cancer.

Within the tumor microenvironment, macrophages are one of the most abundant subsets of the myeloid cell lineage [25, 26]. Accumulation of this cell type at the tumor site is often associated with poor prognosis [27–29]. Tumor-associated macrophages (TAMs) are known to have a diverse set of polarities, with an M2-like phenotype dominating [30, 31]. TAMs are known to promote tumor progression via a multitude of mechanisms, including providing angiogenesis-inducing factors (VEGF, proMMP-9), impairing effector T cells via reactive oxygen species, and recruiting T regulatory cells by producing interleukin (IL)-10, IL-2 and transforming growth factor (TGF)-β [32]. For both metastatic breast cancer and ovarian cancer models, 27HC resulted in decreased T cell abundance, indicating that immune suppression is a putative role for 27HC-myeloid cell-mediated pro-tumor actions [23, 24]. A recent report showed that the use of statins to lower cholesterol led to reduced number of TAMs and better prognosis in human lung adenocarcinoma [33]. This is particularly intriguing as macrophages highly express the enzyme responsible for the synthesis of 27HC, CYP27A1, suggesting that their pro-tumor function may require the conversion of cholesterol to 27HC.

Given the evidence suggesting that 27HC is critical for promoting the pro-tumorigenic properties of myeloid cells, and the abundance and plastic nature of macrophages within the tumor microenvironment, the goals of this work were to (1) establish the effects of 27HC on macrophages and (2) elucidate the mechanisms by which 27HC impacts macrophage regulation of T cell activity.

Materials and Methods

Reagents

27HC (purity >95%) and dendrogenin A (purity >98%) were synthesized by Sai Life (Hyderabad, India). 25HC, 4βHC and Desmosterol were synthesized by Steraloids (Newport, RI) (purity >98%). 22(R)HC was synthesized by Avanti (Alabaster, AL) (purity >99%). 24(S)HC and 24,25-epoxycholesterol (24,25EC) were synthesized by Enzo (Farmingdale, NY) (purity ≥98%). GW3965, T0901317, GSK2033 and 7-ketocholesterol (7KC) were synthesized by Cayman (Ann Arbor, MI) (purity ≥98%). 20(S)HC was synthesized by Tocris (Bristol, UK) (purity >95%). 17β-Estradiol (E2) was synthesized by MP Biomedicals (Irvine, California) (purity ≥98%). 27HC, dendrogenin A, 24(S)HC,

20(S)HC, GW3965, T0901317 and GSK2033 were dissolved in dimethyl sulfoxide (DMSO) and stored in −20°C. 7KC, 4βHC, 22(R)HC, 24,25EC and 25HC were dissolved in ethanol and stored in −20°C. Desmosterol was dissolved in M-pyrol and stored in −20°C. Antibodies for flow cytometry were purchased from BD Biosciences and used at a working dilution of 1:100 in FACS buffer (2% fetal bovine serum (FBS) in phosphate buffered saline (PBS)). Catalogue numbers were as follows: CD3: 555275, CD4: 553729, CD8: 557682. Annexin V-FITC/propidium iodide apoptosis assay was purchased from Thermo Fisher (catalogue number: V13242), and FAM-DEVD-FMK/propidium iodide apoptosis assay was purchased from Immunochemistry (catalogue number: 94).

Cell lines

E0771 and 4T1-luc cells were a gift from Mark Dewhirst (Duke University) and cultured in RPMI supplemented with 10% FBS (Corning), 1% non-essential amino acids (Corning), 1% sodium pyruvate (Corning) and 1% penicillin/streptomycin (Corning). E0771-OVA cells were a gift from Jun Yan (University of Louisville) and grown in the above-described condition. EL4 and E.G7-OVA cells were from American Type Culture Collection (ATCC) and cultured with RPMI medium supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin and 0.05 mM 2-mercaptoethanol. E.G7-OVA cells were additionally cultured with 0.4 mg/ml G418 (Fisher). We did not culture any of these cell lines longer than 3 months after thawing the stocks. Cell lines were tested for mycoplasma.

Preparation of bone marrow-derived macrophages (BMDMs)

Bone marrow cells were collected from mouse tibia and femur. Cells were passed through 70-micron cell strainer and subsequently cultured in the presence of 25 ng/mL of M-CSF (Novoprotein). Complete RPMI media supplemented with M-CSF was replaced every 3–4 days. On day 10 or 14, BMDMs were harvested for further experiments. For subsequent experiments, BMDMs were treated with DMSO or 27HC (solvent at a 1:1000 dilution) for 24hrs. Treatments were washed off prior to co-culturing with T cells.

T cell proliferation and apoptosis assay

CD3+ T cells were enriched from the spleen of wildtype, OT-I or OT-II mice, using Dynabeads Untouched mouse T cells kit (Thermo Fisher) according to the manufacturer's instructions. Naïve CD4+ T cells were isolated from wildtype mice, using mouse Naive CD4+ T Cell Isolation Kit (Miltenyi Biotec) according to manufacturer's instruction. T cells were labeled with the vital dye CFSE according to the manufacturer's instructions (BioLegend). Wildtype T cells activated chemically were treated with 10 nM PMA and 1 μM ionomycin and cultured with washed BMDMs [as described in 34]. OT-I or OT-II T cells activated by antigen presentation were also labeled and cultured with LPS- and OVA-primed BMDMs [35–37]. BMDMs activating T cells from OT-I mice were primed with 50 μg/ml $OVA_{257–264}$ (Bachem) and BMDMs activating T cells from OT-II mice were primed with 50 μg/ml $OVA_{323–339}$ (Sigma). The proliferation of T cells was assessed by flow cytometry 72hrs after co-culture. Some OT-I mouse-derived T cells were harvested 48 or 72hrs after co-culture for RNA extraction (Thermo Fisher) and mRNA expression measurement by qPCR (BioRad). Some OT-I mouse-derived T cells were harvested 48 or 72hrs after co-

culture for apoptosis assay using Annexin V-FITC and propidium iodide (Thermo Fisher), and some OT-I mouse-derived T cells were harvested 24, 48 or 72hrs after co-culture for apoptosis assay using FAM-DEVD-FMK (FAM-FLICA) against active caspase-3/7 and propidium iodide (ImmunoChemistry Technologies). Naïve CD4+ T cells were isolated from wildtype mice, chemically activated and cultured in conditioned media from treated BMDMs, harvested after 24 or 48hrs and assayed for apoptosis (FAM-FLICA + PI) as described above.

T cell cytolytic assay

T cells were isolated from the spleen of OT-I mice and activated for 72hrs by LPS- and OVA-primed BMDMs. E0771 and E0771-OVA or EL4 and E.G7-OVA cells were labeled with CFSE or Far Red cell tracker (Far Red) (Thermo Fisher), respectively, and then plated in a 96-well plate. Subsequently, activated T cells were harvested and counted to be co-cultured with cancer cell lines of interest at various ratios. 24hrs after co-culture, the cytolytic activity of activated T cells was assessed by the ratio between the OVA-expressing lines and the parental cell lines by flow cytometry. The level of granzyme B secreted in the co-culture was measured by ELISA according to the manufacture's instruction (Thermo Fisher).

Gene silencing

For gene knockdown using siRNA, negative control and LXR/ER-complementary siRNA (Sigma, siRNA ID: SASI_Mm01_00189872, SASI_Mm01_00115295) were delivered to BMDMs for 48hrs using DharmaFECT transfection reagent 4 (GE) as previously described [38].

For gene knockdown using shRNA, negative control and a set of 3 LXR-complementary shRNA (Horizon) were delivered to BMDMs using lentiviral particles following the manufacture's instruction at MOI of 20 for 96hrs. The shRNA sequences targeting LXRα were: GTGAGCCTTCGCCATGTGG, GCGCCTGTTACACTGTTGC and GCAGGCGAAGGGCAAACAC. The shRNA sequences targeting LXRβ were: ATGATGGCTAGCTCGGTGA, CTGCACATTAGGCCGATCG and AACCATTCCCAGGCACGGG. Expression of the target genes after the knockdown was assessed using qPCR.

Animal and animal studies

All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois Urbana-Champaign. Wildtype C57BL/6 and BALB/C mice were purchased from Charles River Laboratory. Founder OT-I, OT-II and LysMCre mice were purchased from Jackson Laboratory and bred in-house. Founder CYP27A1$^{fl/fl}$ mice were a kind gift from Donald P. McDonnell (Duke University). Fig. 7A: CYP27A1$^{fl/fl}$;LysMCre^{+} and age-matched LysMCre^{+} mice were orthotopically grafted with 500,000 E0771-OVA cells in Matrigel (Corning) into the mammary fat pad. The resulting tumor growth was measured by caliper 5 times a week. After the tumor size reached ~200 mm^3 in either group, 7 million T cells from OT-I mice were grafted retro-orbitally into each tumor-bearing mouse in that particular group. One day before T cell adoptive transfer, each

tumor-bearing mouse was immunized intraperitoneally with 25 μg of $OVA_{256–264}$ in Complete Freund's Adjuvant (CFA) (Sigma). For Fig. 7B–C: 200,000 E0771 cells were grafted intravenously as described previously [23]. Mice were euthanized 3 weeks post-graft, and exterior, visible metastatic nodules were assessed and scored based number and size. The metastatic lesions were quantified with the following scoring criteria: 1) The metastatic nodules were counted and then scored according to their diameters using photos taken. Specifically, a nodule with diameter greater than or equal to 0.5 in, 0.3 in, 0.2 in and 0.1 in received a score of 4, 3, 2 and 1, respectively. 2). If a metastatic lesion was discovered outside the lung (such as liver), the lesion would also receive a score of 4. For Fig. 7D: BALB/C mice were grafted intravenously with 4T1-luc cells, which are 4T1 cells stably expressing a luciferase plasmid construct. Bioluminescent signal was used to quantify resulting metastatic burden [39]. GW273297X treatment commenced 3d post-graft at 100mg/kg/day [23]. αPD-L1 treatment started at the same time and followed the same regimen as described previously [24].

Data Analysis

RNA sequencing data (RSEM, batched-normalized) for breast invasive carcinoma that were generated from the Cancer Genome Atlas (TCGA, https://www.cell.com/pb-assets/consortium/pancanceratlas/pancani3/index.html) were downloaded from the cBioPortal (http://www.cbioportal.org) using R package cgdsr [40, 41]. Data included 1084 samples in Fig. 3I–N and Supplemental Fig. 3. Correlations between gene expression were assessed by log_2 transformation and Pearson's correlation in Fig 3. I–N and Supplemental Fig. 3, and the best fit line was determined by linear regression between the explanatory (x axis) and the response variable (y axis). Two-tailed t-test followed by Benjamini-Hochberg false discovery rate correction was performed for comparing cytokine secretion by BMDMs in Supplemental Table 1A–B [42]. Two-way ANOVA followed by Bonferroni's multiple comparisons test was used in Fig. 6B. Fisher's exact test was performed for proportion testing in Fig 7. A–C. For Fig. 7D, luminescence was normalized to the mean placebo + α-IgG, and one-way ANOVA was performed followed by the Student Newman-Keuls post-hoc test for significance. For the rest of the results, two-tailed t-test or nonparametric Mann-Whitney U-test was performed to compare the effect of vehicle and 27HC on BMDMs and, subsequently, T cells.

