

The Regulation of SLPI and Its Impact on Metastasis and the Ovarian Tumor Microenvironment in Ovarian Cancer

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Abstract

Understanding the factors that drive epithelial ovarian cancer progression is needed to design effective targeted therapies, especially knowing that it is the deadliest gynecological malignancy. SLPI is a protease inhibitor with anti-inflammatory properties and is highly expressed in ovarian cancers; however, its role in these cancers is unclear. This study found that *Slpi* expression was upregulated by MEK pathway signaling in mouse ovarian cancer cells. The effect of Slpi on ovarian cancer metastasis, survival, and the tumor microenvironment was also investigated. In suspension cultures, *Slpi* knockout cells formed smaller, more abundant aggregates than their *Slpi*-expressing controls, which correlated with increased metastases in vivo. Following intrabursal injections of *Slpi*-overexpressing and control cells, *Slpi*-overexpressing tumors exhibited reduced survival with fewer metastases, more macrophages, eosinophils, and dendritic cells. This research revealed that SLPI increases cancer aggressiveness by creating an immunosuppressive TME and promoting aggregation of metastatic cells.

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List of Abbreviations

AKT	Protein Kinase B
BMDM	Bone Marrow-Derived Macrophage
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
BRCA1/2	Breast Cancer gene 1/2
CCR5	C-C motif chemokine receptor 5
CCNE1	Cyclin E1
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-to-Mesenchymal Transition
EOC	Epithelial Ovarian Cancer
ERK	Extracellular Signal-Regulated Kinase
HGSOC	High-Grade Serous Ovarian Carcinoma
IFN- γ	Interferon Gamma
ILC2	Type 2 Innate Lymphoid Cells
IL-4	Interleukin-4
IL-6	Interleukin-6
IP	Intraperitoneal
Jup	Junction plakoglobin
KRAS	Kirsten rat sarcoma viral oncogene homolog
LGSC	Low-Grade Serous Carcinoma
LPS	Lipopolysaccharide
M-CSF	Macrophage Colony-Stimulating Factor
MEK	Mitogen-Activated Protein Kinase Kinase
MMP	Matrix Metalloproteinase
MOE	Mouse Oviductal Epithelial

NF- κ B	Nuclear Factor Kappa B
NK	Natural Killer Cells
OSE	Ovarian Surface Epithelium
PBMC	Peripheral Blood Mononuclear Cell
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PTEN	Phosphatase and Tensin Homolog
PUMA	P53 Upregulated Modulator of Apoptosis
SCCOHT	Small Cell Carcinoma of the Ovary, Hypercalcemic Type
SEM	Standard Error of the Mean
SLPI	Secretory Leukocyte Protease Inhibitor
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-Like Receptor
TME	Tumor Microenvironment
TP53	Tumor Protein 53
TWIST	Twist Family BHLH Transcription Factor
VEGF	Vascular Endothelial Growth Factor

Contributions

Former master's student in the Vanderhyden lab, Maryam Echaibi:

- Generated data shown in Figures 1 and 4
- Created ID8, MPK, and KPCA.B SLPI-modified cell lines
- Validated primers used for qPCR experiments
- Assisted with tumor, lymph nodes, and peritoneal wash collection for TME analysis described in section 2.12
- Performed RNA extraction for RNA sequencing analysis

Current MD/PhD candidate in the Vanderhyden lab, Dalia Ibrahim:

- Assisted with tumor, lymph nodes, and peritoneal wash collection for TME analysis described in section 2.12
- Completed all the Uniform Manifold Approximation and Projection (UMAP) visualizations shown in Figures 27 - 42
- Assisted in the analysis of flow data generated following treatment of bone marrow-derived macrophages with recombinant SLPI shown in Figure 43

Current Postdoctoral associate in the Vanderhyden lab, Sarah Nersesian:

- RNA sequencing analysis shown was completed in collaboration with Sarah Nersesian.

Current PhD candidate in the Vanderhyden lab, Yalun Zhu

- Generated Figures 2 and 3 using data from the cancer Dependency Map portal

Current Postdoctoral Fellow in the Cook lab, John Abou-Hamad

- Generated western blots shown in Figure 5

Chapter 1: Introduction

1.1. Ovarian Cancer: origins, development, and subtypes

Ovarian cancer is characterized by an uncontrolled division of abnormal cells originating at or near the ovaries¹. It is the biggest contributor to gynecological cancer-related deaths² and is expected to be the fifth deadliest cancer for women in Canada in 2026³ with a high mortality rate characterized by a 5-year survival rate of 44%⁴. Globally, approximately 320,000 new cases of ovarian cancer occur each year, and 200,000 individuals succumb to the disease⁵. In 2020, it represented 3.7% of cancer cases and 4.7% of cancer deaths in women worldwide⁶. Ovarian cancer affects approximately 3,000 people in Canada every year, with about 2,000 deaths^{7,8}. This high mortality rate can mostly be attributed to late-stage presentation in patients⁹. Therefore, the tumor has often metastasized, generally throughout the peritoneal cavity by the time the cancer is diagnosed, and standard treatment strategies such as tumor debulking and chemotherapy become less effective^{10,11}. Not only is there a lack of symptoms during the early stages of ovarian cancer^{9,12}, even when symptoms present, they are generally non-specific gastrointestinal, abdominal, and urinary symptoms, further complicating and often delaying diagnosis¹³.

Since cells have often metastasized at the time of diagnosis, it has been historically difficult to pinpoint the tissues of origin of ovarian cancer. It was originally believed that all ovarian tumors originate from various cell types in the ovary¹⁴. However, we now have a much more nuanced understanding of the tissues of origin of ovarian cancer. Ovarian cancer is a general term that encompasses various subtypes of ovarian cancer characterized by differing pathogenesis, histologic and molecular features, and the subtype is highly dependent on the tissue of origin. The main classifications of ovarian cancer are epithelial ovarian carcinomas, germ cell tumors, and

stromal cell tumors¹⁵. Epithelial ovarian cancer (EOC) is the most common type and is further categorized into various subtypes, i.e., serous, endometrioid, mucinous, and clear cell carcinomas, with high-grade serous ovarian carcinoma (HGSOC) being the most common subtype^{2,15}. HGSOC generally arises from precursor lesions in the fallopian tube epithelium¹⁶. This was discovered through the observation of non-ciliated abnormal cells constituting serous tubal intraepithelial carcinomas that are hallmarked by abnormal nuclear structure, lack of polarization, p53 mutation, apoptotic bodies, mitotic figures, and upregulated Ki-67¹⁶. These lesions are precursors to HGSOC, which gain invasiveness and shed from the distal fallopian tube into the peritoneal cavity, first attaching to the ovarian surface and growing in the ovary before moving to the bowel and other peritoneal surfaces¹⁶. HGSOCs are mostly characterized by mutations or loss of expression of the Breast Cancer 1 and 2 (*BRCA1* and *BRCA2*) genes, the *TP53* tumor suppressor gene¹⁷, *CCNE1*, and *CDK1*, which confer HGSOC with its chromosomal instability and contribute to the poor response of HGSOC tumors to immune checkpoint inhibitors¹⁸.

The second most common type of EOC is endometrioid ovarian cancer. Endometrioid carcinomas and clear-cell carcinomas both present early in patients and are often associated with endometriosis, through emergence from established endometrial cysts, but can also emerge de novo^{11,19}. ECs are characterized by high levels of mutations in the *CTNNB1*, *PTEN*, *ARID1A*, and *PIK3CA* genes^{17,20} and CCCs are characterized by *ARID1A* and *PIK3CA* mutations²¹. Mucinous ovarian carcinoma is diagnosed relatively early and is mostly associated with mutations in *KRAS* and *TP53*, and *HER2* amplification²². Lastly, low-grade serous carcinoma (LGSC) can emerge from previous serous borderline tumors (SBT) or de novo²³. The cells of origin of LGSCs are ambiguous; some believe that LGSCs develop from the ovarian surface epithelium (OSE), some believe that SBTs and LGSCs originate from papillary tubal hyperplasia, and others believe that

the fimbriated end of the fallopian tube adheres to the ovarian surface, leading to ovarian epithelial inclusions that can then become SBTs and then LGSC²³. These tumors are characterized by *KRAS* or *BRAF* mutations^{17,23}.

1.2. Risk factors of ovarian cancer

Risk factors for ovarian cancer include genetic factors, age, and number of lifetime ovulations^{24,25}. The biggest risk factor for ovarian cancer is age. Most cancer diagnoses occur after menopause, and survival rates and prognosis decrease with age²⁴. Since hereditary genetic factors are also a major risk factor, individuals with these risks are typically diagnosed earlier²⁴. These genetic factors include hereditary breast/ovarian syndrome and Lynch syndrome and account for 10-15% of EOCs^{24,26}. Mutations in the *BRCA1* and *BRCA2* genes, which function as double-strand DNA break repair and tumor suppressor genes, are the most common cause of hereditary EOC^{27,28}. Lynch syndrome is a hereditary condition that increases an individual's risk of some types of cancers and is characterized by mutations in DNA mismatch repair genes that act as tumor suppressors, such as mutL homolog 1 (*MLH1*), mutS homolog 2 (*MSH2*), *MSH3*, *MSH6*, postmeiotic segregation increased 1 (*PMS1*), and *PMS2*^{29,30}.

Another important risk factor is the number of lifetime ovulations, which is described by the “incessant ovulation” hypothesis³¹. This hypothesis states that each ovulation presents the possibility of damage to the OSE in two ways: an increased risk of DNA mutation when repeated, and exposure to estrogen-rich follicular fluid, which increases cell proliferation of OSE cells, also potentially increasing risk of DNA mutation^{25,31}. In consequence, factors that suppress ovulation, such as pregnancy, breastfeeding, and oral contraceptive use, reduce the risk of ovarian cancer²⁵. More recent studies have provided evidence for gynecologic risk factors for ovarian cancer such

as endometriosis and polycystic ovary syndrome (PCOS) (now named Polyendocrine Metabolic Ovarian Syndrome (PMOM)), and lifestyle factors such as smoking and possibly obesity^{24,32}.

1.3. Metastasis in ovarian cancer

The metastatic pathway in ovarian cancer differs from well-established mechanisms in other cancers where cancer cells metastasize through blood vessels and therefore must cross through the endothelial layers to enter the circulation (i.e., intravasate) and cross again to exit (i.e., extravasate) to colonize a new environment¹¹. Ovarian cancer adopts a more passive mechanism to metastasize to new sites¹¹. Cancer cells from the tumor at the site of origin shed and travel through the peritoneal fluid to different tissues in the peritoneal cavity, which provide an environment where metastatic cells thrive¹¹.

However, there is increasing evidence that metastasis of ovarian cancer doesn't only happen through arbitrary dissemination throughout the peritoneal cavity³³, but that they have preferential seeding sites, most notably the omentum, a large fatty tissue in the abdomen^{33,34}. There is also evidence of the presence of ovarian tumor cells circulating in the blood of cancer patients, indicating that ovarian cancer can also metastasize through the blood³³. This hematogenous axis of metastasis was shown by Pradeep et al. using a parabiosis model where pairs of mice were anastomosed. They injected ovarian cancer cells into the peritoneal cavity or into the ovary of one mouse and found that most of the parabiont counterparts had metastases within the omentum or mesentery, and this transfer was happening through the blood vessels, not the lymphatic vessels. They attributed this method of metastasis to upregulation of *ERBB3* and *neuregulin 1* and mesenchymal phenotype genes such as *vimentin*, *MMP3*, *COL1A*, *HMGA2*, and *HGF*³⁴.

To metastasize, cancer cells normally undergo an epithelial-to-mesenchymal transition (EMT) to acquire a more migratory and invasive phenotype. This transition is enabled by a loss of E-cadherin, which normally ensures adhesion of cells to each other, and a gain of N-cadherin, and increased expression of E-cadherin inhibitors such as zinc-finger-containing transcriptional repressors Sip1/ZEB2, Snail, and Slug³⁵. E-cadherin is a membrane glycoprotein that links to actin microfilaments in the cytoplasm of cells to maintain their adhesion to each other¹¹. Therefore, loss of E-cadherin allows cells to more readily detach from each other and metastasize¹¹.

1.4. The tumor microenvironment

Ovarian tumors are highly heterogeneous, both within a single tumor (intratumor heterogeneity) and between tumors from different patients (intertumor heterogeneity)^{9,36}. This heterogeneity is due in part to the complexity of the tumor microenvironment (TME), which is composed of various cells such as fibroblasts, endothelial cells, and immune cells, and various other factors such as the extracellular matrix (ECM), MMPs, and growth factors (VEGF, TGF- β , PDGF)⁹. Components of the TME all have a role in modulating tumor development, progression, and chemoresistance⁹. For example, components of the ECM contribute to structural and biochemical changes in the TME and modify the function of cells within the tumor, often optimizing cell proliferation, invasion, and apoptotic evasion⁹. Cancer-associated fibroblasts (CAFs) can arise from stromal cells recruited into the tumor through exposure to inflammatory and pro-fibrotic factors⁹. CAFs are responsible for connective tissue formation and release MMPs that remodel the ECM to optimize tumor progression, invasion, inflammation, and angiogenesis⁹. Tumors can only grow up to a certain size without vascularization; therefore, the TME optimizes angiogenesis (formation of new blood vessels)³⁷ to supply the tumors with energy and oxygen that are needed for growth and dissemination³⁸.

There are various immune cell types that can infiltrate the TME and contribute to tumor growth or have anti-tumorigenic effects. Myeloid progenitor cells give rise to granulocytes, macrophages, and dendritic cells, while lymphoid progenitor cells give rise to T cells, B cells, natural killer (NK) cells, and group 2 innate lymphoid cells (ILC2)³⁹. Each of these cell types is identified by specific markers. For example, CD45 is a marker for leukocytes⁴⁰, CD3 identifies lymphocytes (T cells)⁴¹, and, more specifically, CD8 and CD4 mark the type of T cell (cytotoxic or helper)⁴². Infiltration of immune cells, specifically tumor-infiltrating lymphocytes (TILs), within the tumor can be described by three main classifications: 1) T cell-inflamed or “hot” tumors where there is infiltration of cancer cells and stroma by T cells; 2) immune-excluded, where immune cells remain on the periphery of the tumor cells and stroma; and 3) ‘immune-desert’ or ‘cold’ tumors characterized by a lack of T cells in the tumor and stroma^{43,44}. The presence of TILs is an important prognostic marker and has been linked to prolonged survival in ovarian cancer⁴³. “Hot” tumors limit tumor growth by exhibiting strong antitumor immune responses and are associated with improved clinical outcomes^{44,45}. However, a majority of EOC patients have low TIL infiltration, and these “excluded” or “cold” tumors are markedly resistant to immunotherapy through dysfunctional antigen presentation, abnormal vasculature, and a robust stromal barrier^{43,44}.

1.5. SLPI: function, regulation, and its role in ovarian cancer

Previous work by David Cook in the Vanderhyden lab explored gene expression differences between epithelial and mesenchymal phenotypes in cancer cells and resulted in a gene signature universally associated with the mesenchymal phenotype⁴⁶. Using single-cell RNA-sequencing datasets, Maryam Echaibi used this signature and discovered higher expression of secretory leukocyte protease inhibitor (SLPI) in ovarian cancer cells with a mesenchymal phenotype when compared to cancer cells with an epithelial phenotype, indicating that SLPI may be involved in

metastatic capability (**Figure 1**). The human datasets used were a combination of datasets from various studies^{47–50} and an unpublished scRNA-seq dataset that was sequenced by Dr. David Cook. Data from the Cancer Dependency Map (DepMap) portal was then used to plot SLPI expression against the tissue of origin of all the ovarian cancer cell lines available. The results show that cell lines derived from ascites or the peritoneal cavity, i.e., pleural effusion, abdomen, and large intestine, generally express higher levels of SLPI, further suggesting a potential involvement of SLPI in metastasis (**Figure 2**). Cancer cell lines originating from the ovary seem to polarize into high and low SLPI-expressing groups, which could be indicative of dichotomous phenotypes that have yet to be characterized (**Figure 2**). Previous literature and unpublished results from our lab have also established a correlation between high expression of SLPI and poor survival⁵¹, warranting further investigation into its function.

SLPI is a small protein (11.7 kDa) that is secreted mostly by epithelial cells but can also be secreted by some immune cells⁵². It is found in mucosal tissues, such as saliva and genital secretions⁵², and functions as a protease inhibitor; it has a very important anti-inflammatory

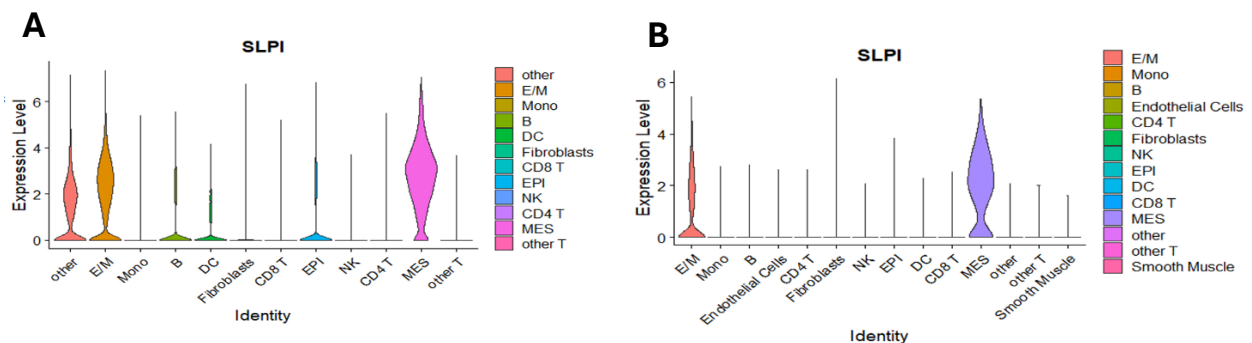


Figure 1. SLPI expression levels in cell populations present in human datasets.