Results

27HC modulates gene expression in macrophages.

Oxysterols are a large group of compounds, with a diverse spectrum of biological activities [43]. Many oxysterols including 27HC have been described as ligands of the liver × receptors (LXRs), the activation of which promotes cholesterol homeostatic machinery, such as cholesterol efflux and cholesterol metabolism [44]. LXR activation, by various ligands including synthetic agonists, has also been implicated in regulating various aspects of the immune system. In general, many reports indicate that LXRs promote an anti-inflammatory phenotype, although these conclusions are largely based on synthetic ligands. This has led the field to believe that LXR agonists are generally anti-inflammatory. However, endogenous oxysterols and other LXR agonists are likely to have diverse, or even opposing actions [44–

46]. It is well established that LXR induction in T cells impairs proliferative expansion and activity [47]. However, the actions of LXRs on the immune function of myeloid cell are less clear. Therefore, to further explore these putative immunomodulatory effects on macrophages, we investigated the effect of 27HC on the expression of genes associated with cholesterol homeostasis and inflammation. In order to simulate an inflammatory state, we evaluated the effects of 27HC in the absence as well as presence of lipopolysaccharide (LPS). LPS would be expected to polarize the macrophages towards an M1-like phenotype.

Bone marrow-derived macrophages (BMDMs) were prepared from female C57BL/6 mice. As expected, a 24-hour treatment of BMDMs with 27HC resulted in an induction of the LXR target genes ABCA1 and ABCG1, regardless of the presence of the inflammatory stimulus LPS (Fig. 1A–B). IL-10, a cytokine previously implicated in immune suppression in the tumor microenvironment [48] was found to be induced by 27HC in the absence of LPS, but modestly suppressed in the presence of LPS, suggesting that 27HC may selectively regulate genes depending on the inflammatory state of BMDMs (Fig. 1C). Similarly, 27HC induced the expression of MYC, a known factor involved in the alternative M2 polarization of macrophages [49], and this induction was most pronounced in the absence of LPS (Fig. 1D). Interestingly, 27HC decreased the expression of antigen-presentation/co-stimulation makers, namely MHCII (H2-Aa, H2-Ab1 and H2-Eb1) and B7 (CD80 and CD86) (Fig. 1E–I). MHCI proteins (H2-K1, H2-D1 and H2-L1) were also decreased by 27HC, regardless of whether the BMDMs were exposed to LPS (Fig. 1J–L). Because macrophages serve as antigen-presenting cells bridging innate and adaptive immunity, such suppression of antigen-presentation and co-stimulation markers indicates that 27HC may decrease the capacity of macrophages to present and activate T cells.

27HC-treated macrophages inhibit T cell expansion.

The regulation by 27HC of genes in BMDMs that are associated with antigen presentation and cross-presentation to T cells (Fig. 1) suggests that 27HC likely influences myeloid cell-T cell crosstalk. Therefore, we investigated whether treating BMDMs with 27HC impacts T cell activation and function. When T cells are activated, they undergo rapid clonal expansion, with proliferative activity therefore, being a biological readout of activation.

For these experiments, we utilized T cells isolated from OT-I or OT-II mice. These mice have been genetically modified so that their T cell receptors (TCRs) recognize specific ovalbumin peptides in the context of the major histocompatibility class (MHC) I and II molecules (H2-K^b or I-A^b) respectively [50]. T cells were enriched from the spleen of these mice, with a purity of 60–80% CD3+ T cells as assessed by flow cytometry (data not shown). When the cells were purified from OT-I mice, 50–60% of all cells were CD8+ (data not shown). Wildtype murine BMDMs were cultured in the presence of the activating agent LPS and ovalbumin peptides ($OVA_{257–264}$ or $OVA_{323–339}$), which serve as defined antigens. Simultaneously, BMDMs were treated with either vehicle or 27HC for 24hrs. At this time, BMDMs were washed to preclude the direct effects of 27HC on T cells, and subsequently co-cultured with CFSE-labeled CD3+ T cells isolated from OT-I or OT-II mice.

Intriguingly, flow cytometry showed that 27HC-treated BMDMs failed to promote antigen-specific T cell expansion, a hallmark of T cell activation. Impaired expansion was observed

regardless of the T cell subtype considered: CD4+ helper T cells (OT-II) or CD8+ cytotoxic T cells (OT-I) (Fig. 2A and B, respectively; representative histograms in Supplemental Fig. 1A). Therefore, 27HC-treated BMDMs result in decreased T cell expansion.

Although the ability of 27HC to regulate MHCII and B7 expression (Fig. 1) is consistent with the decreased expansion of T cells resulting from a deficit in antigen presentation or co-stimulation, it is well known that macrophages can also impair T cell activity via several distinct mechanisms, including those independent of antigen presentation [51]. To assess this possibility, we evaluated the impact of 27HC-treated BMDMs on the expansion of 'chemically' activated T cells. Specifically, BMDMs were treated with vehicle or 27HC, along with or without stimulation signals, LPS and BSA. After the treatments were washed off, those BMDMs were then co-cultured with CFSE-labeled T cells that were activated chemically by phorbol 12-myristate 13-acetate (PMA) and ionomycin [as described in 34]. These activated T cells should be highly proliferative due to the activation of the PKC pathway without the need for specific antigen-presentation. The induction of T cell proliferation by PMA and ionomycin was confirmed in assays with T cells only (verified in each experiment, Fig. 2C–H).

Under these co-culture conditions, we found that 27HC treatment of BMDMs resulted in suppression of the expansion of chemically activated T cells (Fig. 2C). This impaired expansion was observed whether or not the BMDMs were previously stimulated by LPS and BSA (Fig. 2C–D). The suppression was found to be dose-dependent: when higher concentrations of 27HC were used to treat BMDMs, there was a concomitant decrease in subsequent T cell expansion (Fig. 2E). The decreased expansion was also apparent when conditioned media was cultured with naïve CD4+ T cells (Supplemental Fig. 1B). Therefore, the effects of 27HC on the macrophage-mediated impairment of T cell expansion did not require MHC-cognate antigen-TCR ligation, indicating that any macrophage exposed to 27HC within proximity to a T cell could suppress its expansion. Furthermore, this would suggest that 27HC-induced macrophages could impair T cell expansion regardless of how they are activated (as in, even if they were activated by dendritic cells), since the chemical activation strategy targets downstream cell surface interactions.

Given that the enzyme responsible for the synthesis of 27HC, CYP27A1 is highly expressed in macrophages, it was of interest whether an autocrine source of 27HC was sufficient to impair T cell expansion. Therefore, we evaluated the capacity of BMDMs isolated from CYP27A1$^{-/-}$ mice to influence T cell expansion. In support of our hypothesis, we found that compared to BMDMs from wildtype mice, co-culture of T cells with CYP27A1$^{-/-}$ BMDMs resulted in increased expansion (Fig. 2F). This finding is important, given that CYP27A1 is amenable to targeting with a small molecule inhibitor, and one such molecule was previously shown to have anti-metastatic effects [23].

Since we previously demonstrated the requirement of neutrophils and γδ T cells for the pro-metastatic effects of 27HC [23], we were interested in whether these cell types could influence the 27HC-mediated BMDM impairment of T cell expansion. Thus, we co-cultured 27HC-treated BMDMs, bone marrow-derived neutrophils or both cell types with chemically activated T cells. We found that 27HC-treated neutrophils alone could impair T cell

expansion (Fig. 2G). There was no obvious additive or synergistic effect of co-culturing both neutrophils and BMDMs on T cell expansion (Fig. 2G). Culture of T cells from mice incapable of producing γδ T cells (DTCR$^{-/-}$ mice) continued to be suppressed by 27HC-treated BMDMs, suggesting that, at least *in vitro*, this cell type is dispensable for the immunosuppressive activities of 27HC (Fig. 2H). Collectively, these results indicate that (A) γδ T cells are not required, and (B) provide support for our hypothesis that 27HC results in impaired acquired immunity, regardless of what myeloid cell subtype it is working through, and the specific myeloid cell of importance is likely tumor type, tumor site and tumor-stage dependent [23, 24].

Our observations that BMDMs treated with 27HC impaired the ability of chemically activated T cells to proliferate, suggest that antigen-presentation is not required for 27HC-treated BMDMs to inhibit T cell expansion. Therefore, it was of interest as to whether direct contact between these two cell types was required and whether a soluble factor was mediating these effects. To address this question, we treated BMDMs with vehicle or 27HC for 24hrs and then washed the treatments off. BMDMs were then cultured in fresh media for another 72hrs, before we harvested supernatant from them. The conditioned media was subsequently used in culture with chemically activated T cells. Intriguingly, even without the presence of BMDMs, conditioned media alone exerted T cell suppression (representative experiments in Fig. 2I and Supplemental Fig. 1C). This is consistent with our findings that 27HC-pretreated mice continue to have increased metastatic burden post intravenous graft of mammary cancer cells, even when MHCII has been neutralized by an antibody (Supplemental Fig. 2). Thus, the 27HC-macrophage-mediated T cell impairment does not require cell-cell contact, suggesting that the effects of 27HC are mediated by a soluble factor(s).

T cells co-cultured with 27HC-treated macrophages have decreased cytotoxic activity.

Our data indicate that 27HC-treated BMDMs severely impair T cell expansion. However, it was also of interest whether those T cells that did expand had altered functional activity. In this regard, when we controlled for input post exposure to 27HC-treated BMDMs (Fig. 3A), we observed downregulated mRNA expression of cytotoxic T cell effector proteins, granzyme B and perforin (Fig. 3B–C). Subsequent ELISA analysis of T cell-conditioned media confirmed that cytotoxic T cells exposed to 27HC-treated BMDMs had decreased secretion of granzyme B (Fig. 3D). These findings indicate that the effector function of T cells was impaired after exposure to 27HC-treated BMDMs.

To specifically test whether anti-cancer T cell function was impaired by 27HC-treated BMDMs, we performed tumor cytolysis assays. T cells derived from OT-I mice were co-cultured with BMDMs exposed to LPS and OVA peptide, in the presence or absence of 27HC. As expected, 27HC resulted in decreased T cell expansion (Fig. 3E). The resulting T cells were harvested, counted, and then co-cultured with E0771 murine mammary cancer cells (controlling for T cell input) for 24hrs. Two different sublines of E0771 were used: the parental line that was stained with CFSE, and E0771-OVA cells that ectopically express $OVA_{257–264}$ and were stained with Far Red [development of E0771-OVA cells described in 52]. In this way, antigen-specific cytolytic activity could be determined as a ratio between

E077-OVA (Far Red) : parental E0771 (CFSE). A low ratio indicates strong cytolytic activity of T cells, while a high ratio suggests otherwise. Importantly, T cells harvested from co-culture with 27HC-treated BMDMs were less efficient at killing E0771-OVA cells (Fig. 3E–F). In order to ensure that these effects were not specific to E0771 cells, we repeated this experiment using the murine lymphoma cell line EL4 and its $OVA_{257–264}$ expressing derivative, E.G7-OVA [53]. As expected, T cells harvested from co-culture with 27HC-treated BMDMs had significantly reduced capacity to eliminate this cell type, as the ratio between E.G7-OVA and EL4 remained high and unchanged, even with increasing numbers of antigen-specific T cells added to the co-culture (Fig. 3G–H). Collectively, our data indicate that 27HC-treated BMDMs not only impair T cell expansion, but also their functional activity, potentially explaining the robust increase in metastasis observed in 27HC-treated mice [23].

Elevated level of CYP7B1, the enzyme responsible for catabolizing 27HC, expression has been associated with improved progression-free survival among breast cancer patients, and is reflective of intra-tumoral 27HC concentrations [54, 55]. In an effort to demonstrate the relevance of our findings to human health, we surveyed The Human Cancer Genome Atlas (TCGA) as accessed through the Invasive Breast Carcinoma from Pan-Cancer Atlas. We assessed tumoral CYP7B1 mRNA, the enzyme responsible for the catabolism of 27HC, expecting that higher CYP7B1 levels would result in decreased 27HC levels, as recently demonstrated previously for human thyroid tumors [55]. We found a modest, positive correlation between CYP7B1 and CD3G, a component of the T cell receptor CD3 family (Fig. 3I). Likewise, there is a modest, positive correlation between CYP7B1 and both CD8 and CD4 expression (Fig. 3J–K). Finally, CYP7B1 had a modest, positive correlation with both T cell effector proteins, perforin 1, granzyme B, and IFN-γ (Fig. 3L–N). Even after normalizing for CD8A expression, we still observed a weak but significant positive correlation between granzyme B or perforin and CYP7B1 (Supplemental Fig. 3). All breast cancer subtypes were used for these analyses, which would be expected to exhibit heterogeneity. Therefore, data from human breast tumor specimens support our pre-clinical findings that 27HC impairs T cell expansion and function.

27HC alters cytokine secretion in macrophages (BMDMs), potentially contributing to its suppressive effect.

Since direct contact between BMDMs and T cells was dispensable for the suppressive effects of 27HC, we next examined the involvement of potential soluble factors, focusing on those previously reported to mediate the pro-tumor or immunosuppressive effect of macrophages [51, 56]. In this regard, TAMs have been shown to upregulate arginase 1 (ARG1) and indoleamine 2,3-dioxygenase 1 (IDO1) to deprive T cells of arginine and/or tryptophan, and thereby inhibit their activity (T cells being auxotrophic for these amino acids) [57]. However, supplementation with either arginine or tryptophan in our BMDM-T cell co-culture failed to attenuate the immunosuppressive effect of 27HC-treated BMDMs (Fig. 4A, B). CD8+ activity has been shown to be impaired by increased cholesterol content, and given that oxysterols would result in reverse-cholesterol transport in BMDMs, it was possible that nearby T cells were subject to elevated cholesterol [43, 58]. However, expansion of T cells isolated from $LDLR^{-/-}$ mice (and thus lacking the receptor to take up

LDL-cholesterol) continued to be impaired by 27HC-treated BMDMs, although their baseline expansion was also decreased compared to wildtype T cells (Supplemental Fig. 4). It is important to note that $LDLR^{-/-}$ T cells may still be able to internalize cholesterol via scavenger receptors, so it is still possible that cholesterol mediates these effects.

There are recent reports of extracellular vesicles and their miRNA cargo being important mediators of cholesterol homeostasis [59, 60]. Extracellular vesicles, including exosomes are known to be secreted by myeloid cells, and have been implicated in immune suppression [61, 62]. Therefore, it is possible that 27HC-treated BMDMs released extracellular vesicles that mediated the decreased T cell expansion and function. To test this possibility, we isolated extracellular vesicles from vehicle or 27HC-treated BMDMs and cultured them with chemically activated T cells. Although there was an inhibition of T cell expansion by extracellular vesicles isolated from increasing numbers of BMDMs, there was no difference between extracellular vesicles derived from vehicle or 27HC-treated BMDMs (Fig. 4C). Therefore, it is unlikely that the inhibitory effects of 27HC-treated BMDMs are mediated through extracellular vesicles.

Several cytokines secreted by BMDMs have been described to inhibit T cell activation or proliferation. Therefore, we assessed a multiplex panel of murine cytokines at two time points: 24hrs after treatment with vehicle or 27HC and 72hrs after co-culture with T cells. The majority of changes observed with the treatment of 27HC were apparent after 24hrs (Supplemental Table 1A, B). Under basal conditions, many cytokines were below the detection thresholds of the assay, although 13 were detected and of those, only two were significantly regulated by 27HC after 24hrs (MIP-1a and MIP-1b), but these were no longer significantly different after 72hrs in culture (Supplemental Table 1A). When stimulated with LPS, several cytokines reached detectable concentrations after 24hrs, and of those, only IL-1α and IL-1β were significantly regulated by 27HC, although IL-6, IFN-γ, IL-18 and MIP-1b had false discovery rates <0.15 (Fig. 4D and Supplemental Table 1B). TGFβ was not included in the multiplex panel, but of potential relevance, and thus was assessed independently by ELISA (Supplemental Fig. 5). Of those cytokines regulated by 27HC, IL-1α was of interest as it has previously been shown to influence the growth and activity of T cells [63–66], and was consistently upregulated by 27HC under both time points. We confirmed this observation, as addition of IL-1α at a concentration reflective of measured levels in our co-culture, resulted in reduced proliferation of T cells activated by antigen presentation (Fig. 4E). However, siRNA mediated knockdown of IL-1α in BMDMs, did not significantly impair the ability of 27HC-treated BMDMs to inhibit T cell expansion (Fig. 4F). These observations indicate that while IL-1α may play a role in suppressing T cells, it is unlikely to be only one factor among several that mediate the effects of 27HC. It is very possible that 27HC modulates the phenotype of BMDMs through several diverse mechanisms, which converge to exhibit a strong immunosuppressive effect on T cells.

Reduced T cell expansion by 27HC-treated macrophages is mediated by the LXR.

Since 27HC is an abundant oxysterol, and its presence and metabolism have been clinically associated with breast, ovarian and thyroid cancers [24, 54, 55, 67], it was of importance to determine the mechanism by which 27HC alters macrophages to impair T cell expansion

and function. Previously reported pathways involved in macrophage function or cholesterol signaling (PI3K, AKT, NOTCH and CaMKK2) were ruled out through the use of pharmacologic inhibitors of these enzymes (Supplemental Fig. 6). Thus, to provide initial insight into potential mechanisms, we assessed the ability of 12 different oxysterols, cholesterol metabolites and synthetic LXR ligands to inhibit T cell expansion via treatment of BMDMs (Supplemental Table 2). Most oxysterols and cholesterol metabolites tested impaired T cell expansion to varying degrees (Fig. 5A). Dendrogenin A at 1 μM was a notable exception, with no noticeable effect on T cell expansion. This finding is in line with previous reports indicating that this metabolite induces tumor cell differentiation and immune cell infiltration and thus is associated with a good prognosis [68, 69]. Moreover, when BMDMs were co-treated, dendrogenin A exhibited dose-dependent attenuation of the actions of 27HC (Fig. 5A). This indicates that the effects of 27HC are likely receptor-mediated, and that dendrogenin A has the capacity to out-compete 27HC for binding.

27HC is known to bind to two nuclear receptors, ER and LXR, both receptors being implicated in immunomodulatory effects [43, 70]. Since several oxysterols have been shown to modulate the activities of the ERs and LXRs to different extents, we first evaluated capacity of the same panel of oxysterols and cholesterol metabolites (Supplemental Table 2) to activate the ERs and LXRs. We then assessed whether there was a correlation between this receptor activation and impaired T cell expansion. T47D cells, engineered to stably express an ER-reporter (estrogen response element upstream of a luciferase construct) [71], were treated with different doses of the ligands. Several oxysterols were found to have estrogenic activity, including those not previously been reported to do so (Fig. 5B, Supplemental Fig. 7 and Supplemental Table 2). Importantly, there was no notable correlation between the ability to activate ER-mediated transcription and inhibition of T cell expansion (Fig. 5B). Furthermore, no impact on T cell expansion was observed after treatment of BMDMs with either E2, a naturally occurring ER agonist, or ICI 182,780 (ICI, Fulvestrant) an ER antagonist and degrader (Fig. 5B, F). These results indicate that impairment of T cell expansion upon 27HC treatment of BMDMs is unlikely to be ER-mediated.

In order to assess the ability of different ligands to activate the LXRs, we evaluated the extent to which they could induce ABCA1, a well-known LXR target gene, in BMDMs. As expected, 27HC and many of the oxysterols could induce ABCA1 as well as another LXR target gene, ABCG1, while E2 did alter basal expression of ABCA1 (Supplemental Fig. 8). Interestingly, the ability of a ligand to induce ABCA1 expression was moderately and significantly correlated with its ability to inhibit T cell proliferation after treatment of BMDMs (Fig. 5C). This suggests that modulation of the LXR is mediating the effects of 27HC and other oxysterols. To definitively test this, we used complementary strategies of RNA-interference and pharmacologic antagonists.

ERα has been reported to be the predominant subtype in macrophages [72–74]. Therefore, we first evaluated the impact of knocking down ERα on the 27HC-mediated decrease in T cell expansion and function. Although siRNA-mediated knockdown resulted in an over 80% decrease in ERα mRNA, 27HC treatment of these BMDMs continued to inhibit the expansion of chemically activated T cells (Fig. 5D). Similarly, siRNA against ERα in

BMDMs did not impact the ability of 27HC to decrease OT-I T cell proliferation after antigen-mediated activation (Fig. 5E). Furthermore, the cytotoxic capacity of T cells was reduced by 27HC-treated BMDMs, regardless of ERα expression (not shown). We confirmed these findings using the pharmacological antagonist and ER degrader, ICI 182,780 (ICI; Fulvestrant; Fig. 5F) [75]. ICI antagonizes both ERα and β subtypes. As expected, ICI treatment failed to 'rescue' the inhibitory effects of 27HC-treated BMDMs on T cell proliferation or cytotoxic capacity (Fig. 5F and not shown). Collectively, these data strongly indicate that the mechanism by which 27HC modulates macrophages to impair T cell expansion and function is independent of the ER.

Treatment of BMDMs with a synthetic LXR agonist, GW3965, resulted in impaired T cell expansion (Fig. 5A, B), suggesting that this receptor may mediate the effects of 27HC. Both subtypes of LXRs (α and β) are expressed in BMDMs and whether they play specific roles is unclear. Therefore, we elected to knockdown both subtypes simultaneously. mRNA expression shows that siRNA-mediated knockdown of LXRα was marginal in several attempts, while LXRβ knockdown was moderate (around 10% and 50%, respectively, Supplemental Fig. 9A). However, even in the context of this partial knockdown of LXR, the immunosuppressive effect of 27HC-treated BMDMs on chemically activated T cells was significantly attenuated (Fig. 5G). shRNA against the LXRs administered by lentivirus infection of BMDMs (Supplemental Fig. 9B) also lead to the attenuation of 27HC-mediated impairment of OT-I T cell expansion after antigen-activation (Fig. 5H). The involvement of the LXRs was corroborated as co-treatment with the small molecule LXR antagonist, GSK2033 [76–80], restored T cell proliferation when cultured with 27HC-treated BMDMs (Fig. 5I). In this assay, GSK2033 was also able to 'rescue' the inhibitory effect of BMDMs treated with a synthetic ligand of the LXRs, GW3965 (Supplementary Fig. 9C). GSK2033 has been implicated in modulating the activity of other receptors (such as RORγ and ERRα) [81], and this cannot be ruled out here, although the effects of siRNA against the LXRs would support the conclusion that LXRs are mediating the effects of 27HC.