(A) primary and (B) metastatic ovarian cancers. Cancer cells are separated by their epithelial-mesenchymal phenotype: epithelial cells (EPI), mesenchymal cells (MES), and partially mesenchymal cancer cells (E/M). (unpublished).

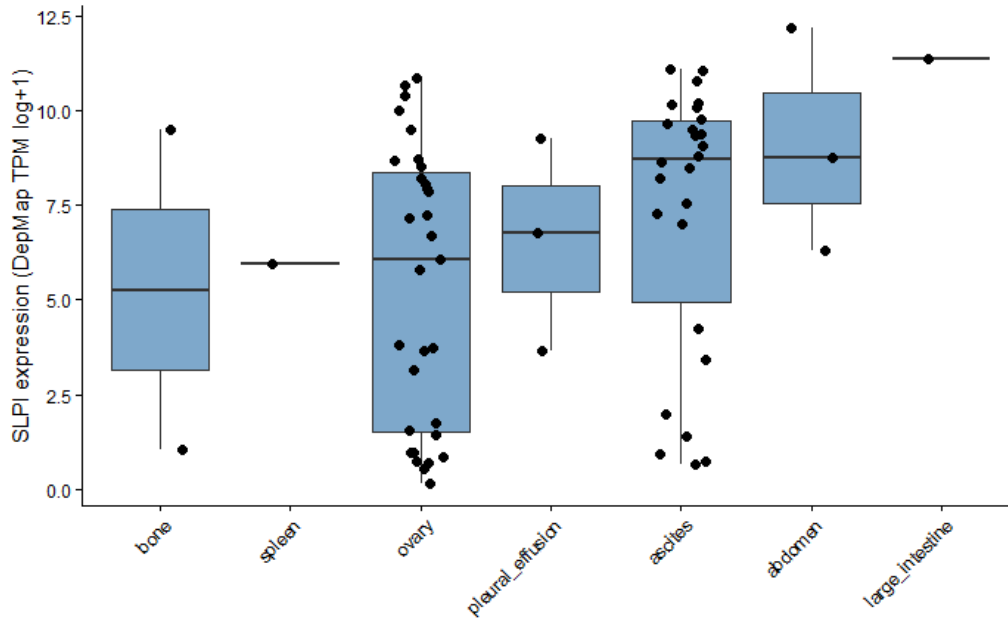


Figure 2. SLPI-expressing cell lines are prominent in the ascites and abdomen, indicating an association with metastatic sites.

Boxplot representing distribution of SLPI in ovarian cancer cell lines retrieved from DepMap and distributed by tissue of origin. Each point represents a different cell line. The middle line shows the median, the top and bottom lines of the box show the third and first quartile, respectively. The whiskers represent 1.5x of the interquartile range (IQR).

function that is involved in a wide set of responses^{53,54}. Through these functions, it plays an important homeostatic role in the innate and adaptive immune systems⁵⁵. SLPI inhibits various substrates, including neutrophil elastase, cathepsin G, chymase, chymotrypsin, trypsin, and tryptase activity, and it can also modulate the expression of matrix metalloproteinases (MMPs)⁵². Some of these proteases, including MMP-9, chymase, and cathepsins B, L, and S, also have the ability to inhibit SLPI, creating a negative feedback loop to maintain homeostasis⁵². Many signaling pathways can upregulate SLPI expression; notably, one of its well-established stimulants are ligands that bind to Toll-like receptors (TLR)⁵⁴. TLRs recognize pathogen-associated molecular patterns (PAMPs), which include pathogen components such as lipopolysaccharide (LPS), viral RNA, and parasites, and damage-associated molecular patterns (DAMPs), which include molecules from damaged cells^{54,56}. Other factors that can upregulate SLPI in epithelial

cells include proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 beta (IL-1 β), as well as transforming growth factor alpha (TGF- α), insulin-like growth factor 1 (IGF-1), progesterone, and corticosteroids⁵⁷. However, not much is known about how SLPI is upregulated in cancer.

SLPI, encoded by the *SLPI/WFDC4* gene, shows strong potential to be a key player in ovarian cancer, as it is very strongly associated with the gene *whey acidic protein four-disulfide core domain 2* (*WFDC2*), coding for the human epididymis protein 4 (HE4), which is a known clinical biomarker of ovarian cancer and is correlated with poor survival in serous ovarian cancer patients⁵⁸. Overexpression of SLPI has been shown to be involved in the pathogenesis of various tumors, including lung, ovarian, cervical, neck, and pancreatic cancers, by increasing progression, invasion, and metastatic capacity⁵⁴. SLPI generally has a tumor-promoting role in ovarian cancer by increasing cell survival, proliferation, and metastasis⁵⁷. Studies debate whether SLPI's role in ovarian cancer is due to its protease inhibitor function or is independent of it. Devoogdt et al. found that SLPI increased proliferation, survival, and anoikis resistance in ovarian cancer cells, as well as tumor formation, dissemination, and invasion in vivo, with or without its protease inhibitor function⁵⁹. They attributed this effect to SLPI's protection of progranulin, which is a growth factor that regulates cell survival, wound repair, and inflammation and is usually degraded by neutrophil elastase^{59,60}. Another study has shown that SLPI inhibited MMP-9, partially through its anti-protease inhibitor function, which enhanced ovarian cancer invasiveness⁶¹. Sun et al. showed that SLPI is packaged in extracellular vesicles secreted by a specific subtype of cancer-associated fibroblasts, which travel to cancer cells and regulate the PI3K-AKT-GSK3, NF- κ B, and MAPK pathways, thereby increasing tumor growth and immunosuppression⁶².

When SLPI expression is plotted against ovarian cancer subtype from which ovarian cancer cell lines were derived, SLPI expression is the highest in cell lines from HGSOCs, undefined serous ovarian cancers, and mucinous ovarian cancers, and is very low in endometrioid and small cell carcinomas of the ovary, hypercalcemic type cell lines (**Figure 3**). This strengthens the rationale to focus on HGSOC models for this study.

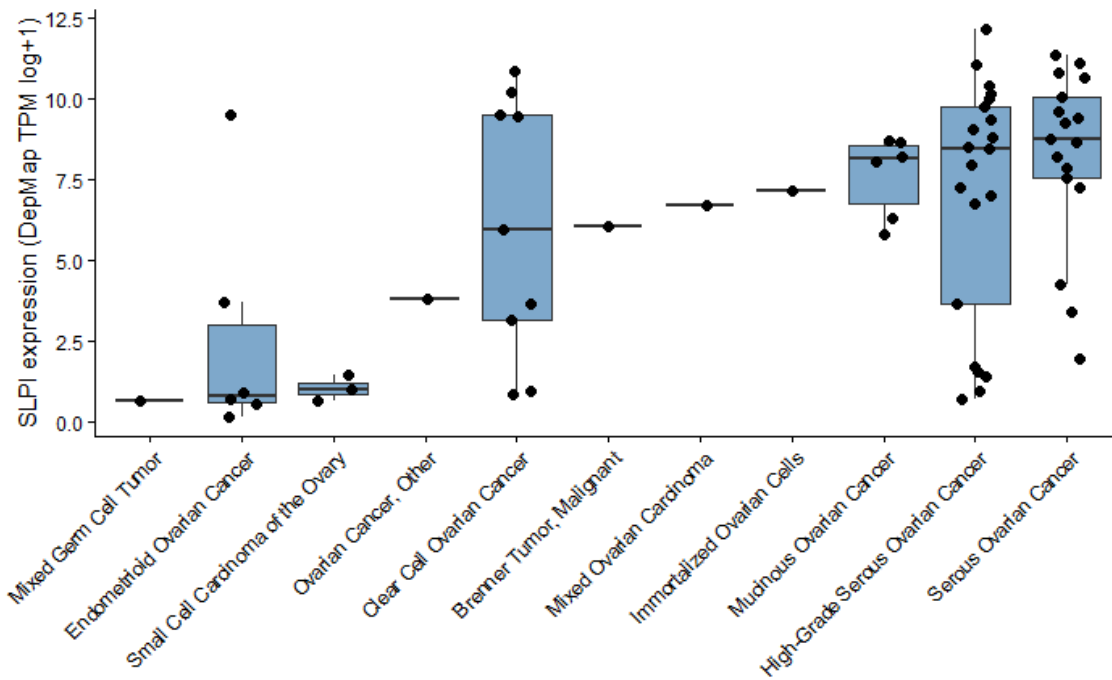


Figure 3. Cell lines expressing higher levels of SLPI are derived from HGSOC, all serous and mucinous carcinomas.

Boxplot representing distribution of SLPI in ovarian cancer cell lines retrieved from DepMap and distributed by subtype. Each point represents a different cell line. The middle line shows the median, the top and bottom lines of the box show the third and first quartile, respectively. The whiskers represent 1.5x of the IQR and outliers are found past the whiskers.

The standard of care for ovarian cancer is surgery to remove the bulk of the tumor, followed by chemotherapy, especially in cases where surgery is not sufficient to fully remove the tumor and the risk of recurrence is high⁶³. The standard chemotherapeutic drugs used since the 2000s are a combination of carboplatin and paclitaxel⁶³. Carboplatin is a platinating agent, similar to cisplatin, which was a previous standard chemotherapeutic drug for ovarian cancer⁶³. These can interact with

DNA, RNA, and proteins because they are nucleophilic. These interactions create lesions such as monoadducts, intrastrand crosslinks, and interstrand crosslinks on DNA strands, which lead to DNA breakage and inhibit replication and transcription^{64,65}. Paclitaxel has an anti-mitotic function that causes apoptosis in cancer cells. It binds to β -tubulin within microtubules, which prevents GTP binding and depolymerization, leading to stable, non-dynamic microtubules. In consequence, chromosomes cannot separate properly during replication, which causes cell cycle arrest and apoptosis^{66,67}.

There is some evidence that SLPI expression is correlated with chemotherapy resistance. Notably, Rasool et al. showed that paclitaxel-resistant cells have higher baseline levels of SLPI secretion compared to their non-resistant parental cell line⁶⁸. When chemosensitive cells were exposed to paclitaxel, there was an increase in SLPI expression. Overexpression of SLPI in cells that were initially sensitive to paclitaxel caused cells to become more resistant to the drug⁶⁸. In vivo, they showed that when SLPI was downregulated, paclitaxel became more effective by slowing tumor development and progression⁶⁸. Wei et al. showed that cisplatin treatment of cells in which SLPI expression was knocked down reduced cell viability relative to the unmodified control in vitro⁶⁹. They also showed that in vivo, tumor sizes were decreased in response to cisplatin treatment, but tumors with SLPI knockout (KO) in combination with chemotherapy treatment had the smallest tumor volume⁶⁹. This was confirmed by immunohistochemistry, which showed that SLPI KO tumors treated with cisplatin had the lowest levels of proliferation and highest levels of apoptosis⁶⁹. These two studies showed that SLPI inhibition can increase the efficacy of these chemotherapeutic drugs and that SLPI expression can increase chemoresistance.

Rationale

Late detection, in combination with the unique and complex TME of HGSOCs, which optimizes tumor growth and immune evasion, make effective treatment especially difficult^{9,12}. Therefore, it is necessary to find better treatment options by characterizing the pathways involved in ovarian cancer and its progression. Few studies have investigated how SLPI expression impacts cancer progression and the ovarian TME. This project aims to characterize the effects of SLPI on chemoresistance, to elucidate the mechanisms that regulate its expression and activation of downstream factors, and to investigate the impact of SLPI on the ovarian TME. Critical to this work is the previous generation, by Maryam Echaibi, of three ovarian cancer cell lines that either overexpress or have a CRISPR-mediated knockout of *Slpi*. These include mouse oviductal epithelial (MOE) and KPCA.B cells, which simulate serous carcinomas originating in the fallopian tube⁷⁰. These cells have modified PI3K/RAS pathways to replicate the tumor phenotype⁷⁰. The third cell line is an ID8 cell line, which arose from spontaneously transformed mouse OSE cells and has a knockout of *Trp53* to replicate the serous ovarian cancer phenotype⁷⁰.

Hypothesis and Objectives

We hypothesize that high SLPI expression in ovarian cancer is associated with a decrease in survival and an increase in chemoresistance, metastasis, and immune suppression. We also hypothesize that upregulation of SLPI is mediated by the MEK pathway.

The objectives of this study were as follows:

- 1) Determine the signaling pathway(s) that control SLPI expression in ovarian cancer cells;
- 2) Identify the impact of SLPI on resistance to standard chemotherapeutic drugs used in ovarian cancer treatment;

- 3) Determine the effect of SLPI modification on cell line metastatic behavior, spheroid formation, and the downstream factors that are involved;
- 4) Investigate how SLPI modulates in vivo survival and the immune composition of the ovarian TME.

Chapter 2: Materials and Methods

2.1. Cell lines

Murine cell lines used in this study originated either from the OSE layer, ID8 and STOSE, or from the oviductal epithelium, MOE and KPCA.B. Specifically, we used these cell lines with the following mutations: ID8 *Trp53*^{-/-}, MOE *Pten*^{shRNA} (MP), MOE *Pten*^{shRNA}*KRas*^{G12V} (MPK), *p53*^{-/-R172H}*Ccne1*^{OE}*Akt2*^{OE}*KRAS*^{G12V} (KPCA.B), and STOSE with no known mutations. The cell lines with modified SLPI expression used in this project were generated by a previous M.Sc. student in the Vanderhyden lab, Maryam Echaibi. She first assessed the baseline levels of SLPI in murine ovarian cancer cell lines compared to normal epithelial cell controls, mouse ovarian surface epithelial (MOSE) and mouse oviductal epithelial (OVE4) cells (**Figure 4**). She then modified MPK, ID8 *Trp53*^{-/-}, and KPCA.B to create three pairs of cell lines with altered SLPI expression. Since MPKs have high baseline levels of SLPI expression (**Figure 4**), SLPI was knocked out in this cell line by transduction with a plasmid in which single guide RNAs (sgRNA) targeted SLPI. The control sgRNA targeted a Renilla sequence, which doesn't interact with the mammalian genome. Since ID8 and KPCA have low levels of SLPI expression initially (**Figure 4**), SLPI was overexpressed in these cell lines by transduction with a lentiviral vector that codes for SLPI. The lentiviral vector alone was used as a control. To select for the cells that had successful transduction, puromycin (Fisher Scientific, #A11138-03) was used to kill cells that didn't acquire antibiotic resistance from the vector. Modified SLPI expression was validated by western blotting (**Figure 5**). MOE *Pten*^{shRNA} *KRas*^{G12V} Control, MOE *Pten*^{shRNA} *KRas*^{G12V} *Slpi*^{-/-}, ID8 *Trp53*^{-/-} *Slpi*^{EV}, and ID8 *Trp53*^{-/-} *Slpi*^{OE} will be named MPK control, MPK *Slpi*^{-/-}, ID8 *Slpi*^{EV} and ID8 *Slpi*^{OE}, in this study, respectively.

Cell lines of human origin were derived either from the ascites of a HGSOC patient, PEO1 (ECACC, #10032308) and PEO4 (ECACC, #10032308) which were purchased from The European Collection of Authenticated Cell Cultures (ECACC), or from an ovarian endometrioid tumor, A2780 S (ECACC, #93112519) and A2780 CP (ECACC, #93112517), which were purchased from Sigma-Aldrich. Both pairs of human cell lines have a chemosensitive and chemoresistant counterpart, with PEO4 acquiring resistance by in vivo treatment of the same patient from whom PEO1 was derived, and A2780 CP acquiring resistance by prolonged cisplatin treatment in vitro. References, mutation(s), cell of origin, and culture medium used to maintain each cell line are summarized below in **Table 1**. Media solutions consisted of DMEM (Gibco, 11995123), α MEM (Gibco, #12571-063), or RPMI (Gibco, #11875176), to which additives were added including Fetal Bovine Serum (FBS) (Gibco, #12483-020), Insulin-Transferrin-Selenium (ITS) (Gibco #41400045 or Sigma, #I1884), murine EGF (R&D systems, #2028-EG-200) and/or β -Estradiol (Sigma, #E2257). All the cells were incubated at 37°C and 5% carbon dioxide (CO₂).

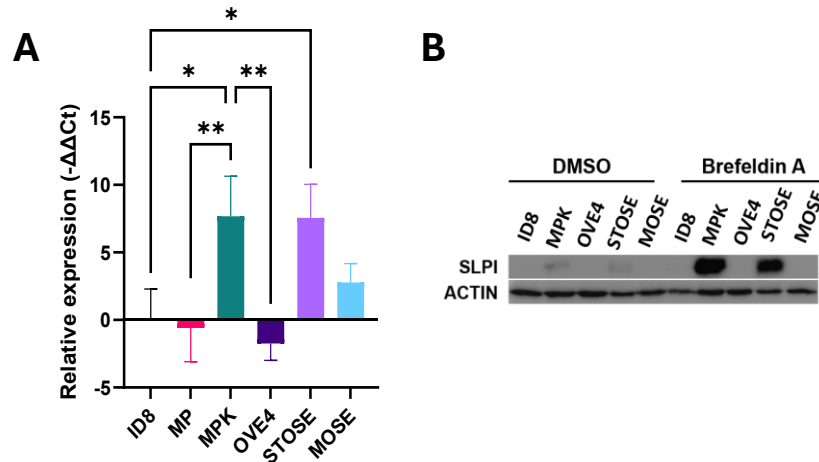


Figure 4. Relative mRNA and protein levels of Slpi in various mouse ovarian cancer cell lines compared to normal epithelial cell controls (MOSE, OVE4).

(A) Expression levels were quantified by RT-qPCR. (B) Protein levels of most cell lines presented in (A) quantified by western blot. Brefeldin A is a Golgi plug that was used to stop secretion of SLPI. Cell lines were compared using a one-way ANOVA (n=3/cell line); mean is shown $\pm$ SEM. * p-value $\leq$ 0.05, ** p-value $\leq$ 0.01. ID8: ID8 *Trp53*^{-/-}, MP: MOE *Pten*^{shRNA} and MPK: MOE *Pten*^{shRNA} *KRas*^{G12V}.

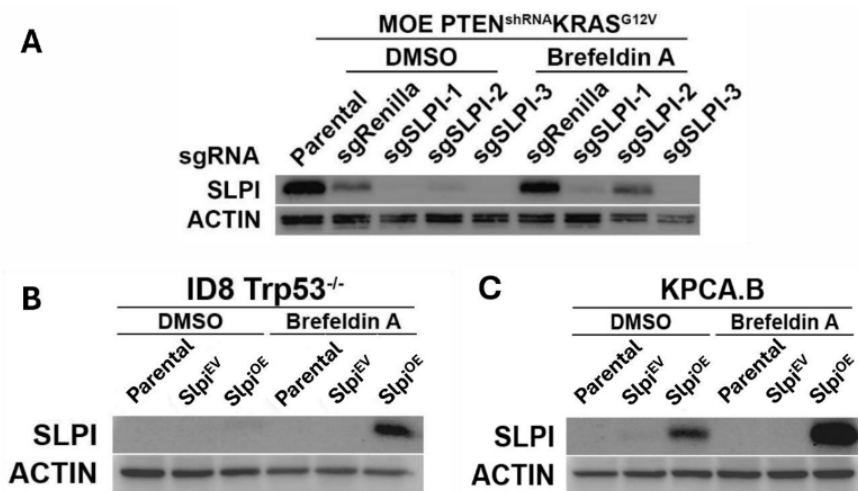


Figure 5. Western blots showing successful modification of Slpi protein levels in mouse ovarian cancer cell lines.

MPK cells (A), ID8-*Trp53*^{-/-} cells (B), and KPCA.B cells (C) were transduced with either sgRNAs or lentiviral vectors to modify SLPI expression levels. (EV: empty vector; OE: overexpression). Cells were treated with Brefeldin A to prevent SLPI secretion or DMSO as a control.

Table 1. Cell line used and respective culture media for murine and human OC models

Cell line	Reference	Known mutations	Origin	Culture medium
Murine models				
MOE <i>Pten</i> ^{shRNA} (MP)	Eddie et al. ⁷¹	<i>Pten</i> ^{shRNA}	Oviductal epithelium	αMEM + 4% FBS + 1x ITS + 2μg/mL EGF + 18.2ng/mL β-Estradiol
MOE <i>Pten</i> ^{shRNA} <i>KRAS</i> ^{G12V} (MPK)	Eddie et al. ⁷¹	<i>Pten</i> ^{shRNA} , <i>KRAS</i> ^{G12V}	Oviductal epithelium	
MOE <i>Pten</i> ^{shRNA} <i>KRAS</i> ^{G12V} Control	-	<i>Pten</i> ^{shRNA} , <i>KRAS</i> ^{G12V}	Oviductal epithelium	
MOE <i>Pten</i> ^{shRNA} <i>KRAS</i> ^{G12V} <i>Slpi</i> ^{-/-}	-	<i>Pten</i> ^{shRNA} , <i>KRAS</i> ^{G12V}	Oviductal epithelium	

Spontaneously Transformed Ovarian Surface Epithelial (STOSE)	McCloskey et al. ⁷²	-	Ovarian surface epithelium	αMEM + 4% FBS + 1x ITS + 2μg/mL EGF
ID8 <i>Trp53</i> ^{-/-} (ID8)	Walton et al. ⁷³	<i>Trp53</i> ^{-/-}	Ovarian surface epithelium	DMEM + 4% FBS + 1x ITS
ID8 <i>Trp53</i> ^{-/-} <i>Slpi</i> ^{EV}	-	<i>Trp53</i> ^{-/-}	Ovarian surface epithelium	
ID8 <i>Trp53</i> ^{-/-} <i>Slpi</i> ^{OE}	-	<i>Trp53</i> ^{-/-} , <i>Slpi</i> ^{OE}	Ovarian surface epithelium	
KPCA.B	Iyer et al. ⁷⁴	<i>Trp53</i> ^{-/-R172H} , <i>Ccne1</i> ^{OE} , <i>Akt2</i> ^{OE} , <i>Kras</i> ^{G12V}	Oviductal epithelium	
KPCA.B <i>Slpi</i> ^{EV}	-	<i>Trp53</i> ^{-/-R172H} , <i>Ccne1</i> ^{OE} , <i>Akt2</i> ^{OE} , <i>Kras</i> ^{G12V}	Oviductal epithelium	DMEM + 4% FBS+ 1x ITS + 2μg/mL EGF + 1x Penicillin Streptomycin (Thermo Fisher, #15140122)
KPCA.B <i>Slpi</i> ^{OE}	-	<i>Trp53</i> ^{-/-R172H} , <i>Ccne1</i> ^{OE} , <i>Akt2</i> ^{OE} , <i>Kras</i> ^{G12V} , <i>Slpi</i> ^{OE}	Oviductal epithelium	
Human models				
PEO1	-	<i>BRCA2</i> , <i>TP53</i>	Ovarian cystadenocarcinoma from ascites	RPMI + 10% FBS

PEO4	-	<i>BRCA2, NF1, TP53</i>	Ovarian cystadenocarcinoma from ascites	
A2780 S	-	<i>ATM, PIK3CA, PTEN, RRAS2</i>	Ovarian endometrioid adenocarcinoma	
A2780 CP	-	<i>ATM, PIK3CA, PTEN, RRAS2</i>	Ovarian endometrioid adenocarcinoma	

2.2. RNA extraction and RT-PCR (quantitative PCR/qPCR)

To measure mRNA expression, cells were first collected, washed, and centrifuged into a pellet. RNA was then extracted from the cell pellets using the RNeasy® Mini Kit (Qiagen, #74106). The manufacturer's protocol was followed, which consisted of a cell lysis step, RNA precipitation, a series of wash steps by centrifugation in a spin column, and finally elution of the RNA. The concentration of the eluted RNA was measured using a NanoDrop (Thermo Fisher) and then used to synthesize cDNA using the Lunascript RT Supermix (New England Biolabs, #M3010X). The prepared LunaScript RT SuperMix and RNA sample mix were placed in a thermocycler at the following conditions: 25°C for 2 minutes, 55°C for 10 minutes, 95°C for 1 minute, and the synthesized cDNA was diluted. To measure relative expression of a given gene, sample cDNA was combined with primers for the genes of interest listed in **Table 2**. These primers were previously validated by Maryam Echaibi. The expression of these genes was normalized to the housekeeping genes *β-actin* and *Ppia* for the murine cancer cells, and *PPIA* and *HPRT* for the human cancer cells.

Table 2. Murine and human primer oligonucleotide sequences from 5' to 3' used for qPCR.

Gene of interest	Species	Forward primer	Reverse primer
<i>Slpi</i>	Mouse	CCATTCGCAAACCAGTGTGG	AGTTTCCAGAGCACACCGAG
<i>SLPI</i>	Human	TGTTGACACCCCAAACCCAA	ATGGCAGGAATCAAGCTTTCAC
<i>β-actin</i>	Mouse	CCTTCCTTCTTGGGTATGGA	ACGGATGTCAACGTCACACT
<i>Ppia</i>	Mouse	AGGGTGGTGACTTTACACGC	GATGCCAGGACCTGTATGCT
<i>PPIA</i>	Human	TGCTGGACCCAACACAAATGGTTC	TGGTGATCTTCTTGCTGGTCTTGC
<i>HPRT</i>	Human	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT

2.3. Protein extraction

To extract protein from cell samples, freshly collected and washed cell pellets were lysed using an optimized lysis buffer containing RIPA buffer, Pepstatin (Sigma, #P5318-5MG), PMSF, Vanadate (NEB, #P0758S), and 20x inhibitor (made of RIPA buffer, 1M NaF, 1M DTT, leupeptin (Thermo, #J61188.MB), aprotinin (Thermo, #78432), benzamidine hydrochloride hydrate, and β -glycerophosphate). Lysed pellets were left at -20°C for at least 24 hours. The lysed cell mixture was then thawed and centrifuged at 13,000 rpm for 15 min at 4°C to separate the protein from the cell debris, which became the pellet. The supernatant, which contains the protein, was collected and put into a new tube, which was then frozen at -80°C for storage. To measure the protein concentration of the samples, 5 μ L of protein was added to 1 mL of Bio-Rad Protein Assay Dye Reagent concentrate (BioRad, #5000006) diluted in water at a 1:5 ratio, and absorbance was read at 595nm using the BioMate3 (Thermo Fisher).

2.4. Western blot

Protein samples were diluted in specific volumes of water to obtain equal concentrations of the different protein samples in a final volume of 15 μ l. Then, 5 μ l of loading buffer containing 2-mercaptoethanol (Sigma-Aldrich, #M7522) was added to all the samples. Protein samples were denatured at 100°C for 5 min. western blot gels were made with a separating layer (made of separating gel, water, 30% acrylamide (Biorad, #1610158), glycerol (only for 15% gel), tetramethylethylenediamine (TEMED, Thermo Fisher, #A12536.AE), and 10% APS) and a stacking layer (made of stacking gel, water, 30% acrylamide (Biorad, #1610158), 10% sodium dodecyl sulfate (Fisher Scientific, #BP166-500), TEMED, and 10% APS). Gel concentration ranged from 10-15%. Samples were loaded into the gel, surrounded by running buffer, and were run at 0.1A for 45 minutes to an hour. The gel was then transferred to a PVDF membrane (Millipore, #IPFL00010) at 100V at 4°C for 1 hour. Following the transfer, the membrane was incubated in bovine serum albumin (Bioshop, #ALB001) for 30 min to 1 hour to block and then probed with the primary antibody, listed in **Table 3**, generally overnight at 4°C or for 1 hour at room temperature. Between incubations with different antibodies, the membranes were washed with TBST for 5 minutes, three times. If needed, membranes were stripped with stripping buffer, a mixture of water, glycine (Thermo Fisher, #56-40-6), Tween 20™ (Thermo Fisher, #J20605AP), and sodium dodecyl sulfate, for 20 min, two to three times. To visualise the blots, chemiluminescence was measured using the ChemiDoc Imaging System (BioRad).

To visualize SLPI, which is a secreted protein, the cells were treated with a protein transport inhibitor to retain SLPI within the cells. Cells were seeded and left to adhere overnight and were treated with either 1:1000 BD GolgiPlug™ Protein Transport Inhibitor (containing Brefeldin A) (BD Biosciences, #555029) or DMSO as a control 4-6 hours before collection. After treatment,

the cells were collected, and protein was extracted as described above. When visualizing SLPI protein levels on a western blot, a 15% acrylamide tris-glycine gel was used.

Table 3. Primary murine and human antibodies used for western blotting.

Ab target	Reactivity	Source	Dilution	Company and Catalogue #
Mouse SLPI	Mouse	Polyclonal Goat IgG	1:1000	R&D Systems, #AF1735
Human SLPI	Human	Polyclonal Goat IgG	1:1000	R&D Systems, #AF1274
pAKT	Human, mouse	Rabbit Monoclonal IgG	1:2000	Cell Signaling, #4060S
AKT	Human, mouse	Polyclonal Rabbit IgG	1:1000	Cell Signalling, #9272
β -Actin	Human, mouse	Mouse monoclonal IgG	1:2000	Sigma, #A2228

2.5. Proliferation assay

Proliferation was assessed in 4 cell lines: MPK Control, MPK *Slpi*^{-/-}, ID8 *Slpi*^{EV}, and ID8 *Slpi*^{OE}. These cell lines were plated at low densities of 0.075 x 10⁵ cells/well for ID8 cells and 0.125 x 10⁵ for MPK cells in a 6-well plate to prevent premature confluency. After 24, 48, 72, and 96 hours, they were lifted using trypsin, collected, and counted using the Vi-CELL XR Cell Viability Analyzer (Beckman Coulter). Three technical replicates at each time point were collected for each of at least three biological replicates. The cells were incubated in standard growth conditions, that is 37°C, 5% CO₂.

2.6. Treatment of cells with AKT/MEK inhibitors

To determine which pathway contributes to regulation of SLPI expression, intermediates of two KRAS signaling pathways were inhibited, and resulting SLPI expression was measured. Concentrations of 1.5 x 10⁵ MPK cells/well, 1 x 10⁵ STOSE cells/well, 2 x 10⁵ A2780S and A2780 CP cells/well, and 2.5 x 10⁵ of PEO1 and PEO4 cells/well were plated in a 6-well plate. Cells were

incubated overnight in standard culture conditions to adhere and were then treated with 10 μ M inhibitors of MEK (Selleck Chemicals, Mirdametinib (PD0325901) #S1036) and AKT (Selleckchem, MK-2206 #S1078). Control wells were treated with 1:1000 DMSO. Following treatment for 24 hours, cells were collected, protein expression was measured using western blotting, and mRNA expression levels were quantified using qPCR.

2.7. Drug treatments

To evaluate the effect of SLPI on chemoresistance, viability of various cell lines following chemotherapy treatment was measured. To compare chemoresistance in SLPI-modified cell lines and their controls, the following mouse cell lines were used: MPK Control, MPK *Slpi*^{-/-}, ID8 *Slpi*^{EV}, ID8 *Slpi*^{OE}, KPCA.B *Slpi*^{EV}, and KPCA *Slpi*^{OE}. Cells were plated at different concentrations in a 96-well plate depending on their baseline rate of proliferation, i.e., 10,000 cells/well of ID8 cells, 20,000 cells/well of MPK cells, and 15,000 cells/well of KPCA.B cells. Once the cells adhered, they were treated with various concentrations of carboplatin (0, 25, 50, 75, 100, 150, and 200 ng/ μ L) and varying concentrations of paclitaxel (0 to 200 ng/ μ L) diluted in media for 48 hours. This experiment was also completed using the human cell lines PEO1, PEO4, and SLPI KD PEO4 cells. These cells were plated at a concentration of 10,000 cells/well and, once adhered, were treated with various concentrations of carboplatin (0, 25, 50, 75, 100, 150, and 200, 250 ng/ μ L) and paclitaxel (0 to 25 ng/ μ L) for 48 hours. The cell cultures were assessed using AlamarBlue™ cell viability assays by incubating the cells with a complete media solution containing 10% Alamar Blue Reagent (Thermo Fisher, DAL1100) at 37°C for 3 hours. IC₅₀s were determined using a dose-response nonlinear regression curve.

2.8. siRNA knockdown of SLPI

SLPI knockdown was done in PEO4 cells due to their high baseline levels of SLPI. PEO4 cells (1.75×10^5 /well) were plated in a 6-well plate and left to adhere overnight. 1x transfection buffer (5x GenMute™ Transfection Buffer (SignaGen, #SL100572) diluted with water) and a small volume of GenMute™ siRNA Transfection Reagent (SignaGen, #SL100568) was added to the aliquoted siRNA. Three siRNAs for SLPI were chosen: Silencer™ Select Pre-Designed siRNA (4392420 (ID: s194801)), Silencer™ Select Pre-Designed siRNA (4392420 (ID: s224651)), Silencer™ Select Pre-Designed siRNA (4392420 (ID: s224652)), and one negative control was used: Silencer™ Select Negative Control No. 1 siRNA. The mixture was incubated at room temperature for 15 min. The siRNA transfection mix was added dropwise to the target cells in media. Following 5 hours of incubation at 37°C, 1 mL of media was added to each well. The transfection media was replaced with fresh media after overnight incubation at 37°C. Cells were collected 48 hours after transfection.

2.9. Spheroid formation assay

To evaluate the effect of SLPI on the ability of MPK and ID8 cells to form spheroids in vitro, we plated 10,000, 5,000, 1,000, and 500 MPK *Slpi*^{-/-}, MPK Control, ID8 *Slpi*^{EV} and ID8 *Slpi*^{OE} cells in a Corning® 96-well Clear Flat Bottom Ultra-Low Attachment Microplate (Corning, #3474). For subsequent replicates, 10,000 cells/well was determined to be the optimal concentration for consistent technical replicates. To visualize the wells, 5-6 images were taken on the EVOS M5000 (Invitrogen) at 4x magnification. Spheroids were counted and measured in ImageJ. Spheres under 50 µm in diameter were excluded. To evaluate spheroid formation in a semi-solid media environment, cells were plated in 1% carboxymethylcellulose (CMC) in media. Results for the CMC experiment were based on one image taken in the middle of each well.

2.10. Low attachment cell viability assay

Cells of interest were plated at a concentration of 10,000 cells/well in 200 μ L in a Corning® 96-well Clear Flat Bottom Ultra-Low Attachment Microplate (Corning, #3474). To count the cells after 48 and 96 hours of culture, the volume within a well was collected in an Eppendorf tube, and the tubes were centrifuged at 500g for 4-5 minutes. The media was aspirated without disrupting the pellet, and the pellet was washed with PBS once. After aspirating the PBS, cells were incubated in trypsin (Corning, # 25-052-CI) at 37°C for 5 minutes to separate spheroids into a single-cell suspension. Media was then added to neutralize the trypsin. The total volume was transferred to a column, and the cells were counted using the Vi-CELL XR Cell Viability Analyzer (Beckman Coulter).