27HC-treated macrophages impair T cell function by promoting T cell apoptosis

Mechanistically, it was important to investigate whether the impaired T cell activity was a result of impairment of initial activation or post-activation proliferation, as myeloid-derived suppressor cells and macrophages have been previously shown to inhibit T cell activation [82, 83]. Consistent with this notion, we observed down-regulation of several markers of T cell activation (CD25, CD27 and CD69) 48 and 72hrs after co-culture with 27HC-treated BMDMs (Fig. 6A–C). However, down-regulation of CD62L expression was also observed (Fig. 6D). Shedding of CD62L is commonly observed in activated T cells [84]. A lower level of CD62L in T cells cultured with 27HC-treated BMDMs would suggest that those cells were indeed being activated, despite decreased expression of early activation markers and effector proteins (Fig. 6A–C, 3B–D). While mRNA expression is not necessarily correlated to surface protein concentration, one explanation for this apparent contradiction and the consistent, unidirectional change in gene expression is that 27HC-treated BMDMs induce T cell death, thereby leading to global downregulation of gene expression [85–88]. To investigate this possibility, we labeled OT-I T cells 48 or 72hrs after co-culture with Annexin V-FITC and propidium iodide. Annexin V binds to phosphatidylserine, a membrane lipid

that becomes externalized and accessible during apoptosis. Propidium iodide (PI) enters cells with disrupted membranes and binds DNA, indicative of cell necrosis. Through this assay, we found that at 48hr time point, there was no significant difference in live cell (Annexin V−/PI−) and necrotic cell (Annexin V−/PI+) populations (Fig. 6E). However, at both 48 and 72hr time points, 27HC-treated BMDMs induced a significantly greater number of Annexin V+/PI+ (indicative of late apoptotic/necrotic) T cells (Fig. 6E and Supplemental Fig. 10A). It is important to note that the 27HC group also had lower levels of early apoptotic cell population (Annexin V+/PI−; Fig. 6E), which could be a result of increasing number of cells moving into late apoptosis. Given that Annexin V is capable of entering ruptured membrane and binding to phosphatidylserine in necrotic cells and PI also interacts with DNA of cells during late apoptosis, we could not definitively tell whether the Annexin V+/PI+ population was a result of late apoptosis, necrosis or both [89, 90]. In order to conclusively identify the type of BMDM-driven T cell death, we alternatively labeled T cells, harvested from 3 time points (24, 48, 72hrs) post co-culture with vehicle- or 27HC-treated BMDMs, with fluorescent caspase-3/7 inhibitor probe FAM-DEVD-FMK (FLICA-FAM) and propidium iodide. FAM-DEVD-FMK covalently binds to activated caspase-3/7 through the fluoromethylketone (fmk) moiety [91, 92]. Because caspase-3 is an executioner in both the intrinsic and extrinsic apoptosis pathways but has no role in necrosis, presence of active caspase-3 in T cells after co-culture with 27HC-treated BMDMs more clearly distinguishes between apoptosis and necrosis. Specifically, FLICA+/PI− and FLICA+/PI+ population indicate early and late apoptosis respectively, while FLICA−/PI+ suggests necrosis. Consistent with the Annexin V/PI assay, results from this approach support the conclusion that 27HC-treated BMDMs induce T cell apoptosis between 24 and 48 hours after co-culture (Fig. 6F, Supplemental Fig. 10B). Notably, T cells in contact with 27HC-treated BMDMs entered late apoptosis at a much higher rate and, 72 hours after co-culture, there were less than half the live cells when cultured with 27HC-treated BMDMs compared to control (Fig. 6F). Since splenic T cells include different forms such as memory T cells and thus may not be representative of a normal T cell immune response, we isolated CD4+ naïve T cells, chemically activated them, and cultured them in the presence of conditioned media from either vehicle or 27HC treated BMDMs. Under these conditions, naïve T cells had increased late apoptotic cells after 48hrs of exposure to conditioned media from 27HC-treated BMDMs (Fig. 6G), mirroring results from pan-splenic T cells. Therefore, our results suggest that 27HC modulated BMDMs promote accelerated T cell apoptosis, where the T cells rapidly (within 48hrs) progressed from an early into a late apoptotic stage. Admittedly, at 72hrs, a small yet significant increase in a population of necrotic T cells was observed after T cells were co-cultured with 27HC-treated macrophages, and therefore we cannot rule out the possibility that caspase-3/7-independent pathways could also play a role in mediating 27HC-driven T cell dysfunction.

The immune checkpoint molecule, PD-L1 is known to induce apoptosis in effector T cells [93]. Therefore, we assessed PD-L1 mRNA in BMDMs treated with 27HC (same experiment as in Fig. 1). No changes in PD-L1 transcript were observed (Fig. 6H). Intriguingly however, there was a five-fold increase in soluble PD-L1 in conditioned media from 27HC-treated BMDMs, as assessed by ELISA (Fig. 6I). The biological relevance of soluble PD-L1 is still unclear, but emerging reports suggest that it too can induce apoptosis

[94], suggesting that this may be the putative factor mediating the pro-apoptotic effects of 27HC.

Absence or inhibition of CYP27A1 results in increased antigen-specific T cell cytolysis, decreased tumor growth and enhanced efficacy of immune checkpoint inhibition.

To determine whether the impaired T cell expansion and function upon BMDM treatment with 27HC occurred within the complex *in vivo* microenvironment, we orthotopically grafted E0771-OVA cells into mice that had CYP27A1 genetically knocked out specifically in the myeloid immune lineage ($CYP27A1^{flfl}$;$LysMCre^{+}$) and respective control mice ($CYP27A1^{+/+}$;$LysMCre^{+}$) (genotyping and selective knockout demonstrated in Supplemental Fig. 11). A myeloid-specific knockout approach was adopted, because introduction of exogenous 27HC would likely have direct effects on cancer cells, potentially confounding interpretation our results [22, 24]. After tumors were established, we then grafted all mice with OT-I T cells and measured resulting tumor growth; OT-I T cells were expected to specifically target OVA-expressing cells after presentation and activation by myeloid cells. Since we hypothesized that loss of myeloid cell CYP27A1 would result in increased T cell function, the OT-I T cells were grafted at a suboptimal concentration and would not be expected to fully eradicate the tumor. As expected, loss of myeloid CYP27A1 resulted in an increased number of mice whose tumors grew at a reduced rate post OT-I T cell graft compared to those grown in CYP27A1 replete mice (using a quadrupling of tumor volume as our threshold) (Fig. 7A and Supplemental Fig. 12A–B).

We have previously shown that 27HC robustly increases the metastatic colonization and growth of breast cancer cells in a myeloid cell dependent manner [23]. Given the results of the current study indicating that 27HC-treated BMDMs significantly impair T cell expansion and anti-cancer function, we therefore wanted to determine whether loss of myeloid cell expression of CYP27A1 would itself result in altered metastatic colonization and growth. Therefore, mice were grafted intravenously with E0771 cells, and subsequent metastatic lesions were quantified 3 weeks later. Intriguingly, significantly reduced lung metastatic burden was observed in $CYP27A1^{flfl}$;$LysMCre^{+}$ mice compared to control mice (Fig. 7B and Supplemental Fig. 12C). Furthermore, the only mice exhibiting macro-metastases outside the lung were from CYP27A1 intact controls (Fig. 7C). Since LysM promoter is active in all myeloid cells, one cannot ascribe the effects solely to the loss of CYP27A1 to macrophages, but rather to all types of myeloid cells. This is likely the case, as neutrophils also displayed inhibitory effects when treated with 27HC (Fig. 2G).

Immune checkpoint inhibition is, at best, modestly effective for the treatment of metastatic breast cancer, even within the patient cohort that is approved for its use. Since myeloid cells highly express CYP27A1 and are abundant in breast tumors and metastatic lesions, we speculated that inhibition of 27HC synthesis may increase the efficacy of this therapeutic strategy. Thus, we grafted mice intravenously with the highly aggressive 4T1 model of TNBC and allowed lesions to establish. At this point, mice were randomized and treated with αPD-L1 (checkpoint inhibitor), GW273297X (a small molecule inhibitor of CYP27A1), or both. As expected, treatment with αPD-L1 alone had no significant effects on metastatic burden (Fig. 7D). In this model, exogenous 27HC treatment robustly increased

metastatic burden, regardless of whether mice were treated with αPD-L1, indicating that the pro-metastatic effects of 27HC are likely a combination of enhanced colonization [23] as well as growth (Fig. 7D). Interestingly however, treatment with GW273297X as a single agent modestly reduced metastatic burden (Fig. 7D). Importantly, co-treatment with GW273297X and αPD-L1 further reduced metastatic burden, suggesting that myeloid-cell synthesized 27HC does impede the actions of checkpoint inhibition.

Discussion

Despite recent improvements in the treatment of breast cancer, advanced-stage and metastatic disease remain major contributors to breast cancer mortality. Although success using checkpoint inhibition in advanced TNBC have been reported and is now approved in combination with nab-Paclitaxel for a subset of patients, its overall response rate in breast cancer remains low [6]. Therefore, developing a clear understanding of the checkpoint-independent immune suppression biology within breast cancer is critical to the future development of curative therapies. The current work indicates that the actions of cholesterol metabolites such as 27HC on myeloid cells results in severe T cell impairment, both in terms of expansion as well as function.

Clinically, elevated cholesterol has been shown to be associated with breast cancer recurrence, while patients taking cholesterol-lowering medication (statins) have increased time to recurrence [95]. In preclinical models, a high cholesterol diet increases tumor growth and metastasis of mammary cancer [95]. However, our previous work has found that the pathophysiology of cholesterol on breast cancer progression is largely attributed to its primary metabolite, 27HC, as the growth and metastatic effects of a high cholesterol diet are absent in CYP27A1$^{-/-}$ mice [22, 23]. 27HC promotes primary tumor growth through a cancer-cell intrinsic mechanism by being an ER agonist and activating the ERs [22]. However, exogenous 27HC does not require the expression of ERs in cancer cells to robustly increase metastatic burden in mice [22, 23]. Engagement of the LXR by 27HC did result in increased EMT and modest increases in metastatic capacity of mammary cancer cell lines [22, 96]. However, we have shown that 27HC also acts in a cell extrinsic manner to robustly promote metastatic colonization [23]. Specifically, it was found that myeloid cells were required for these effects. Immune depletion of neutrophils ablated the pro-metastatic effects of 27HC, suggesting involvement of this cell type. This is perhaps because neutrophils dominate the myeloid cell landscape at the lung metastatic site of mice [97, 98]. On the other hand, the ovarian tumor microenvironment required 27HC, which appeared to mediate its effects through MDSCs [24]. Thus, it is likely that while 27HC exerts its effects on all myeloid cells, the extent to which it does so is dependent on the tumoral and microenvironmental context. Therefore, we explored the biology of 27HC in the prototypical myeloid cell, the macrophage.