2.11. Survival study of mice bearing SLPI-modified cell lines

To evaluate the impact of SLPI on survival in an orthotopic tumor model, we used an SLPI overexpression model using the ID8 cells and an SLPI knockout model using the MPK cells. Before injection into mice, cell lines were tested for mycoplasma using the Mycoplasma PCR Detection Kit (Cedarlane, # G238(AG)). MPK control and MPK *Slpi*^{-/-} cells were resuspended in 100 μ L of PBS, and 0.15×10^6 cells were injected under the bursal membrane of eight 10-week-old female syngeneic FVB/N mice. Surgeries were performed by John Abou-Hamad, Olivia Piccolo, and Alyssa Murray. Similar injections of ID8 *Slpi*^{EV} and ID8 *Slpi*^{OE} cells were made into syngeneic C57BL/6 mice. The mice were monitored every two days and were euthanized using CO₂ at humane endpoint, which was determined by the presence of characteristics of pain or distress, such as impaired movement, respiratory issues, anemia, ascites buildup, facial grimace, or severe piloerection. Length of survival, overall body weight, tumor weight, ascites volume, total weight of all visible metastases, and spleen weight were recorded.

2.12. Tumor, lymph nodes, and peritoneal wash collection and flow cytometry for TME analysis

To investigate the immune composition of tumors with and without the expression of SLPI, we generated orthotopic tumors by injecting 0.15×10^6 ID8 *Slpi*^{EV} and ID8 *Slpi*^{OE} cells under the bursal membrane of syngeneic mice, as described previously⁷². Cells were injected into eight 8-week-old female syngeneic C57BL/6 mice for each cell line. Surgeries were performed by Dalia Ibrahim and Sara Asif. All mice were euthanized 79 days following injection of tumor cells, an estimated 6 days before the known median humane endpoint for the ID8 model with the shorter survival, *Slpi*^{OE}. Once euthanized, tumors, peritoneal wash, and lymph nodes were collected. Tumors were first dissected using a razor blade into small pieces in 2.5 mL of digestion solution from the Tumor Dissociation Kit (Miltenyi Biotec, #130-096-730) and then completely dissociated using the gentleMACS Octo Dissociator with heaters (protocol tumor 37°C_m_TDK_1), following the manufacturer's protocol. To remove red blood cells, cells were resuspended in ACK Lysis Buffer for 3 minutes. For the peritoneal wash (PW), 5mL of PBS-EDTA 2mM was injected intraperitoneally, abdominal massage was performed for 5 min, and the peritoneal fluid was collected and passed through a 70 µm cell strainer in a tube. ACK Lysis Buffer was used to remove red blood cells if needed. Lymph node tissue was disrupted through a 70µm filter to obtain single cells and washed with PBS. The viability control sample was killed with 90% ethanol. Samples were then incubated with an Fc-blocking antibody (BD Biosciences, #553142) diluted in PBS for 10 minutes. Cells and the viability control were stained with viability stain, and then samples were stained with extracellular antibodies listed in **Tables 4 and 5** and incubated at 4°C for 30 min. Fixation/permeabilization buffers (eBioscience™, #00-5523-00) were then applied to the samples at 4°C for 45 min. Cells were stained with intracellular antibodies listed in **Tables 4 and 5** and

were incubated overnight at 4°C. The Cytex[®] Aurora Spectral flow cytometer was used to obtain data, and it was analyzed using the FlowJo software (v10.10.1).

Table 4. Panel of fluorophore-conjugated flow cytometry antibodies used to characterize immune lymphoid populations.

Target	Fluorochrome	Dilution factor	Company and Catalogue #
Viability	Zombie NIR	500	Biolegend, #423106
Extracellular Antibodies			
CD45	BUV496	300	BD Biosciences, #569673
CD3	BB700	200	BD Biosciences, # 742099
CD4	BUV661	300	BD Biosciences, #612974
CD8	BUV615	300	BD Biosciences, #613004
CD44	BV650	300	BD Biosciences, #740455
CD25	BV605	300	BD Biosciences, #563061
CD62L	BUV737	300	BD Biosciences, #612833
iCOS	PE-Cy5	200	Biolegend, # 107708
CD69	PE-Cy7	300	BD Biosciences, #552879
CD103	BV711	200	Biolegend, #121435
CD19	BV510	500	BD Biosciences, # 562956
CD20	APC-Fire750	200	Biolegend, #152118
NK1.1	BV421	100	Biolegend, # 108732
DX5	AF700	100	Thermo Fisher, # 56-5971-82
ST2	PE	200	BD Biosciences # 566311
PD-1	BV750	200	Biolegend, #135263
PD-L1	BUV395	200	BD Biosciences #568307

LAG3	BUV805	100	BD Biosciences, # 748540
TIGIT	PE-Dazzle594	100	Biolegend #142110
KLRG1	BUV563	200	BD Biosciences, #741343
Intracellular antibodies			
FoxP3	eFluor450	300	Thermo, #48-5773-82
GATA3	AF488	200	BD Biosciences, #560163
CTLA4	PE-Fire 810	200	Biolegend, #106335
Ki67	BV786	200	BD Biosciences, #563756

Table 5. Panel of fluorophore-conjugated flow cytometry antibodies used to characterize immune myeloid populations.

Target	Fluorochrome	Dilution factor	Company and Catalogue #
Viability	Zombie NIR	500	Biolegend, #423106
Extracellular Antibodies			
CD45	BUV496	300	BD Biosciences, #569673
CD3/CD19/NK1.1	PE-Cy5	500	Biolegend, #561006, 115510, 108716
F4/80	BV421	500	BD Biosciences, #565411
CD11b	BUV661	200	BD Biosciences, #612977
Siglec-F	PerCP-eFluor710	200	Thermo, #46-1702-82
CD206	BV650	200	Biolegend, #141723
CD86	BV786	200	BD Biosciences, #740900
Gr1	BV605	200	BD Biosciences, #563299
CD11c	BUV563	200	BD Biosciences, #749040
CD103	BV711	200	Biolegend, #121435

CCR7	BUV805	200	BD Biosciences, #742065
CCR5	BUV615	200	BD Biosciences, #752321
CD80	BV510	200	BD Biosciences, #740130
ST2	PE	200	BD Biosciences, #566311
MHC-II	BUV737	300	Invitrogen, #367-5321-82
PD-L1	BUV395	200	BD Biosciences, #568307
PD-1	BV750	200	Biolegend, #135263
Intracellular antibodies			
Arginase-1	PE-Cy7	200	Invitrogen, #25-3697-82
GATA3	AF488	200	BD Biosciences, #560163

2.13. Extraction, differentiation and treatment of bone marrow-derived macrophages with recombinant SLPI

To evaluate the effect of SLPI on macrophage polarization, we collected undifferentiated monocytes from the bone marrow of three healthy 12-week-old male C57BL/6 mice. One femur was extracted from each mouse, and the cells from the bone marrow were flushed using a 5mL syringe into a dish containing PBS + 2% FBS + 1X PenStrep (Thermo Fisher, #15140122). The cells were transferred to a 50 mL tube and centrifuged. To lyse the red blood cells in the sample, ACK Lysis Buffer (Thermo Fisher, #A10492-01) was added for 3 min and then quenched with media. The media used for these cells consisted of DMEM + 10% FBS + 1X PenStrep and was filtered. The ACK lysis buffer was removed by centrifugation, and cells were resuspended and counted using the Invitrogen™ Countess™ 3 Automated Cell Counter (Invitrogen). Cells were plated at a concentration of 0.35×10^6 cells/well in a total volume of 2 mL/well. Cells were treated with 20 ng/mL of M-CSF (Thermo Fisher, #AF3150210UG) every 3 days for 6 days to

differentiate monocytes into macrophages. On day 6, media was changed, and macrophages were polarized into either M1 macrophages by treatment with 50 ng/mL of IFN- γ and 20 ng/mL of M-CSF, or into M2 macrophages by treatment with 20 ng/mL of IL-4 and 20 ng/mL of M-CSF, or maintained as M0 macrophages by treatment with only 20 ng/mL of M-CSF for 48 hours. To evaluate the effect of SLPI on polarization, one well receiving M0 and M1 polarizing treatment for each replicate was simultaneously treated with 2 μ g/mL of recombinant mouse SLPI (Abxexa, #abx069004) on day 6 (summarized in **Figure 6**). After 48 hours, macrophage samples were lifted and collected using PBS + 5mM EDTA. They were then washed with PBS and plated in a 96-well V-bottom plate. Flow cytometry staining and analysis were completed as described in the previous section (section 2.12). Antibodies used in this experiment are listed in **Table 6** below.

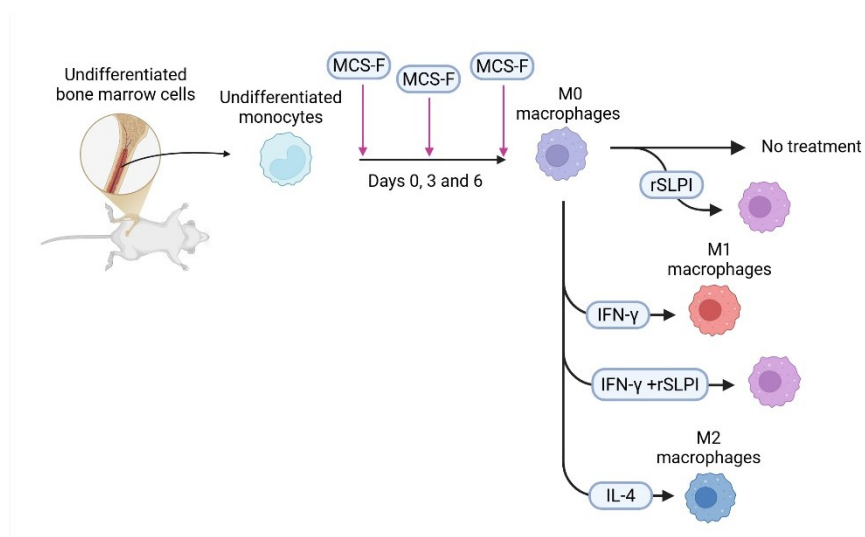


Figure 6. Flow chart describing in vitro investigation of the effect of rSLPI on murine macrophages.

Table 6. Panel of fluorophore-conjugated flow cytometry antibodies used to characterize macrophage polarization following in vitro treatment with rSLPI.

Target	Fluorochrome	Dilution factor	Company and Catalogue #
Viability	Zombie NIR	1000	Biolegend, #423106

Extracellular Antibodies			
F4/80	BUV615	500	BD Biosciences, #751454
CD11b	BUV661	200	BD Biosciences, #612977
CD11c	BUV563	200	BD Biosciences, #749040
CD86	BV711	200	BD Biosciences, #740709
MHC-II	BUV737	200	Invitrogen, #367-5321-82
CD206	Alexa fluor 647	200	BD Biosciences, #565250
CD80	BV510	200	BD Biosciences, #740130
Ly6C	BV605	200	BD Biosciences, #563011
PD-L1	PE	200	BD Biosciences, #558091
Intracellular antibodies			
Arginase-1	PE-Cy7	200	Invitrogen, #25-3697-82
iNOS	FITC	200	BD Biosciences, #610330
Ki67	BUV421	200	BD Biosciences, #565929

2.14. RNA sequencing analysis

The RNA used for sequencing was previously extracted from the mouse SLPI-modified cell lines, MPK Control, MPK *Slpi*^{-/-}, ID8 *Slpi*^{EV}, ID8 *Slpi*^{OE}, KPCA.B *Slpi*^{EV}, and KPCA *Slpi*^{OE}, using a standard RNA extraction protocol described earlier in section 2.2. RNA samples were sent to Genome Quebec, where a polyA-enriched RNA (stranded) library was prepared, and Illumina NovaSeq PE100 - 25M reads sequencing was performed.

Chapter 3: Results

3.1. Elucidating the pathway(s) regulating SLPI expression

Measurement of relative SLPI mRNA expression in various ovarian cell lines (**Figure 4A**) showed that SLPI is most likely regulated by KRAS, a member of the rat sarcoma viral oncogene family (RAS), which is a well-documented oncogene and is one of the most commonly mutated genes in cancer⁷⁵. It functions as a GTPase (guanosine triphosphatase) that can switch between an active state where it is bound to GTP and an inactive state where it is bound to GDP⁷⁶. It is important in relaying external signals to intracellular molecules and therefore is important in cell growth, division, and survival⁷⁶⁻⁷⁸. This was hypothesized because the MPK cell line has much higher SLPI expression compared to the MP cell line, which is identical except for the mutated, constitutively active KRAS found in the MPK cells. When KRAS is activated, it acts primarily through the MEK or AKT signaling pathways⁷⁵. To determine which downstream pathway is responsible for regulating SLPI, AKT and MEK activity were inhibited pharmacologically, and the resulting SLPI expression was measured. Two mouse cell lines with high expression of SLPI were tested: 1) MPK cells having a mutated constitutionally active KRAS (*KRas*^{G12V} mutation), and 2) STOSE cells with no known mutations, but known to have elevated phospho-ERK levels⁷⁹. The cells were treated with either an AKT inhibitor, a MEK inhibitor, or DMSO as a control. For the visualization of protein levels by western blot, cells were treated with Brefeldin A to prevent secretion of SLPI. Following treatment with a MEK inhibitor, SLPI protein and relative mRNA expression were almost completely absent in both MPK ($p = 0.045$) and STOSE ($p = 0.0023$) (**Figure 7A, B, C, D**). Following inhibition of AKT activity, *Slpi* mRNA expression was significantly increased in STOSE ($p = 0.0433$) and trending towards an increase in MPK ($p =$

0.0643) (**Figure 7A, C**). This is consistent with SLPI protein expression, which is visibly higher in the AKT-inhibited samples and is especially notable in the samples also treated with Brefeldin A to prevent SLPI release from the cells (**Figure 7B, D**). These results suggest that the MEK pathway is responsible for upregulating SLPI, while AKT signaling downregulates its expression, at least in these mouse cell lines. To investigate if this pattern is consistent in human ovarian cancer cell lines, this experiment was repeated in four human cell lines, PEO1, PEO4, A2780 S, and A2780 CP. First, we measured baseline *SLPI* expression in our cell lines and found that PEO4 exhibits high baseline levels of *SLPI* mRNA levels while PEO1, A2780S, and A2780 CP have low levels of *SLPI* relative to PEO4 (**Figure 8**). Following AKT inhibition in the human cell lines, no differences in *SLPI* mRNA expression were observed; however, *SLPI* expression was significantly increased following MEK inhibition in PEO1, PEO4, and A2780S cells (**Figure 9A, B, C**). These results suggest that MEK activity is downregulating *SLPI* expression in these human ovarian cancer cell lines, contrary to what was observed in the murine cell lines. PEO1 and PEO4 do not have KRAS mutations, and A2780s have PI3K and RRAS2 mutations^{80,81}. Therefore, the regulation of SLPI expression may be species- or cell line-dependent. Its expression in human ovarian cancer cell lines and mouse cell lines does not appear to be dependent on a KRAS mutation but could be affected by a *KRAS* gene amplification, or amplification/mutation of another factor in the KRAS signaling cascade.

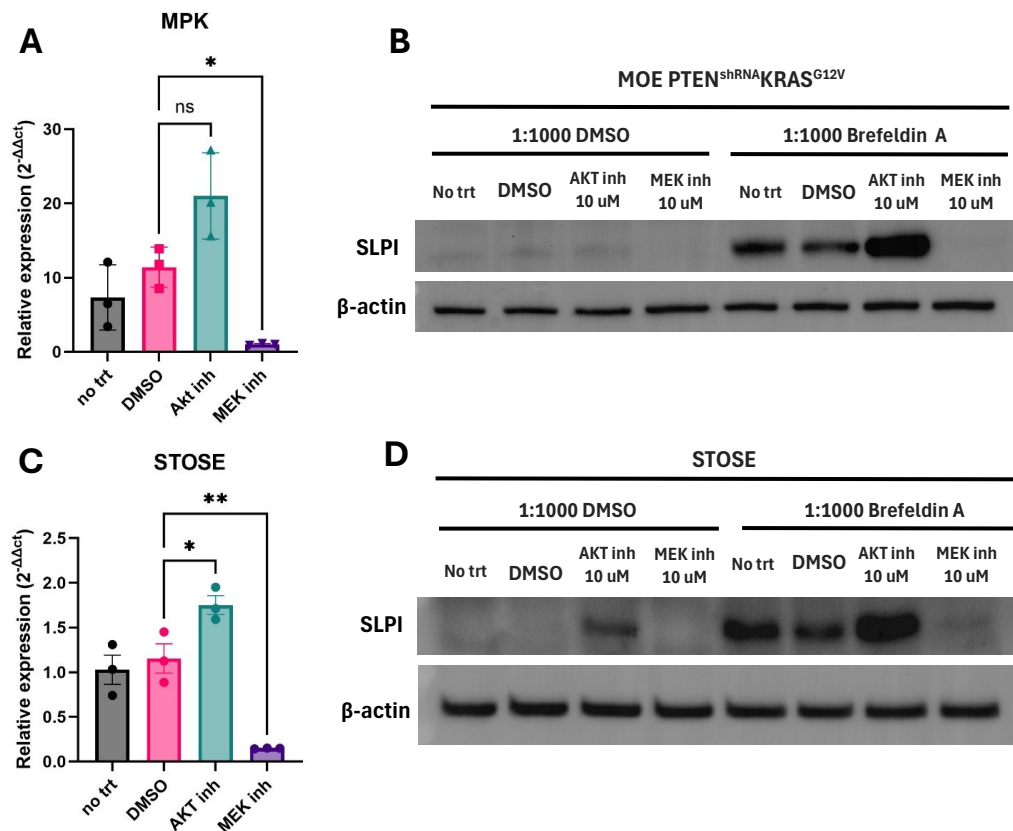


Figure 7. Relative expression of *Slpi* in murine ovarian cancer cells following AKT or MEK inhibition.

(A) *Slpi* mRNA expression in MPK, (B) SLPI protein expression in MPK, (C) *Slpi* mRNA expression in STOSE, (D) SLPI protein expression in STOSE, were measured using qPCR and western blotting. Cells were treated with 10 μ M of AKT or MEK inhibitor or 1:1000 DMSO. Expression levels were compared using a one-way ANOVA ($n=2-3$), mean $\pm$ SEM. * $p \leq 0.05$, ** p -value ≤ 0.01

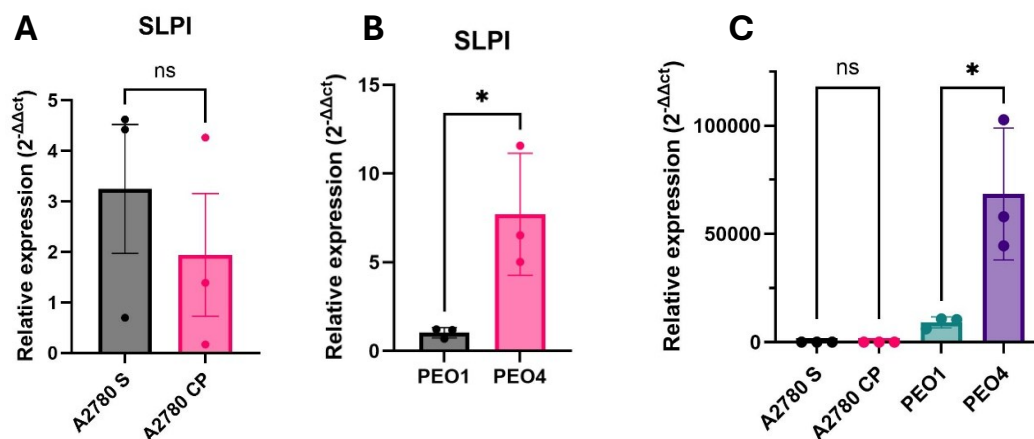


Figure 8. Relative mRNA levels of *SLPI* in human ovarian cancer cell lines and their chemoresistant pair.

(A) *SLPI* mRNA expression in the A2780 pair, (B) *SLPI* mRNA expression in the PEO1/4 pair (C) Relative *SLPI* mRNA expression in both pairs. Cell line pairs were compared using an unpaired t-test (n=3). The mean is shown +/- SEM. *p-value ≤ 0.05 .

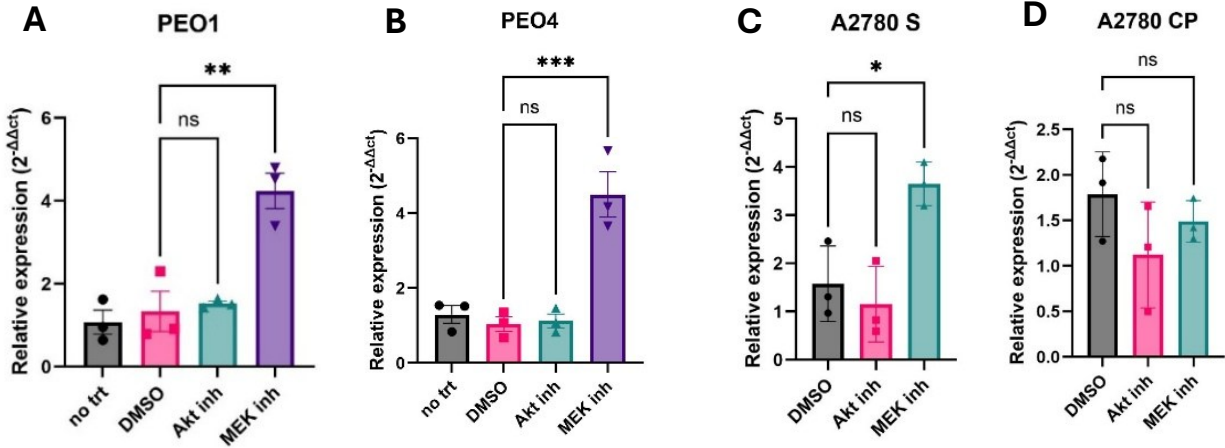


Figure 9. Relative mRNA expression of *SLPI* in human ovarian cancer cells following AKT or MEK inhibition.

PEO1 (A), PEO4 (B), A2780 S (C), A2780 CP (D) cells were treated with 10 μ M of AKT or MEK inhibitor. Expression levels were compared using a one-way ANOVA (n=3), mean +/- SEM. *p ≤ 0.05 , **p ≤ 0.01 , ***p-value ≤ 0.001 .

3.2. Assessing the effect of *SLPI* on proliferation

Following *Slpi* modification of our cell lines, we first set out to determine if the level of *SLPI* expression affected their rate of proliferation. Both cell lines exhibited exponential growth, with the MPK cell lines plateauing at 72 hours and the ID8 cell lines being confluent but still growing at 96 hours. While the MPK *Slpi*^{-/-} cells had proliferated slightly more compared to the MPK Control at 48 hours (p = 0.0153) and 72 hours (p = 0.0677) and the ID8 *Slpi*^{OE} cells had proliferated slightly less compared to its control at 72 hours (p = 0.0068) and 96 hours (p = 0.0005), the overall differences between the *SLPI*-modified cell line curves and their control cells were not significant or were significant, but modest (**Figure 10A, B**). Therefore, any future observed differences

between SLPI-modified cell lines and their controls in these models are likely independent of differences in proliferation rate.

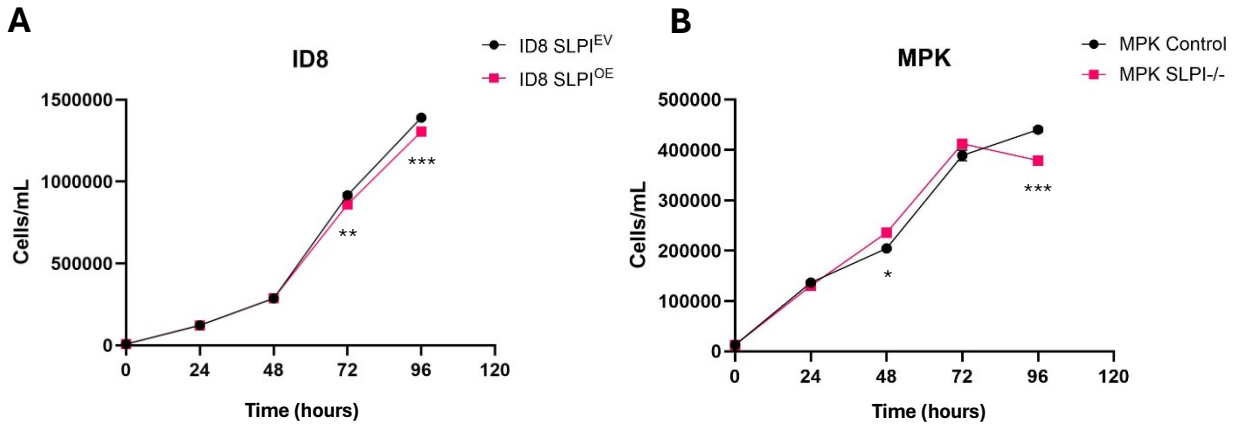


Figure 10. Knockout and overexpression of *Slpi* do not impact cell proliferation.

(A) Proliferation of ID8 *Slpi*^{EV} and ID8 *Slpi*^{OE} cells across time (B) Proliferation of MPK Control and MPK *Slpi*^{-/-} cells across time. A two-way ANOVA with Šídák's multiple comparisons test was used to compare the curves. Each point is shown as mean $\pm$ SEM (n=3). * $p \leq 0.05$, ** $p \leq 0.01$, *** p -value ≤ 0.001 .

3.3. Assessing the effect of SLPI on chemoresistance

3.3.1. SLPI-modified cell lines generally show no difference in response to chemotherapy

To determine if SLPI expression has any impact on the sensitivity or resistance of ovarian cancer cells to chemotherapy, we started by evaluating the responses of our SLPI-modified murine cell lines to chemotherapy. We treated MPK Control, MPK *Slpi*^{-/-}, ID8 *Slpi*^{EV}, ID8 *Slpi*^{OE}, KPCA.B *Slpi*^{EV}, and KPCA *Slpi*^{OE} cells with carboplatin and paclitaxel to determine the IC₅₀ for each cell line. After carboplatin treatment of MPK, ID8, and KPCA.B cells, there were no significant differences in IC₅₀ values of the SLPI-modified cell lines and their control (MPK Control IC₅₀ = 31.023 ng/ μ L, MPK *Slpi*^{-/-} IC₅₀ = 40.143 ng/ μ L, ID8 *Slpi*^{EV} IC₅₀ = 53.983 ng/ μ L, ID8 *Slpi*^{OE} IC₅₀ = 38.313 ng/ μ L, KPCA.B *Slpi*^{EV} IC₅₀ = 87.31 ng/ μ L, and KPCA *Slpi*^{OE} IC₅₀ = 98.027 ng/ μ L) (Figure 11A, C, E, G). After paclitaxel treatment, there were no significant differences in MPK

and ID8 cell lines between the SLPI-overexpressing cells and their controls (MPK Control IC_{50} = 0.115 ng/ μ L, MPK *Slpi*^{-/-} IC_{50} = 0.112 ng/ μ L, ID8 *Slpi*^{EV} IC_{50} = 0.014 ng/ μ L, ID8 *Slpi*^{OE} IC_{50} = 0.0085 ng/ μ L), however, the IC_{50} of the KPCA *Slpi*^{OE} cell line was significantly higher than its control (KPCA *Slpi*^{EV} IC_{50} = 0.243 ng/ μ L, KPCA *Slpi*^{OE} IC_{50} = 0.085 ng/ μ L), suggesting that SLPI expression significantly increased sensitivity of KPCA.B cells to paclitaxel (**Figure 11B, D, F, H**). Interestingly, the curve for each cell line following treatment with carboplatin follows a standard inhibitory dose-response curve; however, the curves following paclitaxel treatment start with a plateau at low concentrations, which is followed by a sudden drop in viability, followed by another plateau and finally another slight drop to reach 0% viability. This indicates that there may be a subset of cells that are resistant to lower doses of paclitaxel. This plateau phenomenon in response to paclitaxel has been shown in various cancer models, including ovarian cancer^{82,83}. Specifically, Lopes et al. demonstrated that paclitaxel-mediated ovarian cancer cell-killing reached a plateau due to the presence of a resistant and a sensitive, most likely mitotic, fraction of cells in vitro⁸³. Overall, these results suggest that SLPI does not play a major role in ovarian cancer response to the two drugs most commonly used in its treatment.

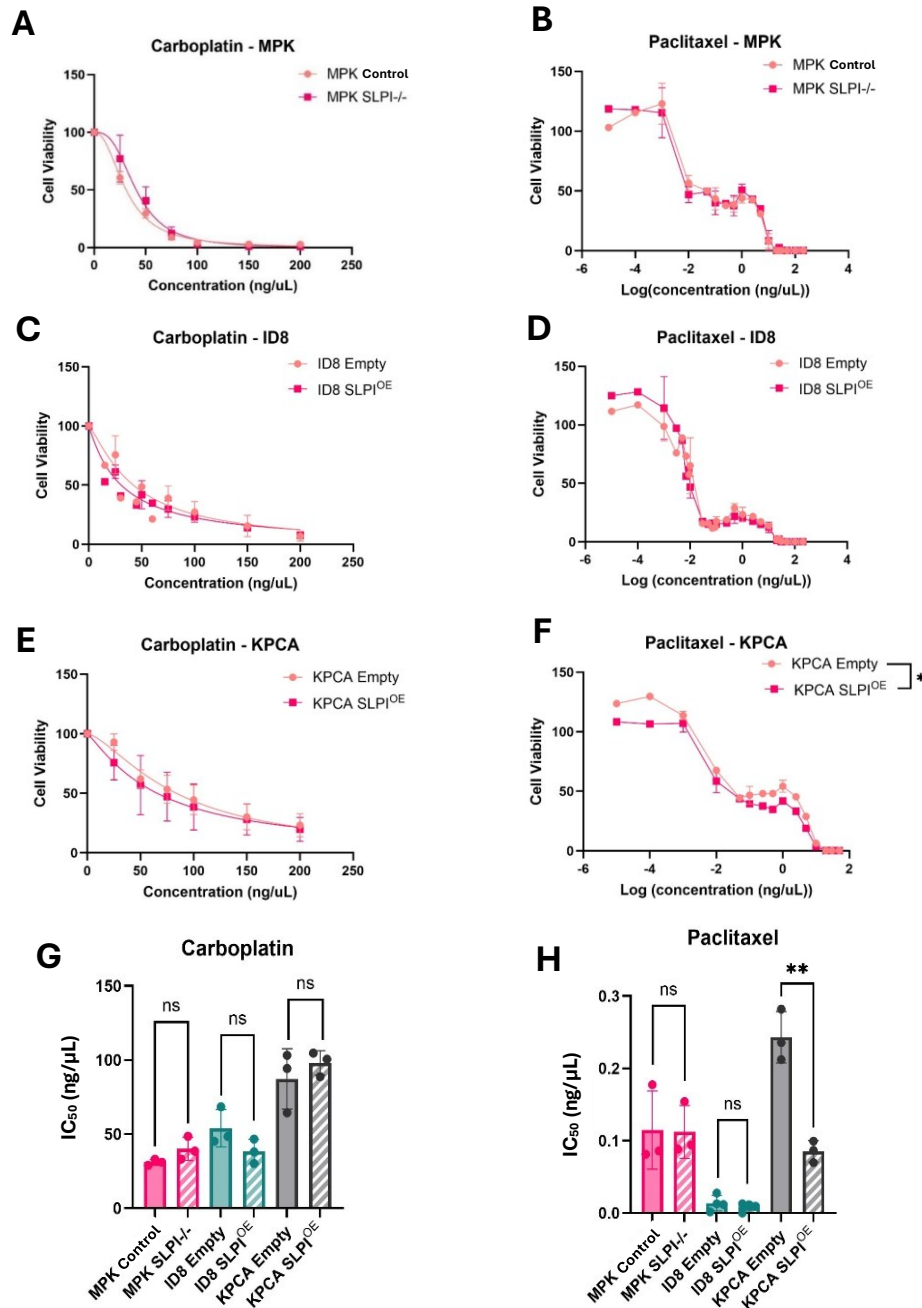


Figure 11. Effect of Slpi expression on murine ovarian cancer cell line viability in response to chemotherapy.

(A), (C), (E) Cell viability (%) of Slpi-modified MPK (n=3), ID8 *Trp53*^{-/-} (n=4) and KPCA.B (n=3) cells in response to treatment with 0 to 200 ng/μL carboplatin. (B), (D), (F) Cell viability (%) of Slpi-modified MPK (n=4), ID8 *Trp53*^{-/-} (n=5), and KPCA.B (n=4) cells in response to treatment with 0 to 50 ng/μL of paclitaxel (log-transformed). IC₅₀ doses from MPK, ID8 *Trp53*^{-/-} and KPCA.B cells treated with carboplatin (G) or paclitaxel (H). Curves were compared using a two-way ANOVA (mixed-effects model) with Geisser-Greenhouse correction. IC₅₀s were compared using an unpaired t-test (n=3-4). *p ≤ 0.05, **p ≤ 0.01.

3.3.2. SLPI is correlated with the chemoresistant phenotype in PEO4

To investigate the effect of SLPI on chemoresistance in human models, we chose two pairs of human ovarian cancer cell lines that have chemoresistant and chemosensitive counterparts. One of these pairs is PEO1 (sensitive) and PEO4 (resistant), originating from the ascites of a high-grade serous ovarian cancer patient and developing chemoresistance to platinum drugs *in vivo*. The other pair is A2780 S (sensitive) and A2780 CP (resistant), originating from a tumor of an ovarian endometrioid cancer patient where resistance was induced by prolonged treatment with platinum drugs *in vitro*. We first aimed to measure differences in *SLPI* mRNA expression in these two pairs to determine if the chemoresistant phenotype is associated with increased SLPI expression. While we found low *SLPI* expression in both A2780 S (mean $2^{-\Delta\Delta Ct} = 3.247$) and A2780 CP (mean $2^{-\Delta\Delta Ct} = 1.941$) and no significant differences between them (**Figure 8A**), chemoresistant PEO4 cells had a 7-fold higher level of *SLPI* expression (mean $2^{-\Delta\Delta Ct} = 7.704$) than their chemosensitive counterpart, PEO1 (mean $2^{-\Delta\Delta Ct} = 1.031$) (**Figure 8B**). This presents initial evidence that SLPI expression may be correlated with the chemoresistant phenotype in certain models such as PEO4. Since PEO1/4 and A2780 S/CP originate from different subtypes of ovarian cancer and developed their chemoresistance in different manners, we can hypothesize that SLPI only contributes to chemoresistance in specific subtypes, such as HGSOC, and that this mechanism only happens through the development of chemoresistance *in vivo*. Before expanding on this observation using additional cell lines, we first sought to determine the contribution of SLPI to chemoresistance in the PEO4 cells. We first validated the chemoresistant and chemosensitive phenotype of PEO1/PEO4 cells by treating both cell lines with varying concentrations of carboplatin and paclitaxel and evaluating cell viability after 48 hours of treatment. This confirmed that PEO4 was indeed chemoresistant to carboplatin since cell viability barely decreased with increasing

carboplatin concentrations, contrary to PEO1, where cell viability reached close to 0% at 200 ng/ μ L of carboplatin (**Figure 12A**). PEO4 is also slightly more resistant to paclitaxel than PEO1, but both cell lines were sensitive to taxane drugs (**Figure 12B**).