Here, we found that antigen-primed, 27HC-exposed macrophages not only failed to promote the expansion of T helper cells and cytotoxic T cells, likely through induction of apoptosis, but also severely impaired the cytolytic capacity of cytotoxic T cells against cancer cells. We further showed that the suppression of T cells by 27HC-treated macrophages is not limited to the antigen-presentation pathway, as unprimed macrophages could also suppress

chemically activated T cell expansion. The general suppression of T cells by 27HC-modulated macrophages suggests even though T cells are already active, their proliferation and effector functions could still be severely impaired by 27HC-exposed macrophages in the tumor microenvironment. This is in line with a previous report that an oxysterol mixture could promote ‘M2’-like macrophages [99].

The ability of media-conditioned with macrophages treated with 27HC to suppress T cells indicates that certain soluble factors mediate the effects. Through our experiments, we ruled out obvious metabolic regulators (arginine and tryptophan) and extracellular vesicles. We have also identified several cytokines that are regulated by 27HC in time-dependent manners. Of those that are consistently altered by 27HC and known to result in T cell impairment, the involvement of IL-1α [63–66] was specifically tested, with our results indicating that this cytokine alone was not mediating the effects of 27HC.

27HC-treated BMDMs were found to increase T cell apoptosis. Conditioned media from 27HC-BMDMs also increased apoptosis in naïve T cells, in line with our other evidence suggesting that a soluble factor was responsible for the T cell suppressive effects of 27HC. Since the immune checkpoint molecule, PD-L1 is known to induce apoptosis in effector T cells [93], we explored whether this factor was regulated by 27HC. Its transcript levels were not altered in BMDMs post-treatment with 27HC (Fig. 6). To our surprise however, soluble PD-L1 was significantly increased in conditioned media from 27HC-treated BMDMs. The soluble form of PD-L1 is an active area of study, with several isoforms (splice variants) being described [100–102], and its precise biological functions and relevance being unclear. However, it has been found to induce apoptosis, similar to its membrane-bound form [94]. This suggests that soluble PD-L1 may be the putative factor mediating the pro-apoptotic effects of 27HC. Our results provide strong rationale for future studies exploring whether this is indeed mediating the effects of 27HC, how 27HC alters its secretion but not its mRNA expression, and whether 27HC alters different splice variants. The specific soluble factor(s) from myeloid cells responsible for mediating the suppressive effects of 27HC remain to be determined.

Our results are supported by preclinical models as metastatic lesions from mice treated with 27HC had decreased CD8+ T cells [23]. Furthermore, human patient tissue from breast cancer tumors with decreased CYP7B1 expression and therefore expected increased 27HC concentrations [55] had decreased expression of CD3G, CD4, CD8, perforin and granzyme B ([23] and Fig. 3I–M). This highlights the relevance of our results to human health and provides context to a recent report indicating that the recruitment of monocytes and subsequent epigenetic silencing of CYP7B1 results in the accumulation of intratumoral 27HC in breast cancer, and corroborated by another report that breast tumors had increased 27HC compared to normal-adjacent material [103, 104].

There have been several reports indicating that the synthesis/secretion of oxysterols is altered upon inflammatory stimuli [105, 106], and in turn, oxysterols exert modulatory activities on the immune system and/or inflammation [43, 107]. Mechanistically, we have shown that, macrophage LXRs, but not ERs, are required for the immunosuppressive effect of 27HC, as knocking down LXRs or blocking them with a pharmacological antagonist

attenuates the suppressive effects of 27HC on T cells. Further supporting this finding are revelations of a positive correlation between the ability of different oxysterols, cholesterol metabolites and synthetic LXR agonists to induce the LXR target gene ABCA1, and their ability to suppress T cell expansion. The involvement of LXR in the immunosuppressive activities of 27HC provides a contrast to certain inflammatory actions of 27HC being mediated through the ER, at least with respect to cardiovascular disease [108], although LXR activation by 27HC has been implicated in the protective effects of vitamin D on atherosclerosis in a preclinical model [109]. Estrogen has been shown to increase hematopoietic stem cell proliferation [110] and myelopoiesis [70]. 27HC was also found to increase hematopoietic stem cell mobilization but not proliferation [110]. Estrogen was found to enhance the immunosuppressive activities of MDSCs [70]. However, here, we show that despite 27HC being an ER ligand, it is its activities through the LXRs in myeloid cells that result in T cell suppression.

Several previous studies have demonstrated that oxysterols and the LXR are involved in management of the innate immune system [99, 111–114]. Interestingly, in apparent contrast with previous reports indicating that macrophages lacking LXRs had increased apoptosis [115], or were more susceptible to LPS-induced liver injury and resistant to inflammatory responses caused by treatment with α-galactosylceramide or concanavalin-A [113], treatment of mice and humans with synthetic LXR agonists decreased MDSC abundance within tumors by decreasing their [MDSC] survival [116]. Specifically, MDSCs isolated from tumors of LXR-treated mice were less able to suppress T cell expansion compared to those isolated form vehicle-treated mice [116]. This apparent discrepancy with our work indicating that myeloid cells treated directly with LXR agonists impair T cell function may be due to either: (1) Differential kinetics and compensatory mechanisms, it is possible that MDSCs isolated from *in vivo* treated mice lose their LXR signal and overcompensate before returning to baseline. (2) Different myeloid cell types may behave differently. Our data indicate that 27HC induces an immune suppressive phenotype on both macrophages and neutrophils, and as such ‘MDSCs’ may not behave similarly. (3) LXR agonists may exert their effects in a ‘selective’ fashion, similar to selective estrogen receptor modulators. In other words, different LXR agonists may behave differentially, in a context-dependent \ specific manner. Even though the exact immunomodulatory pathway downstream of LXR is currently unknown, previous reports have shown that the LXR agonists suppress NLRP3 inflammasome activation, this downregulation being associated with poorer clinical outcome in hepatocellular carcinoma [117–119]. Regardless of mechanism, our work presented here, along with emerging evidence of cancer-cell intrinsic roles for the LXRs [22, 96], would suggest that caution should be taken prior to the development of LXR as an immune-modulatory target for the treatment of solid tumors. Furthermore, these results provide a strong rationale for studies to elucidate the precise signaling events downstream of LXR.

One potential limitation to our study is the reliance on BMDMs, which may not be reflective of macrophages or other myeloid cells within the tumor microenvironment. Indeed, microenvironmental factors can significantly alter macrophage response. Since 27HC is likely one of these factors, it is still relevant that naïve BMDMs are ‘entrained’ by this oxysterol. Furthermore, bone marrow-derived neutrophils treated with 27HC also exhibited an anti-T cell response (Fig. 2). Finally, our *in vivo* data (Fig. 7) and correlational data from

human tumors (Fig. 3) support an immune-suppressive role for 27HC within the tumor microenvironment.

Collectively, we have shown that 27HC-treated macrophages strongly impair the expansion of T cells, suggesting that inhibition of 27HC synthesis should improve the efficacy of immune therapy. Indeed, in a preclinical model of metastatic breast cancer, blocking 27HC synthesis with a small molecule inhibitor of CYP27A1 resulted in a positive response to αPD-L1. The expression profile of CYP27A1, which is abundant in myeloid cells the liver and within cancer cells of high-grade tumors [22, 103, 120] suggests that targeting this enzyme will have a low risk of unwarranted side effects. Our work also reinforces the broader strategy of reeducating myeloid cells to be pro-immune and anti-cancer, rather than ablating them altogether [11, 23, 121–123].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS:

- The cholesterol metabolite, 27-Hydroxycholesterol (27HC), works on myeloid cells to suppress T cell activity
- 27HC-LXR activity mediates myeloid cell-driven immune suppression
- Myeloid cell-driven T cell inhibition likely results from accelerated T cell apoptosis
- Pharmacologic inhibition of 27HC synthesis enhances immune check-point inhibition
- The immune suppression is likely a dominant mechanism by which 27HC promotes breast cancer progression.

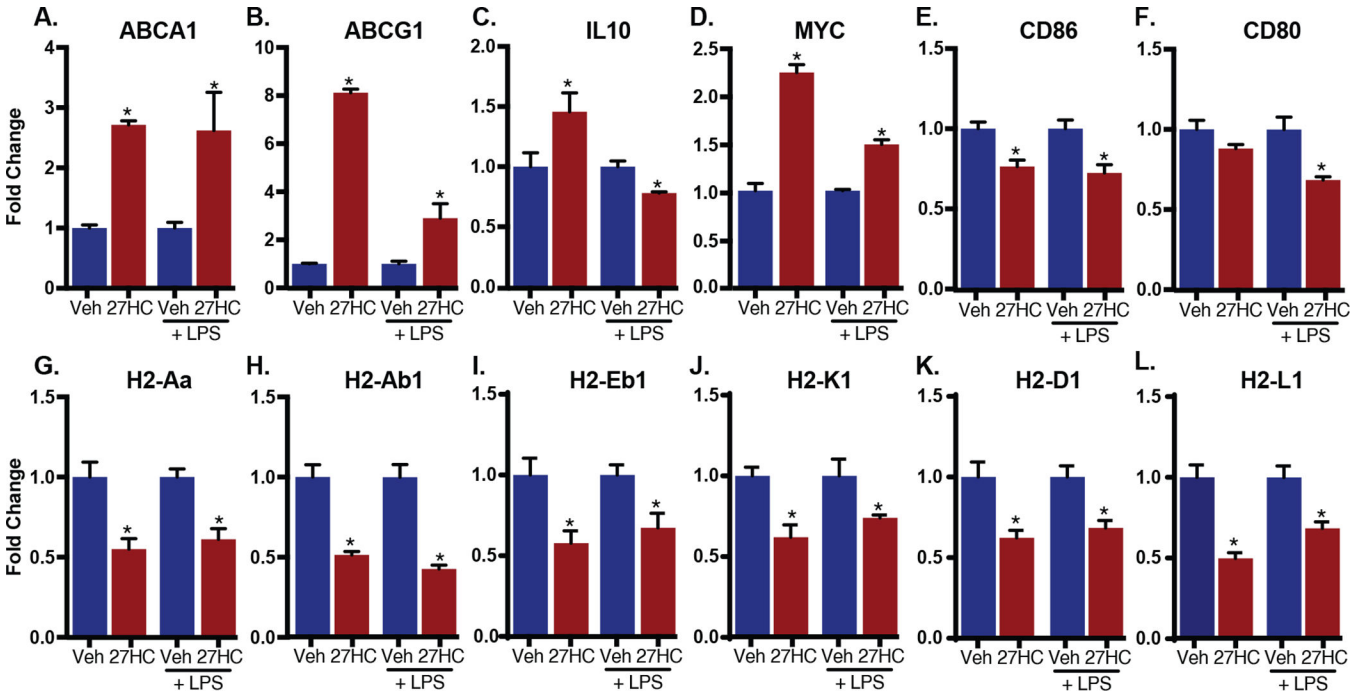

Figure 1: 27HC results in altered expression of genes associated with macrophage function and interaction with T cells.