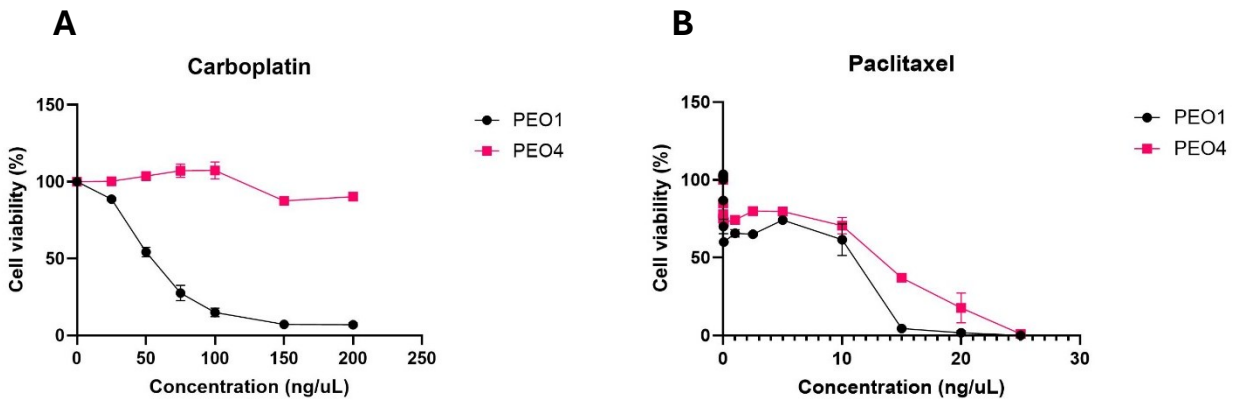


Figure 12. Validation of chemosensitivity and resistance in the human cancer cell lines, PEO1 and PEO4.

Percentage of PEO1 and PEO4 cells that are viable after treatment for 48 hours with 0 to 200 ng/ μ L carboplatin (A) or 0 to 50 ng/ μ L paclitaxel (B). A two-way ANOVA (or mixed-effects model) was used to compare the curves. Each point is shown as mean $\pm$ SEM (n=3).

3.3.3. SLPI levels are not upregulated in response to carboplatin treatment

Previous publications have shown that treatment of ovarian cancer cells with chemotherapeutic drugs, specifically paclitaxel, can induce the expression and release of SLPI⁶⁸. We set out to determine if this was the case in our models and if carboplatin could also induce SLPI expression. To do this, we chose the PEO1 and PEO4 cells since we know they have different levels of baseline *SLPI* expression; therefore, we could capture any changes in SLPI expression. Both cell lines were treated with a low (50 ng/ μ L) or a high dose of carboplatin (200 ng/ μ L) for 24 hours and compared to untreated controls. Following treatment, relative *SLPI* mRNA expression was measured, and no significant differences were found (**Figure 13**). This indicates that exposure to carboplatin treatment does not upregulate expression of *SLPI* in our cell lines. The

data thus far suggest that *SLPI* does not contribute to chemoresistance in either murine or human ovarian cancer cells, at least in the cell lines used in this study.

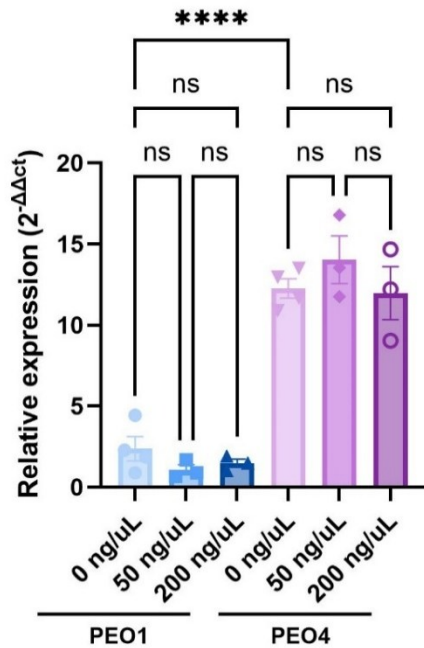


Figure 13. Relative mRNA levels of *SLPI* in PEO1 and PEO4 following carboplatin treatment. Treatment groups were compared using a one-way ANOVA (n=3-4). The mean is shown +/- SEM. ****p-value ≤ 0.0001

3.3.4. Knockdown of *SLPI* expression in PEO4 does not modify chemoresistance

In a final experiment to establish if *SLPI* is involved in a chemoresistant response, *SLPI* expression was knocked down in one of our cell lines. For this, we chose the cell line PEO4 because it has high *SLPI* expression and is chemoresistant, which would allow us to determine if knockdown of *SLPI* would affect its chemoresistant phenotype. Once we validated successful knockdown of *SLPI* in PEO4 cells using 3 siRNAs (**Figure 14A, B**), we treated the *SLPI* knockdown (KD) cells and their siRNA control with various concentrations of carboplatin and paclitaxel and measured cell viability after 48 hours of treatment. We simultaneously did this with PEO1 as a control for carboplatin's cytotoxicity. All the PEO4 cells, with and without *SLPI* knockdown, remained resistant to carboplatin, and resistance to paclitaxel also did not change

(Figure 15A, B). This is further confirmation that SLPI alone is not involved in conferring cancer cells with a chemoresistant phenotype, although this does not eliminate the possibility that SLPI may be part of a larger network of factors contributing to chemoresistance. We therefore shifted our attention to other tumor features that might be affected by SLPI.

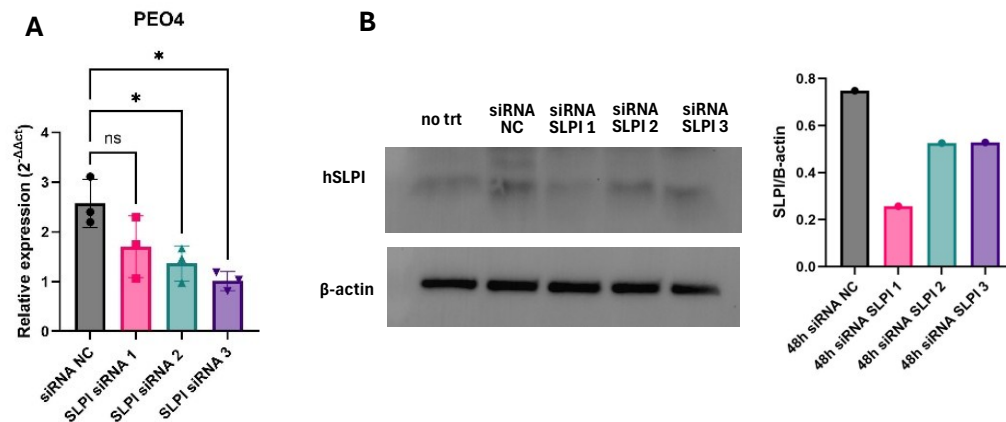


Figure 14. Validation of SLPI knockdown in PEO4 following treatment with siRNAs.

(A) Relative mRNA expression of *SLPI* in PEO4 following treatment with siRNAs for 48hrs, (B) Western blot and quantification of protein levels of SLPI in PEO4 following treatment with siRNAs for 48hrs (n=1). Transcript levels were compared using a one-way ANOVA (n=3), mean $\pm$ SEM. * $p \leq 0.05$. NC: negative control

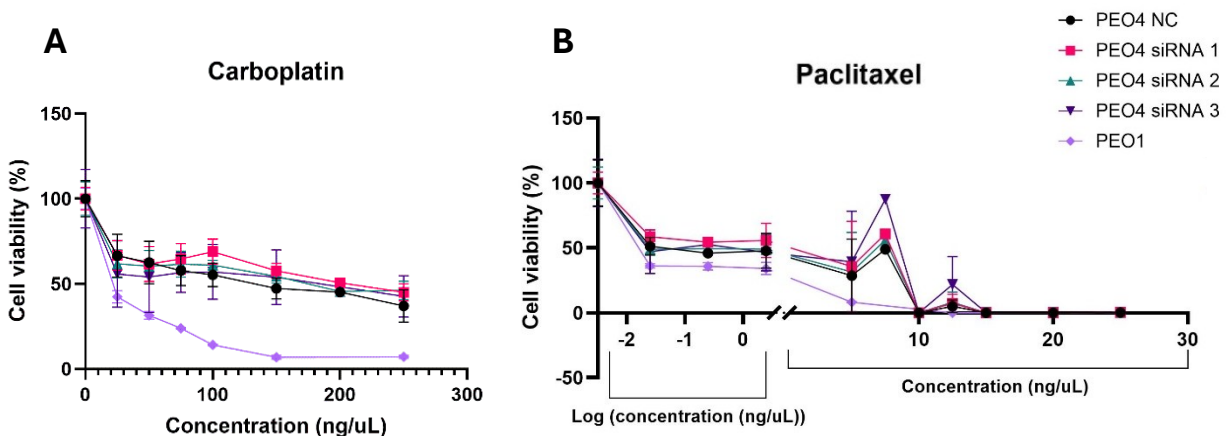


Figure 15. Effect of SLPI knockdown on PEO4 cell viability in response to chemotherapy.

(A) Cell viability of PEO4 control and PEO4 cells treated with 3 different siRNAs to knock down SLPI (n=3), in comparison to PEO1 control cells (n=1) following treatment with various concentrations of carboplatin (0 to 250 ng/μL) or (B) paclitaxel (0 to 25 ng/μL, partially log transformed). The curves were compared using a two-way ANOVA with Dunnett's multiple comparisons test.

3.4. Characterization of SLPI's effect on metastasis formation

3.4.1. Mice bearing MPK *Slpi*^{-/-} tumors have increased metastases

To characterize SLPI's effect on tumor progression and overall survival, we performed intrabursal injection of cells with and without modified *Slpi* expression, notably MOE *Pten*^{shRNA} *KRas*^{G12V} cells with and without *Slpi* knockout (MPK control and MPK *Slpi*^{-/-}). Once the mice reached humane endpoint, they were euthanized, and various characteristics of tumor progression such as number of days elapsed since injection, overall body weight, tumor weight, ascites volume, spleen weight, and total metastases weight were recorded. Endpoint in this model was generally due to the growth of the tumors to the point when they began to constrict breathing and other functions. There were no significant differences in median survival in the MPK *Slpi*^{-/-} vs. its control (61 days for MPK control and 58 days for MPK *Slpi*^{-/-}, p-value = 0.4948) (**Figure 16A**). Of all other parameters measured at endpoint, only total metastasis weights were significantly different (**Figure 16B, C, D, E, F**). MPK *Slpi*^{-/-} mice had significantly more metastases compared to their control (**Figure 16B**). This contradicts our hypothesis and some of the literature suggesting that SLPI contributes to metastasis⁸⁴. Since SLPI is a secreted protein, it has the potential to act in an autocrine or paracrine manner. To better dissect SLPI actions in tumors, we first examined its potential for autocrine actions by determining if the difference in metastasis is due to SLPI's ability to decrease anoikis resistance in cancer cells.

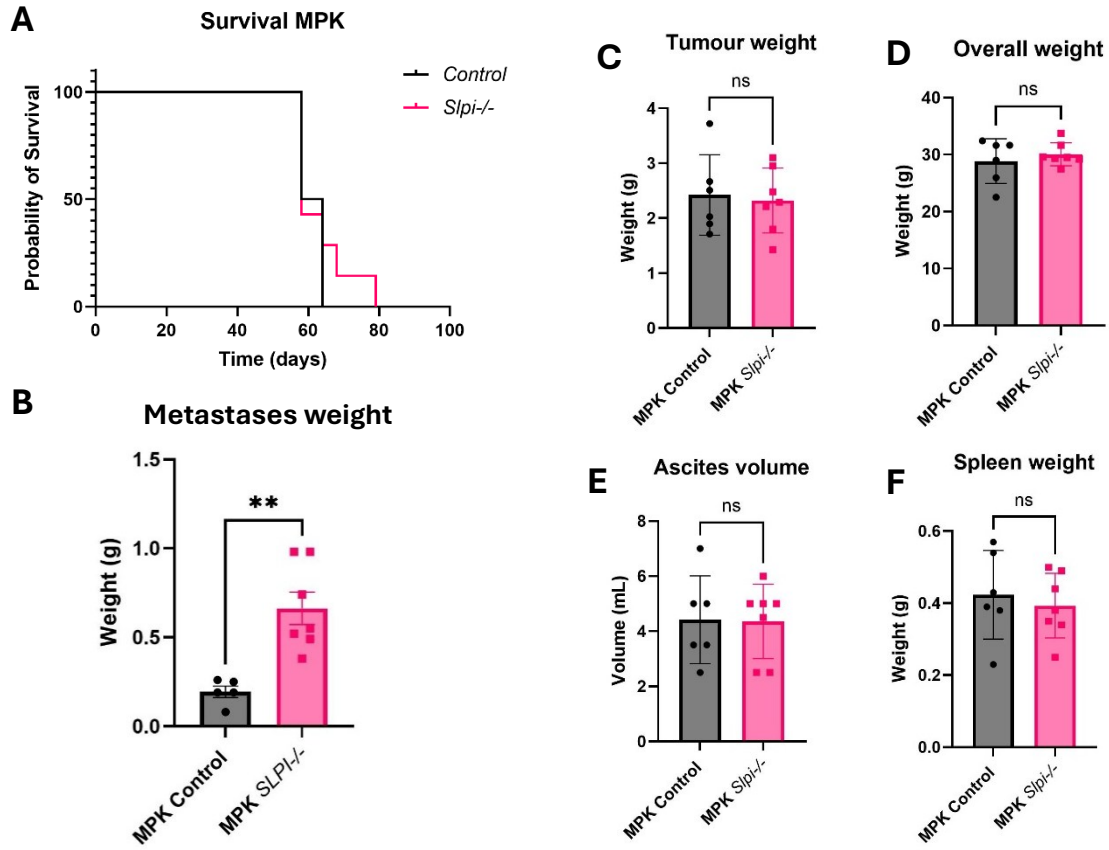


Figure 16. SLPI^{-/-} tumors exhibit increased metastasis weight.

(A) Kaplan-Meier plot showing the survival of mice bearing SLPI-modified MPK tumors. (B) Total weight of all metastases, (C) primary tumor weight, (D) overall body weight, (E) ascites volume and (F) spleen weight at humane endpoint. Statistical analysis was done using a Logrank (Mantel-Cox) test for (A) and an unpaired t-test (n=5-7) for (B-F), mean $\pm$ SEM. ** $p \leq 0.01$.

3.4.2. SLPI is associated with the formation of fewer but larger spheroids

Anoikis is a type of cell death that occurs when cells lose their adhesion to ECM or other cells⁸⁵. Resistance to anoikis is crucial for cancer cells to metastasize and survive in environments with a lack of attachment⁸⁵. To investigate SLPI's effect on spheroid formation in suspension and anoikis resistance, we first seeded MPK Control, MPK *Slpi*^{-/-}, ID8 *Slpi*^{EV}, and ID8 *Slpi*^{OE} cells in ultra-low attachment plates and evaluated the size and number of spheroids formed at different

time points. ID8 cells did not form spheroids, so we were unable to evaluate the effect of SLPI in this capacity. This could be explained by ID8 cells being a more metastatic cell line in vivo, meaning that when we inject these cells orthotopically into mice, they metastasize and accumulate ascites more quickly. These cells are perhaps more resistant to anoikis without the need to form spheres to enhance their survival.

For the MPK cells, we counted and measured spheroid size manually at 3, 5, and 7 days after plating. The number of spheroids was consistently increased in the MPK *Slpi*^{-/-} group in comparison to the control group at Day 3, 5, and 7 (**Figure 17A, E**); however, spheroid size was generally smaller in the MPK *Slpi*^{-/-} group (**Figure 17B**). Variability between replicates makes the pooled data seem insignificant (**Figure 17C, D**). Since there were more metastases (greater metastases weight) in the mice with MPK *Slpi*^{-/-} tumors, we expected to see larger and more numerous spheroids made by the MPK *Slpi*^{-/-} cells in suspension, which would indicate that the cells survive better in suspension and the spheroids are proliferating. The ability of MPK *Slpi*^{-/-} cells to generate more spheroids aligns well with this expectation and the increased metastases in the mouse model. The reduced size of the spheroids was unexpected, especially since the proliferation of these cell lines was not different when grown in monolayer cultures (**Figure 10B**). However, the smaller size could also reflect a decrease in cellular aggregation or an overall reduced ability for the cells to remain viable in suspension, so we next set out to test those possibilities.

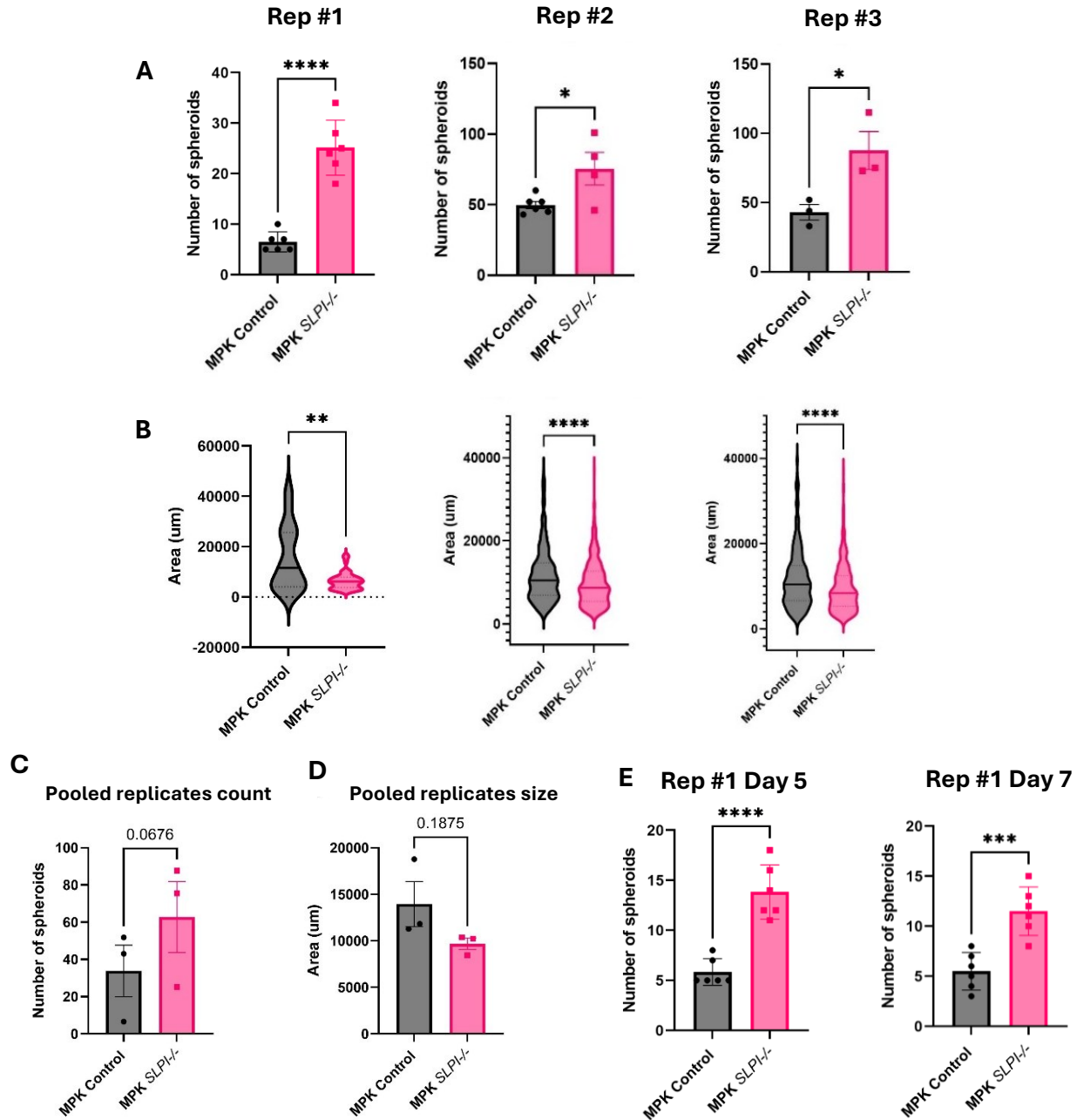


Figure 17. Number and size of MPK *Slpi*-modified spheroids in suspension.

(A) Number of MPK Control and *Slpi*^{-/-} spheroids formed in suspension for three replicates at Day 3. (B) Size of MPK Control and *Slpi*^{-/-} spheroids formed in suspension for three replicates at Day 3. (C) Number and (D) size of pooled replicates of MPK Control and *Slpi*^{-/-} spheroids at Day 3. (E) Number of MPK Control and *Slpi*^{-/-} spheroids formed in suspension for replicate 1 at Days 5 and 7. Spheroids were classified as spheres of cells measuring more than 50μm in diameter. Statistical analysis was performed using an unpaired t-test (n=3); mean +/- SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

3.4.3. SLPI does not have a strong effect on cell viability in suspension

To investigate if there are differences in anoikis resistance between MPK *Slpi*^{-/-} and ID8 *Slpi*^{OE} cells and their controls, we plated these cells in ultra-low attachment plates and measured cell viability at different time points. At 48 hours, the number and frequency of viable cells were decreased in the MPK *Slpi*^{-/-} group compared to their control (**Figure 18A**). At 96 hours, the percentage of viable cells was also decreased in the MPK *Slpi*^{-/-} group (**Figure 18C**). Generally, cells without *Slpi* expression in this model were less viable in suspension and therefore exhibited decreased resistance to anoikis, potentially explaining their smaller size in comparison to MPK Control spheroids. These results suggest that expression of SLPI increases cell viability in suspension. The differences between ID8 *Slpi*^{OE} cells and their control were variable between biological replicates and generally not significant (**Figure 18B, D**). Thus, there is no robust pattern that shows SLPI has a strong effect on cell viability in ID8 cells in suspension.

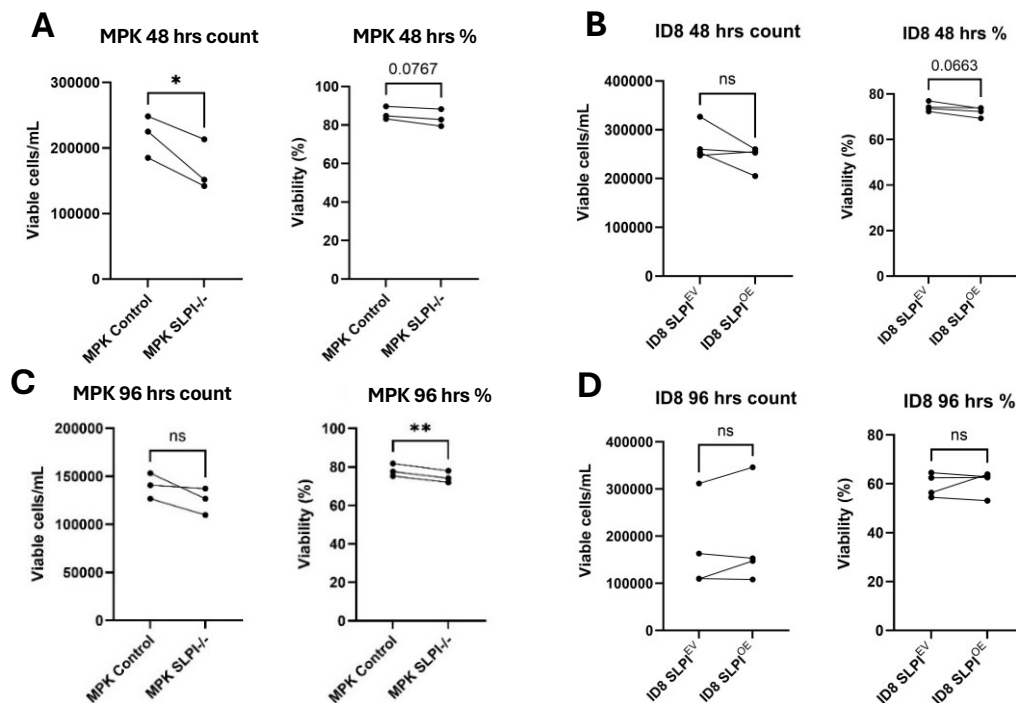


Figure 18. Cell count and percentage of SLPI-modified MPK and ID8 cells and their controls that are viable in suspension after 48 and 96 hours.

(A) Viable cell count and percentage of MPK control and *Slpi*^{-/-} cells at 48 hours, (B) Viable cell count and percentage of ID8 *Slpi*^{EV} and *Slpi*^{OE} cells at 48 hours, (C) Viable cell count and percentage of MPK control and *Slpi*^{-/-} cells at 96 hours, (D) Viable cell count and percentage of ID8 *Slpi*^{EV} and *Slpi*^{OE} cells at 96 hours. Statistical analysis was done using a paired t-test (n=3-4). *p ≤ 0.05, **p ≤ 0.01

3.4.4. Investigation of SLPI's effect on aggregation-independent spheroid formation in suspension cultures

To determine if the difference in spheroid formation by MPK cells with/without *Slpi* expression in suspension cultures is due to cellular aggregation or the ability of the cells in a spheroid to grow and proliferate, we used an ultra-low attachment plate and plated the cells in a mixture of media and carboxymethylcellulose (CMC) to create a semi-solid environment in which the cells cannot move freely in the media. We also used this model to see if our ID8 model could form spheroids in a semi-solid low-attachment environment, but again these cells were unable to form spheroids. Although there was no difference in the ability of MPK cells to initially form spheroids as seen on Day 3, MPK *Slpi*^{-/-} cells formed fewer spheroids on Days 5 and 7. (**Figures 19, 20**). This is in contrast to their viability in suspension in media alone and suggests that the size

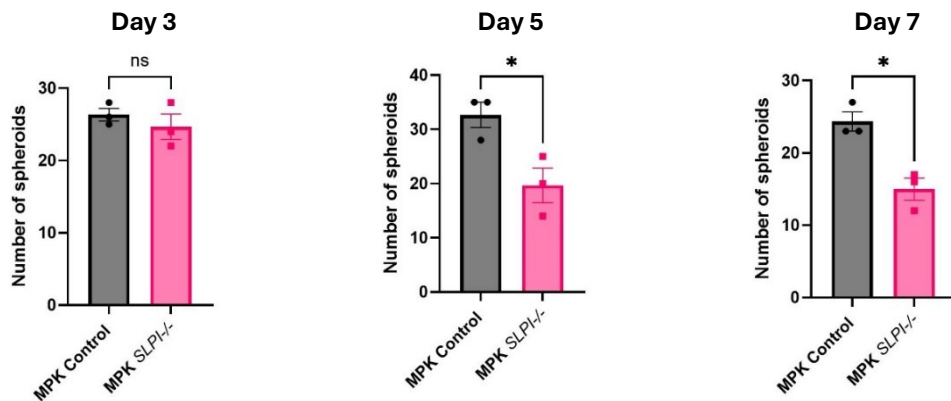


Figure 19. Number of spheroids in SLPI-modified MPK cells in 1% carboxymethylcellulose 3, 5, and 7 days after seeding.

Statistical analysis was performed using an unpaired t-test (n=1); mean +/- SEM. *p ≤ 0.05

difference observed when cells were cultured in liquid media (**Figure 17**) is not due to cellular aggregation alone. Additionally, MPK *Slpi*^{-/-} cells need the ability to move freely to come into contact with each other to form spheroids, and they survive better in liquid environments like the ascites, potentially explaining why there is an increase in total metastases weight in vivo.

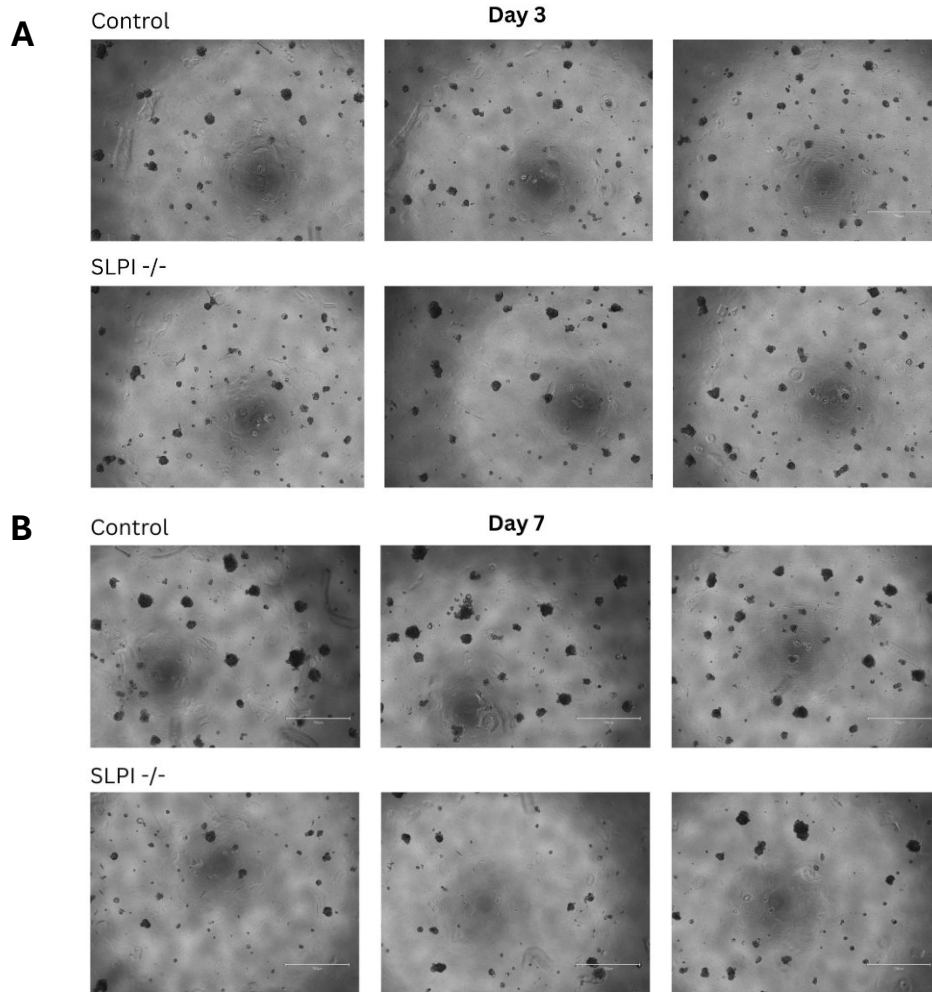


Figure 20. Images of SLPI-modified MPK cells in 1% carboxymethylcellulose on Day 3 (A) and Day 7 (B).

Images were taken at 4x magnification (n = 1).

3.4.5. Investigation of downstream signaling pathways responsible for SLPI's autocrine actions

3.4.5.1. SLPI does not upregulate downstream pAKT

Previous studies have shown that SLPI is able to downregulate or upregulate downstream pAKT/AKT, depending on the cell type^{69,86}. AKT activity is known to promote cell viability and anoikis resistance in ovarian cancer cells⁸⁷, so we aimed to investigate if this could explain the beneficial effects of SLPI on anoikis resistance, which could lead to the observed decrease in cell viability in our MPK *Slpi*^{-/-} spheroids. We first set out to measure baseline levels of pAKT in our SLPI-modified murine cell lines and observed no apparent differences in our SLPI-modified ID8 and MPK cell lines when compared to their relevant controls (**Figure 21**). The presence of higher pAKT levels in the MPK lines compared to ID8 is likely reflective of the knockdown of PTEN, an inhibitor of the AKT pathway, in the MPK cell line⁸⁸. However, it is important to determine pAKT levels in relation to AKT levels, which were not measured in this western blot.

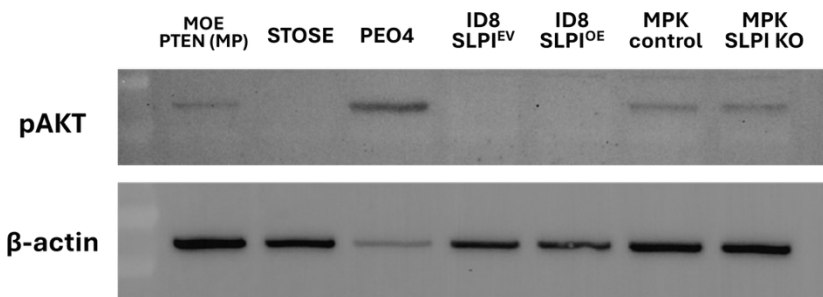


Figure 21. Baseline pAKT levels in control and SLPI-modified murine ovarian cancer cell lines.

Western blot showing protein levels of pAKT and β-actin in various cell lines (n = 1).

3.4.5.2. Adhesion Panel

We performed an RNA sequencing analysis to investigate differential expression of cellular adhesion genes between the SLPI-modified cell lines and their relevant controls to explore possible explanations for the differences we observed in spheroid formation. Spheroid formation is

generally initiated by aggregation of single cells mediated by ECM fibers⁸⁹. This is followed by an increase in cadherin-cadherin binding between cells that creates strong cell-to-cell adhesion⁸⁹. We hypothesized that MPK *Slpi*^{-/-} cells produce more ECM proteins, allowing them to form more spheroids initially but lack factors that would allow them to survive and grow in suspension. Unfortunately, issues with sample consistency arose in the ID8 and KPCA models, making MPK the only model with trustworthy data for this analysis. It is important to note that interpretation needs to be taken lightly, as these are based on transcript levels, not protein levels or functional readouts. Additionally, RNA for this analysis was extracted from cells attached to an adherent cell culture plate. Therefore, extrapolation of these results to explain SLPI's effect on spheroid formation is hypothesis-based.

ECM-associated genes

Expression of collagens: *Slpi* expression was negatively correlated with the expression of most collagens, significantly with *Colla2*, *Col6a2*, *Col4a2*, *Col6a1*, but was significantly positively correlated with *Col4a3* in MPK (**Figure 22**). The collagen transcripts in these cells are relatively high (100-170 transcripts per million (TPM)), so the changes are reliable. While culturing MPK *Slpi*^{-/-} cells in attachment cultures, we noticed that they are much more adherent to the plate than the MPK Control. This could be explained by the increased production of collagens, which would create stronger and more abundant interactions between the cancer cells and the plate. This may also explain the sudden drop in cell count in MPK *Slpi*^{-/-} at 96 hours when measuring proliferation (**Figure 10B**), which could be reflective of difficulty in retrieving all the MPK *Slpi*^{-/-} cells adhered to the plate. Since cancer cells need ECM fibers for initial aggregation

of spheroid formation, we hypothesize that the increase in collagen transcripts in MPK *Slpi*^{-/-} cells allows them to form abundant spheroids in suspension.