Murine BMDMs were treated with vehicle (DMSO) or 10 μM 27HC in the absence or presence of LPS. Transcripts were detected and quantified by qPCR and displayed as mean ± SEM. Data are presented as normalized to respective vehicle control. Asterisks (*) indicate statistical significance from the respective vehicle control (N=3/group for all samples treated with LPS, N=6/group for all samples treated with vehicle only, N=5 for all samples treated with 27HC only). (two-tailed t-test, $p < 0.05$)

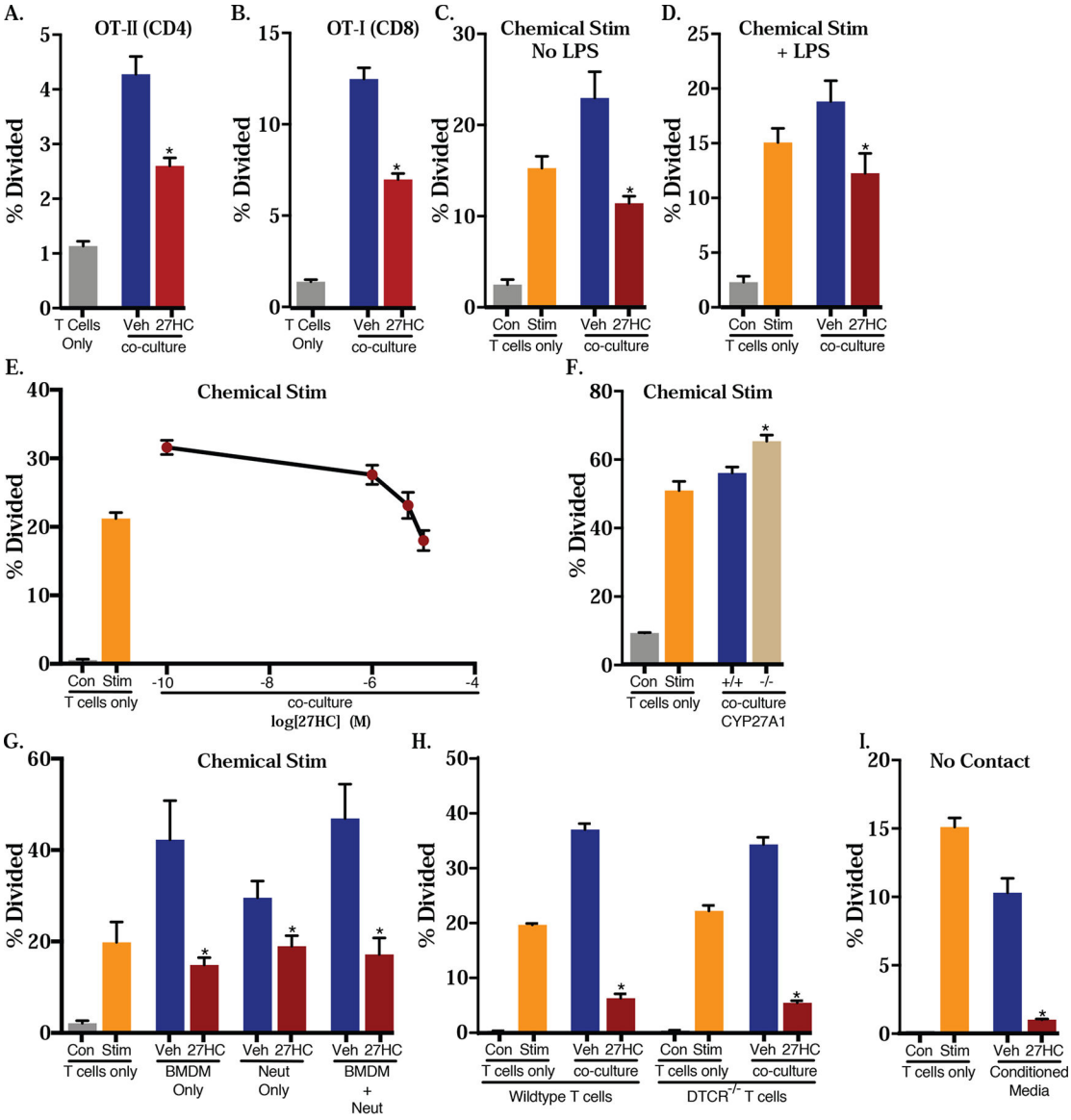

Figure 2: 27HC-treated macrophages decrease T cell activation and expansion.

Murine BMDMs were cultured with OVA peptide in the presence or absence of indicated ligands in A and B. The media was then replaced and BMDMs were co-cultured with CFSE-labeled T cells from **(A)** OT-II mice (where TCRs of CD4+ T cells are engineered to recognize OVA), or **(B)** OT-I mice (where TCRs of CD8+ T cells are engineered to recognize OVA) for 72hrs. Subsequent T cell proliferation was quantified by flow cytometry for CFSE dilution. For subsequent panels (**C-I**), T cells from wildtype mice were activated by chemical stimulation with 10 nM PMA and 250 nM ionomycin and co-cultured with BMDMs. **(F)** BMDMs from wildtype or CYP27A1$^{-/-}$ were used for co-culture with T cells. **(G)** The same inhibitory effects of 27HC-treated BMDMs were observed with 27HC-treated neutrophils (PMNs). **(H)** γδ T cells are dispensable for the effects of 27HC-treated BMDMs on T cell expansion; T cells isolated from DTCR$^{-/-}$ mice do not have γδ T cells. **(I)** Direct contact between 27HC-treated BMDMs and T cells is not required for its inhibitory effects.

Conditioned media from treated BMDMs was used to culture chemically activated T cells. Data are presented as mean ± SEM and asterisks (*) indicate statistical significance from the respective vehicle control (N=3 for all T cell only controls. For co-culture conditions, N=4/group in (A-D), N=5/group in (E), and N=6/group in (F-I)). (two-tailed t-test, $p < 0.05$)

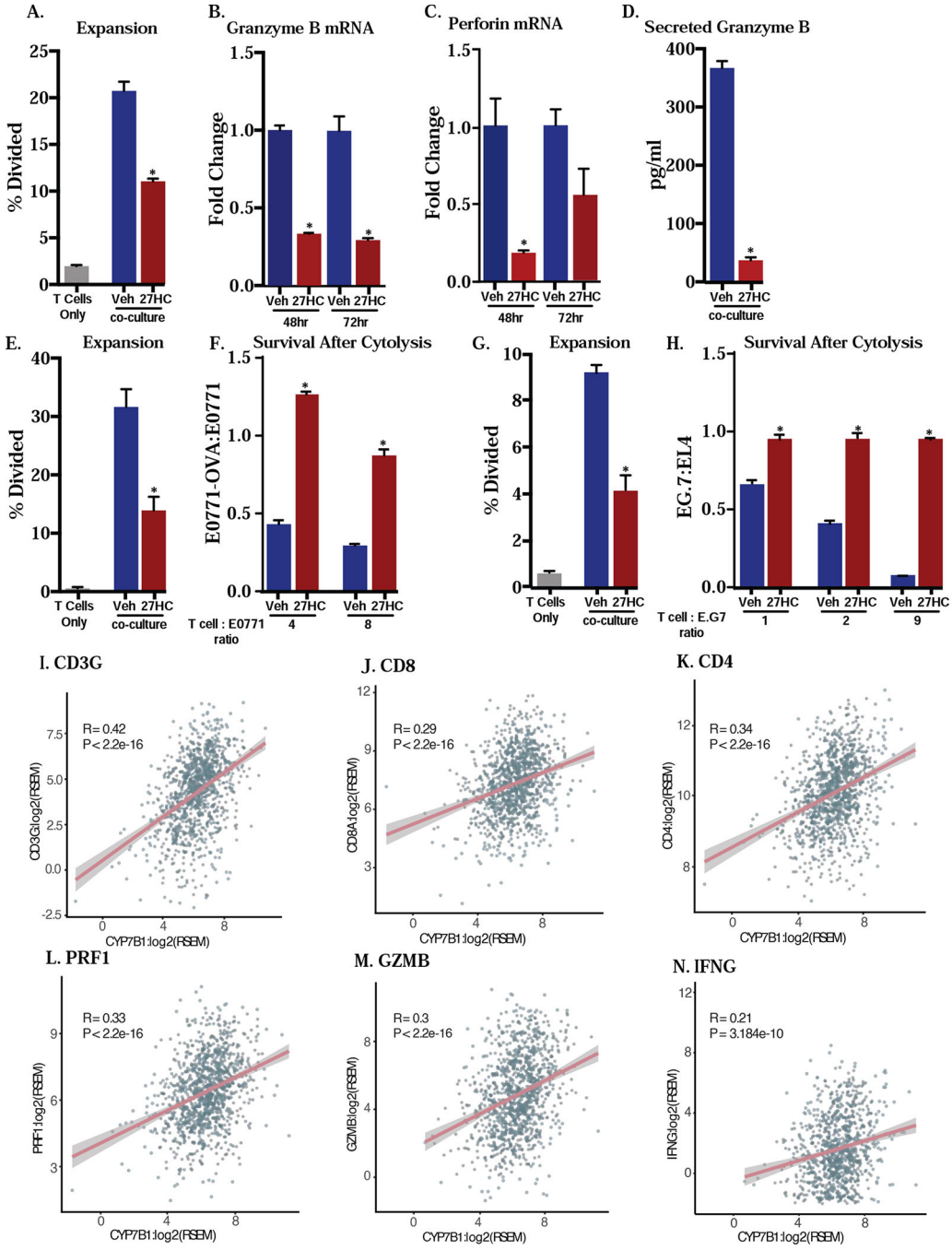

Figure 3: T cells co-cultured 27HC-treated macrophages have decreased cytotoxic activity.

T cells co-cultured with BMDMs treated with 27HC for 72hrs have decreased expansion **(A)** and the resulting expanded T cells also have decreased granzyme B and perforin mRNA expression through time after co-culture as measured by qPCR **(B-C)** and secretion of granzyme B as measured by ELISA **(D)**. Data in (A-C) are presented as normalized to respective vehicle control. **(E** and **G)** When OT-I T cells are co-cultured with BMDMs treated with indicated ligands as well as OVA, 27HC treatment results in decreased expansion, and these T cells have decreased cytolytic activity against **(F)** E0771 mammary

cancer cells or **(H)** EG.7-OVA lymphoma cells. Both of these lines were engineered to express OVA. Specifically, the parental cell lines (E0771 or EL4) were labeled with CFSE and derivative cell lines (E0771-OVA and E.G7-OVA) were labeled with Far Red. Equivalent cell number of the respective parental and derivative cell lines were plated and co-cultured with OT-I T cells activated by vehicle- or 27HC-treatd BMDMs at indicated ratios. The cytolytic activity of T cells was assessed via flow cytometry as represented by the ratio of derivative (Far Red)/parental (CFSE) cell lines. **(I-N)** Human RNA sequencing data from TCGA Pan Cancer for invasive breast carcinoma indicated a moderately positive and significant correlation between CYP7B1, an enzyme responsible for catabolizing 27HC, expression and the expression of 6 T cell genes (CD3G, CD8A, CD4, perforin 1, granzyme B, INF-γ) that are related to immune competence. mRNA expressions were log2 transformed and Pearson's correlation was assessed (N=1059, 1081, 1082, 1082, 1077 and 905, respectively). Data in (A-H) are presented as mean ± SEM and asterisks (*) indicate statistical significance from the respective vehicle control (two-tailed t-test in (A-H), $p < 0.05$). A: N=3/group for control and N=6/group for co-culture conditions, B: N=3–4/group, C: N=3–4/group, D: N=5–6/group, E: N=4/group, F: N=4/group, G: N=4/group, H: N=5/group.

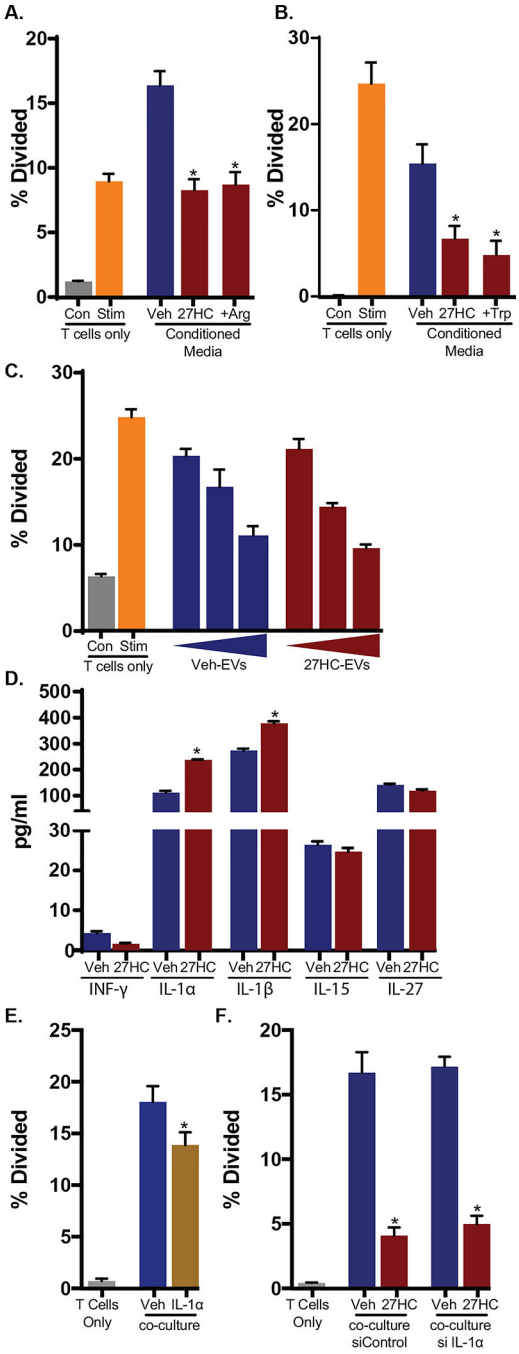

Figure 4: T cell suppression resulting from 27HC treated macrophages is not mediated by common mechanisms.

(A) Arginine (Arg) depletion by BMDMs is not likely the cause of impaired T cell expansion as supplementation with exogenous Arg failed to rescue the phenotype (N=3 for all controls, N=6/group for co-culture conditions). BMDMs were treated with vehicle or 27HC. Those cells were then co-cultured with chemically activated wildtype T cells. In the co-culture, 0.2 g/L of Arg or equivalent volume of PBS was supplemented. T cell expansion was measured by dilution of CFSE as assessed by flow cytometry. **(B)** Tryptophan (Trp) depletion by BMDMs is not likely the cause of impaired T cell expansion as

supplementation with 0.005 g/L of exogenous Trp failed to rescue the phenotype (N=3 for all controls, N=4/group for co-culture conditions). **(C)** Extracellular vesicles derived from BMDMs are not likely the cause of impaired T cell expansion. Direct treatment of T cells with extracellular vesicles decrease expansion, but there was no difference between extracellular vesicles isolated from vehicle or 27HC-treated BMDMs (N=3). Extracellular vesicles were isolated from the conditioned media of vehicle- or 27HC-treated BMDMs at three increasing densities, using Total Exosome Isolation Reagent (Thermo Fisher, Cat no. 4478359) and then cultured with chemically activated T cells at three increasing doses. The increasing densities of starting BMDMs were 1x, 2x, or 3x the number of macrophages used in other experiments. T cell expansion was measured. **(D)** A cytokine array found modest changes in certain cytokines secreted from LPS-primed vehicle- or 27HC-treated BMDMs (full panels indicated in Supplemental Table 1) (N=3/group). **(E)** In line with previous reports, IL-1α, at the same concentrations induced by 27HC as determined in (D), resulted in decreased T cell expansion (N=5/group, Mann-Whitney U test). **(F)** IL-1α is not likely a sufficient cause of impaired T cell expansion as siRNA knockdown within BMDMs failed to reverse the effects of 27HC (N=3 for all controls, N=4–5/group for co-culture conditions). Data are presented as mean ± SEM and asterisks (*) indicate statistical significance from the respective vehicle control. (two-tailed t-test unless otherwise specified, $p < 0.05$)

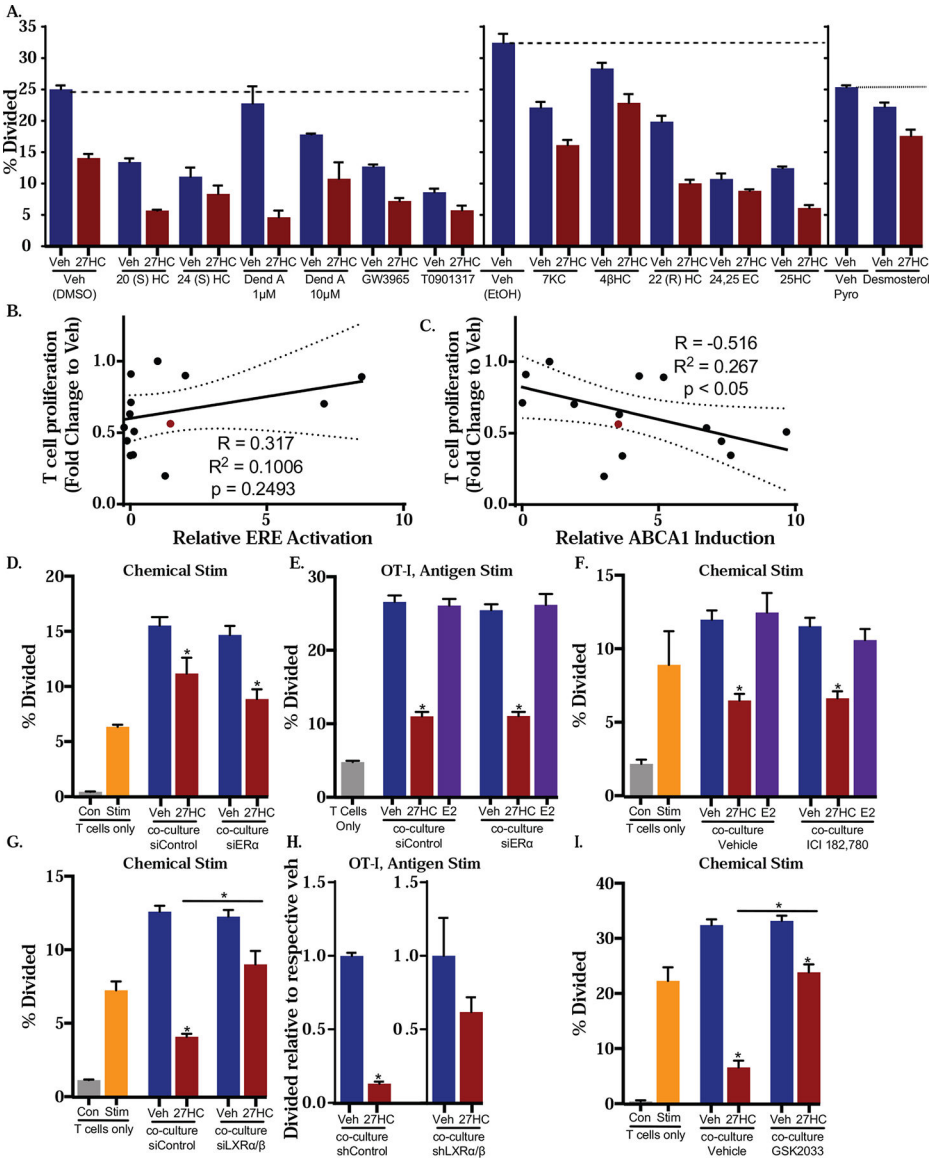

Figure 5: LXR mediates the myeloid cell immunosuppressive effect of 27HC.

(A) BMDMs were treated with 12 cholesterol metabolites, LXR ligands or indicated vehicle controls for 24hrs prior to co-culture with chemically activated T cells for 72hrs, and proliferation was assessed. **(B-C)** Two cohorts of BMDMs were treated with ligands for 24hrs. Expression of ABCA1 mRNA was assessed in the first cohort. BMDMs from the second cohort were co-cultured with chemically activated T cells for 72hrs post treatment, at which point T cell proliferation was measured. **(B)** T47D cells stably transfected with a ERE luciferase reporter were treated as in (A) and the activation of ER was measured by luciferase activity. Pearson's correlation between T cell proliferation and ER activity is presented. **(C)** Pearson's correlation between T cell proliferation and induction of ABCA1 mRNA is presented. **(D)** BMDMs were transfected with siControl or siERα for 48hrs and then treated with vehicle or 27HC. After 24hrs, the treatments were washed off, and media was replaced. Those BMDMs were co-cultured with chemically activated T cells for 72hrs.

(E) BMDMs transfected with siControl or siERα for 48hrs were primed with OVA and treated with vehicle or 27HC for 24hrs. BMDMs were then co-cultured with CD3+ T cells from OT-I mice for 72hrs and proliferation assessed. **(F)** BMDMs were treated with vehicle, 27HC, ICI (an ER antagonist), E2, ICI+27HC or E2+27HC. BMDMs were then co-cultured with chemically activated wildtype T cells as in (D). **(G)** BMDMs were transfected with siControl or siLXRα/β for 48hrs. Those BMDMs were then harvested, treated and co-cultured with chemically activated T cells as in (D). **(H)** BMDMs were transduced with lentiviral shControl or shLXRα/ββplasmids for 96hrs and then treated and co-cultured as in (E). Data presented as normalized to respective control. **(I)** LXR antagonist (GSK2033) attenuates effects of 27HC. BMDMs were treated and co-cultured with T cells as in (D). All T cells were labeled with CFSE and the proliferation was assessed by flow cytometry. Data are presented as mean ± SEM and asterisks (*) indicate statistical significance from the respective vehicle control (N=3 for all T cell only controls. For co-culture conditions: N=4 for A, E, H and I. N=3 for B. N=5–6 for D and F. N=6 for G. (two-tailed t-test, $p < 0.05$). For luciferase activity in (C), N=4)

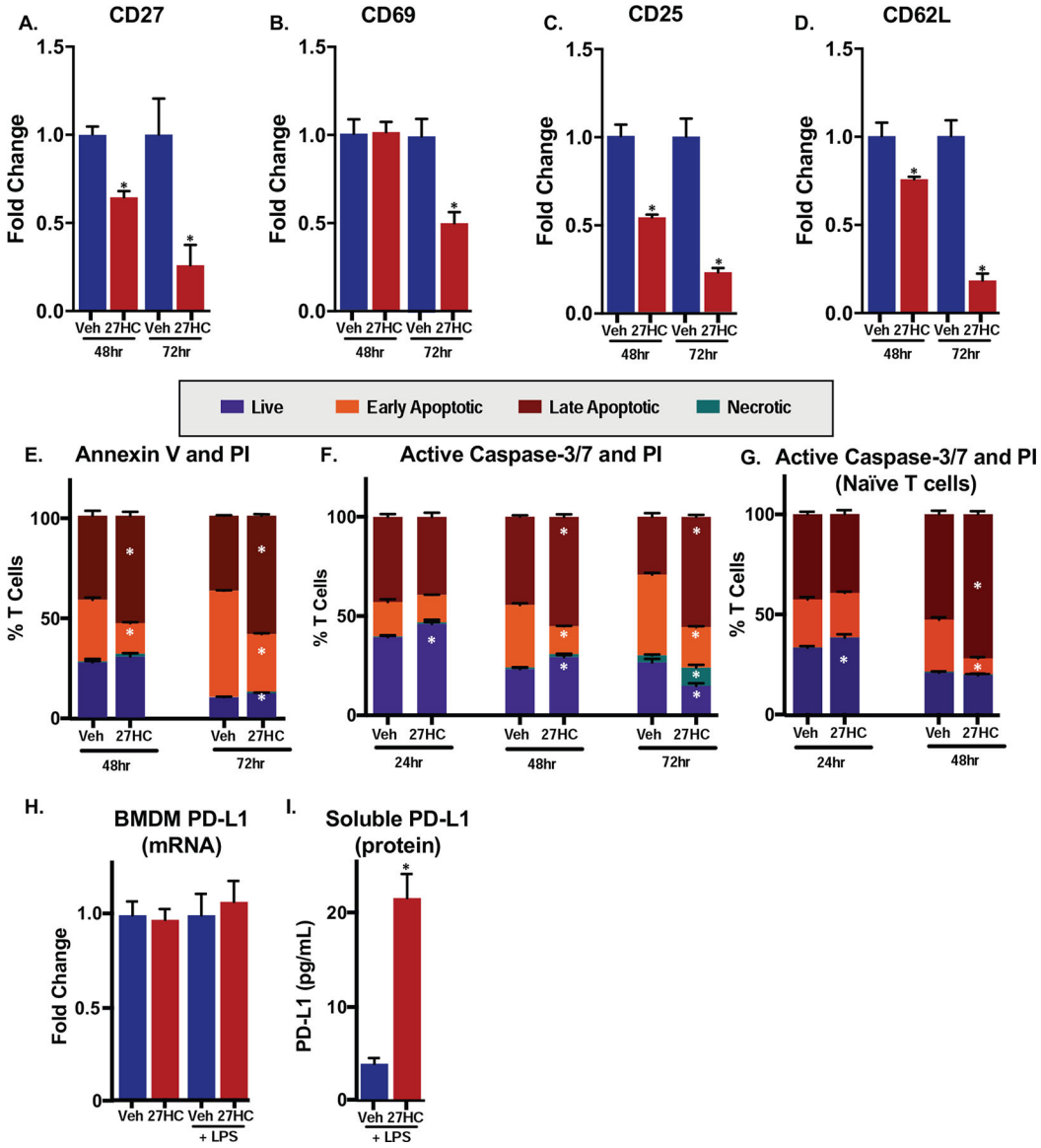

Figure 6: 27HC-treated macrophages induce T cell apoptosis.

(A-D) Gene expression of markers of T cell activation was downregulated when T cells are co-cultured with BMDMs treated with 27HC. qPCR on T cells, 48 or 72hrs after co-culture (N=3–4/group). Data are presented as normalized to respective vehicle control. **(E)** OT-I T cells 48 or 72 hours after co-culture with vehicle- or 27HC-treated BMDMs exhibited enhanced apoptosis. T cells were harvested from the co-culture at two different time points and then were labeled with Annexin V-FITC and PI. Flow cytometry analysis was performed to measure the cell populations (N=4/group). **(F)** OT-I T cells in co-culture with 27HC-treated BMDMs exhibit increased apoptosis. T cells were harvested from the co-culture (as in E) at three different time points and then labeled with FLICA-FAM, a caspase-3/7 inhibitor that covalently binds to active forms of caspase-3/7, and PI. Flow cytometry analysis was performed to measure the cell populations (N=4/group). **(G)** Naïve CD4+ T cells were isolated from the spleen, chemically activated and cultured with conditioned

media from vehicle or 27HC treated BMDMs. Subsequent analysis was similar to **(F)** (N=4/group). **(H)** PD-L1 mRNA expression in BMDMs; see Fig. 1 for details. **(I)** Soluble PD-L1 is increased in media from LPS-primed BMDMs treated with 27HC for 24hrs (similar experimental setup to Fig. 4D (N=4/group). Data are presented as mean ± SEM, and asterisks (*) indicate statistical significance from the respective vehicle control. (two-tailed t-test in (A-D), two-way ANOVA followed by Bonferroni's multiple comparison test in (E) and (F), $p < 0.05$)

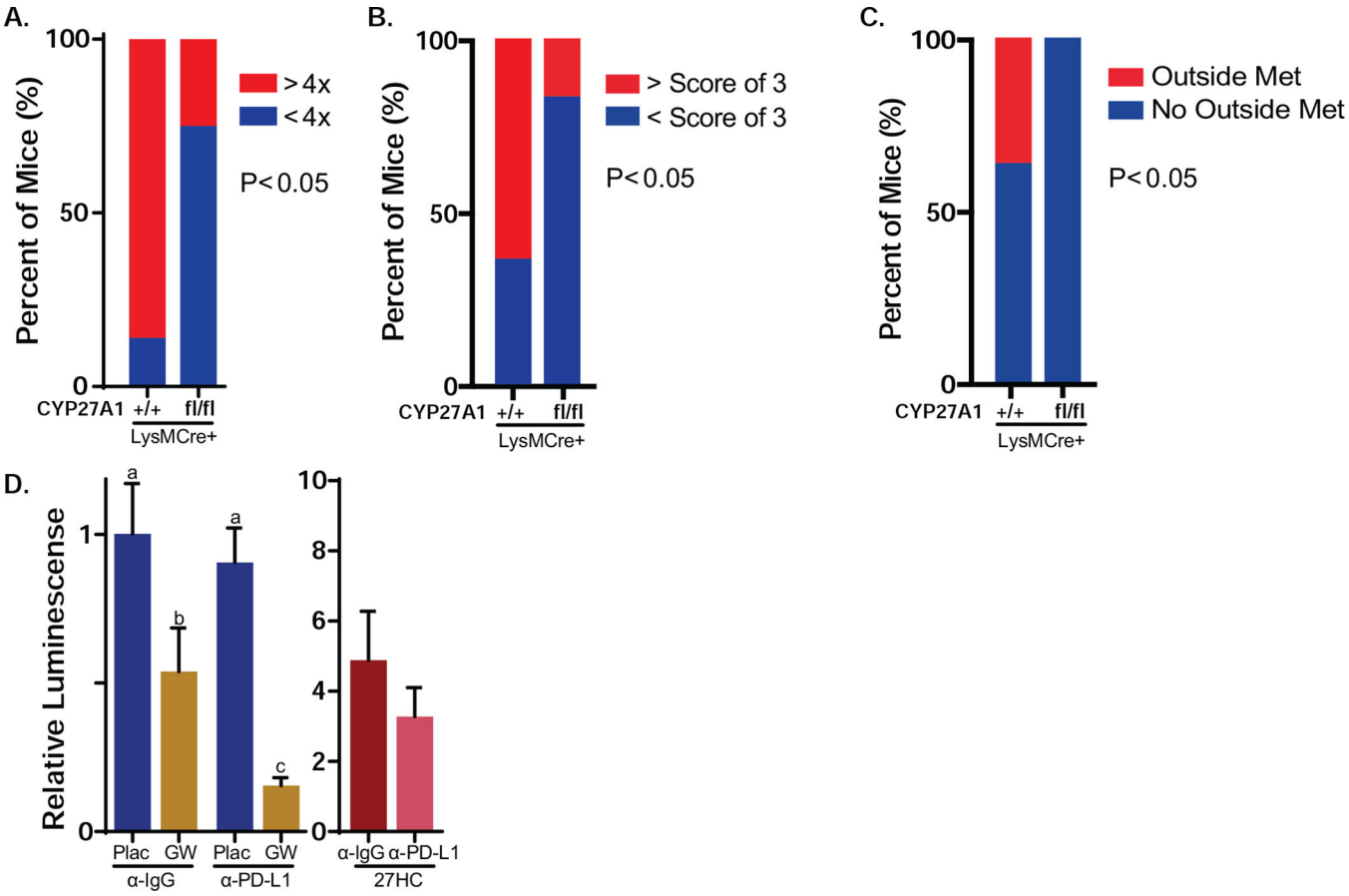

Figure 7: Absence or inhibition of CYP27A1 promotes T cell function, decreased tumor growth and enhanced efficacy of αPD-L1.

(A) Mice with myeloid cell knockout of CYP27A1 (CYP27A1$^{fl/fl}$;LysMCre^{+}) exhibited lower level of antigen-specific primary tumor growth. CYP27A1$^{fl/fl}$;LysMCre^{+} mice (N=8) and their replete controls (N=7) were grafted orthotopically with E0771-OVA cells and tumors allowed to grow to ~200 mm^3. Mice then received an adoptive transfer of CD3+ T cells isolated from OT-I mice. The difference between final tumor volume and the established tumor volume at adoptive transfer was calculated as a ratio between the two. The ratio used for comparison of response between two groups was 4. Macrophage-selective knockout illustrated in Supplemental Fig. 11). Growth curves are depicted in Supplemental Fig. 12A–B. **(B)** Myeloid cell CYP27A1 knockout mice exhibited lower level of metastatic burden, after intravenous graft of E0771 cells (CYP27A1$^{fl/fl}$;LysMCre^{+} mice, N=12; CYP27A1$^{+/+}$;LysMCre^{+} mice N=11. The mice were euthanized 3 weeks after injection and metastatic nodules counted and then scored according to their diameters using photos taken. Specifically, a nodule with diameter greater than or equal to 0.5 in, 0.3 in, 0.2 in and 0.1 in received a score of 4, 3, 2 and 1, respectively. If the metastatic lesion was discovered outside lung (e.g. liver), the lesion would also receive a score of 4. Representative pictures of metastatic lungs are in Supplemental Fig. 12C. **(C)** CYP27A1$^{fl/fl}$;LysMCre^{+} mice exhibited decreased extra-plural metastases. Many controls, but no myeloid cell CYP27A1 knockout mice, had metastases outside the lung (same mice as in B). **(D)** Small molecule inhibition of CYP27A1 reduced metastatic growth and improved checkpoint inhibitor efficacy. BALB/C

mice were grafted intravenously with 4T1-luc cells, and lesions allowed to establish for 3d. At this time, mice were treated with indicated ligands, including the CYP27A1 inhibitor, GW273297X (GW). αPD-L1 refers to a monoclonal antibody against murine PD-L1. (N=10/group except for 27HC treatments which had an N of 7–8/group). Data in (A-C) are presented as contingency table bar graph and data in (D) are presented as mean ± SEM (Fisher's exact test in (A-C), one-way ANOVA followed by Newman-Keuls multiple comparison test for the first 4 groups in (D), $p < 0.05$).