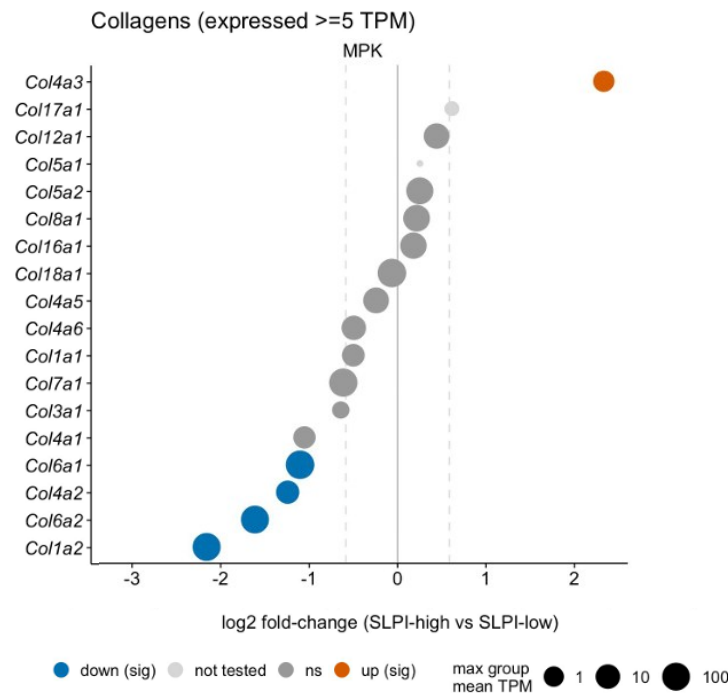


Figure 22. Bubble plot of the collagen-associated genes that are negatively and positively correlated with *Slpi* in the MPK cell line.

Dots on the right of the solid line are positively correlated with SLPI expression (orange), while dots on the left are negatively correlated with SLPI expression (blue).

ECM remodeling - MMP/TIMP balance and crosslinking: While SLPI has anti-proteolytic properties, this analysis showed that the MMP:TIMP ratio does not shift with *Slpi*. However, there are specific genes that increase with *Slpi* expression, such as *Loxl2*, *Adam12*, and *Bmp1*, while other ADAMTS proteases go down with *Slpi*, *Adamts9* and *Adamts7* (**Figure 23**). Lox (lysyl oxidase) enzymes and *Bmp1* (bone morphogenetic protein-1) are generally associated with cross-linking, while ADAMTS are metalloproteases that are responsible for remodelling ECM⁹⁰⁻⁹². The increase in *Loxl2* and *Bmp1* with *Slpi* indicates that there may be an increase in cross-linking, increasing ECM stiffness, and decreasing turnover of ECM components. We

therefore hypothesize that the MPK *Slpi*^{-/-} cell lines would create a more dynamic and flexible ECM, allowing for more effective metastasis formation in vivo than the MPK Control cells, which is consistent with the observed increase in metastases when the cells are injected in vivo (Figure 16B).

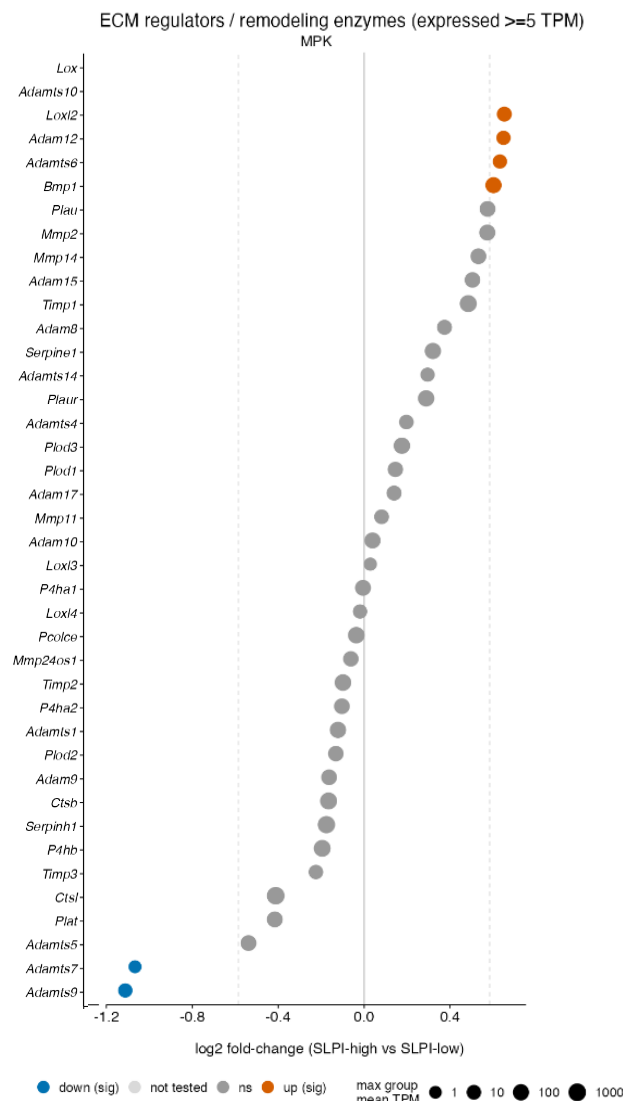


Figure 23. Bubble plot of the ECM regulators/remodelling enzyme-associated genes that are negatively and positively correlated with *Slpi* in the MPK cell line. Dots on the right of the solid line are positively correlated with SLPI expression (orange), while dots on the left are negatively correlated with SLPI expression (blue).

Cell-cell junction genes

An increase in expression of the desmosomal genes, *Pkp1* and *Jup*, with *Slpi* in the MPK model was observed (**Figure 24**). *Pkp1* codes for the plakophilin-1 protein, while *Jup* codes for the plakoglobin protein⁹³. These proteins are both part of the desmosomal plaque, which joins desmosomal cadherins to the keratins of the intermediate filament network of cells, making these proteins extremely important for desmosome structure⁹⁴. Desmosomes are junctions between mostly epithelial cells that are important for cell adhesion and cell–cell communication⁹⁴. We hypothesize that, due to lower expression of these genes in MPK *Slpi*^{-/-} cells, they are not able to form larger spheroids with robust cell-cell adhesion after initial collagen-mediated aggregation. In fact, desmosomal component genes, including *Pkp1* and *Jup*, have been shown to be important in spheroid formation. Specifically, desmoplakin (*Dsp*) and *Pkp1* were upregulated in ID8 cells that could form spheroids and which lost that capacity after KD of *Dsp*⁹⁵. Additionally, reduction of plakoglobin leads to a loss of spheroid formation or loss of spheroid compaction in breast cancer cells⁹⁶.

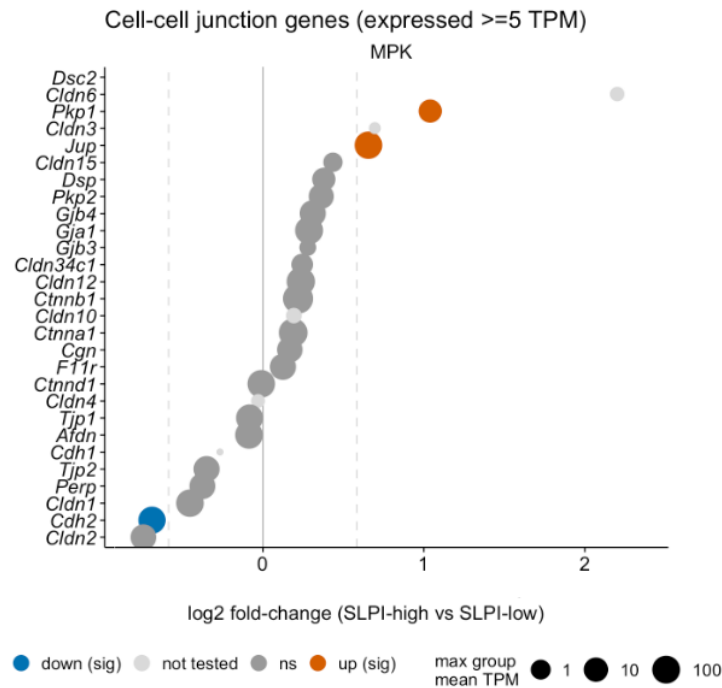


Figure 24. Bubble plot of the cell-cell junction and focal-adhesion signaling-associated genes that are negatively and positively correlated with *Slpi* in the MPK and KPCA cell lines.

Dots on the right of the solid line are positively correlated with SLPI expression (orange), while dots on the left are negatively correlated with SLPI expression (blue).

Cell-adhesion molecules (CAMs)

Lastly, the RNA-seq analysis showed a decrease in most of the cell-adhesion molecule genes, specifically the Immunoglobulin-superfamily CAMs (IgSF CAMs) with *Slpi*. The most significantly negatively correlated genes were *Vcam1* and *Icam1* (**Figure 25**). While *Icam1*'s role in ovarian cancer is debated, it is known to guide immune cells to sites of inflammation⁹⁷⁻⁹⁹. One study showed that ICAM1 expression was correlated with immune cell infiltration, including CD4+ T cells, M1 macrophages, dendritic resting cells, and neutrophils, in multiple cancer types⁹⁷. *Vcam1* is normally expressed on endothelial cells that bind and guide leukocytes to sites of inflammation¹⁰⁰. When expressed on cancer cells, it has been associated with increased immune infiltration of tumors in gastric cancer, but it is generally thought to be tumorigenic in many cancers¹⁰¹. These genes are very important for immune cell guidance and adhesion, suggesting that SLPI may contribute to an immunosuppressive environment through regulation of these IgSF CAMs. We hypothesize that these changes in expression contribute to SLPI's modulation of the immune response in the TME, which we aimed to characterize next.

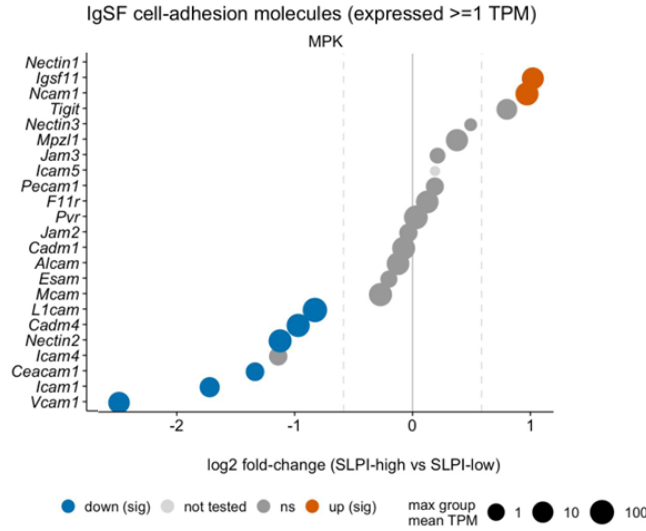


Figure 25. Bubble plot of the cell-adhesion molecules-associated genes that are negatively and positively correlated with *Slpi* in the MPK and KPCA cell lines.
Dots on the right of the solid line are positively correlated with SLPI expression (orange), while dots on the left are negatively correlated with SLPI expression (blue).

3.5. SLPI's effect on survival and modulation of the tumor microenvironment in the ID8 model

3.5.1. Survival of mice bearing SLPI-modified ID8 *Trp53*^{-/-} tumors

To determine the effect of SLPI on survival and tumor progression in a different mouse model, we performed intrabursal injections of ID8 *Slpi*^{EV} and ID8 *Slpi*^{OE}. As we did with the MPK model, once the mice reached humane endpoint, they were euthanized, and various characteristics of tumor progression such as number of days elapsed since injection, overall body weight, tumor weight, ascites volume, spleen weight, and total metastases weight were recorded. Endpoint in this model was generally due to debilitating accumulation of ascites fluid, which made the mice hunch and made walking difficult. Overexpression of *Slpi* in this model significantly shortened median survival compared to its control (103 days for ID8 *Slpi*^{EV} and 85 days for ID8 *Slpi*^{OE}, p-value = 0.0195) (**Figure 26A**). Consistent with the MPK model, all parameters measured at endpoint were

not significantly different between the *Slpi* -overexpressing cell line and its control except for total metastasis weight (**Figure 26B, C, D, E, F**). ID8 *Slpi*^{OE} mice had significantly lower total metastasis weight compared to their control (**Figure 26B**). In this model, the reduced metastases suggest that they are not contributing to the difference in survival, which was shorter. Since the shortened survival of the ID8 *Slpi*^{OE} cannot be explained by increased metastatic burden or tumor weight at endpoint, we hypothesized that SLPI modulates the immune composition of the tumor microenvironment to become tumor-promoting and immunosuppressive.

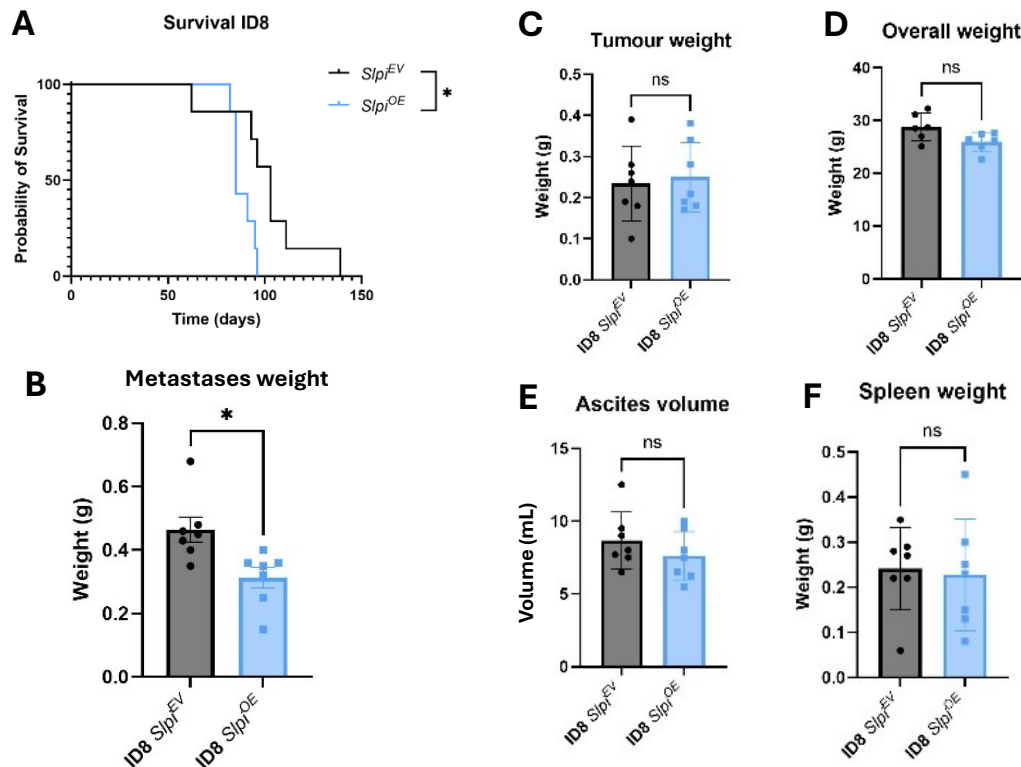


Figure 26. SLPI overexpression shortens in vivo survival and is associated with decreased metastases weight in the ID8 model.

(A) Kaplan-Meier plot showing the survival of SLPI-modified ID8 *Trp53*^{-/-} tumor-bearing mice. (B) Metastases weight, (C) tumor weight, (D) overall weight, (E) ascites volume and (F) spleen weight at humane endpoint. Statistical analysis was done using a Logrank (Mantel-Cox) test for (A) and an unpaired t-test (n=6-7) for (B-F), mean +/- SEM. **p ≤ 0.01.

3.5.2. Characterization of the immune composition of the tumor microenvironment of ID8 *Trp53*^{-/-} SLPI-modified tumors

To investigate the immune composition of tumors with and without *Slpi* overexpression, we generated tumors with ID8 *Slpi*^{EV} and ID8 *Slpi*^{OE} cells by injecting them intrabursally into syngeneic mice to give rise to orthotopic tumors. Tumors, peritoneal wash, and lymph nodes were collected at an estimated 6 days before the known median humane endpoint of ID8 *Slpi*^{OE} mice and analyzed by flow cytometry. At the time of collection, there was no significant difference in tumor weight (mean tumor weight: 0.1817 g for ID8 *Slpi*^{EV} and 0.2520 g for ID8 *Slpi*^{OE}).

Lymphoid cells in tumors: We did not observe any significant differences in the count of total leukocytes between *Slpi*-overexpressing and control tumors, nor any differences in cell counts and frequency of leukocytes of total T cells (CD3+), CD4+ T cells, B cells (CD19+), NK cells (NK1.1+DX5+), ILC2 (ST2+GATA3+), and cell count of cytotoxic T cells (CD8+) (**Figure 27**). The only exception was the frequency of CD8+ T cells, which was lower in the ID8 *Slpi*^{OE} group compared to control tumors, suggesting a reduction in anti-tumor cytotoxicity in the presence of SLPI (p-value = 0.0404) (**Figure 27**). In the UMAP representations, there are, visually, no notable shifts in the CD4+ T cell population, the CD8+ T cell population, and the NK cell population (**Figure 28 and 29**). The lack of significant differences was validated by quantification of the frequency of the following markers: GATA3, ST2, KLRG1, Ki67, CD62L, CD44, CD25, iCOS, CD103, PD-1, CTLA-4, CD69, TIGIT, and PD-L1. None of these markers in the CD4, CD8, and NK cell populations were significantly different between the ID8 *Slpi*^{OE} and ID8 *Slpi*^{EV} groups, with one exception. This phenotypic difference was observed in CD8+ T cells, in which there was a trend toward higher levels of the CD44 marker in the ID8 *Slpi*^{OE} tumors (**Figure 28C**), suggesting more antigen-activated T cells and memory T cells¹⁰². Visually, the UMAP of the B cells showed

a shift in one subset of the B cell (population to the left) (**Figure 30A**). However, none of the markers we used to characterize the B cell populations were significantly different when comparing the tumors; therefore, this shift involves unknown markers (**Figure 30B**).

Tumors – Leukocytes (Lymphoid populations)

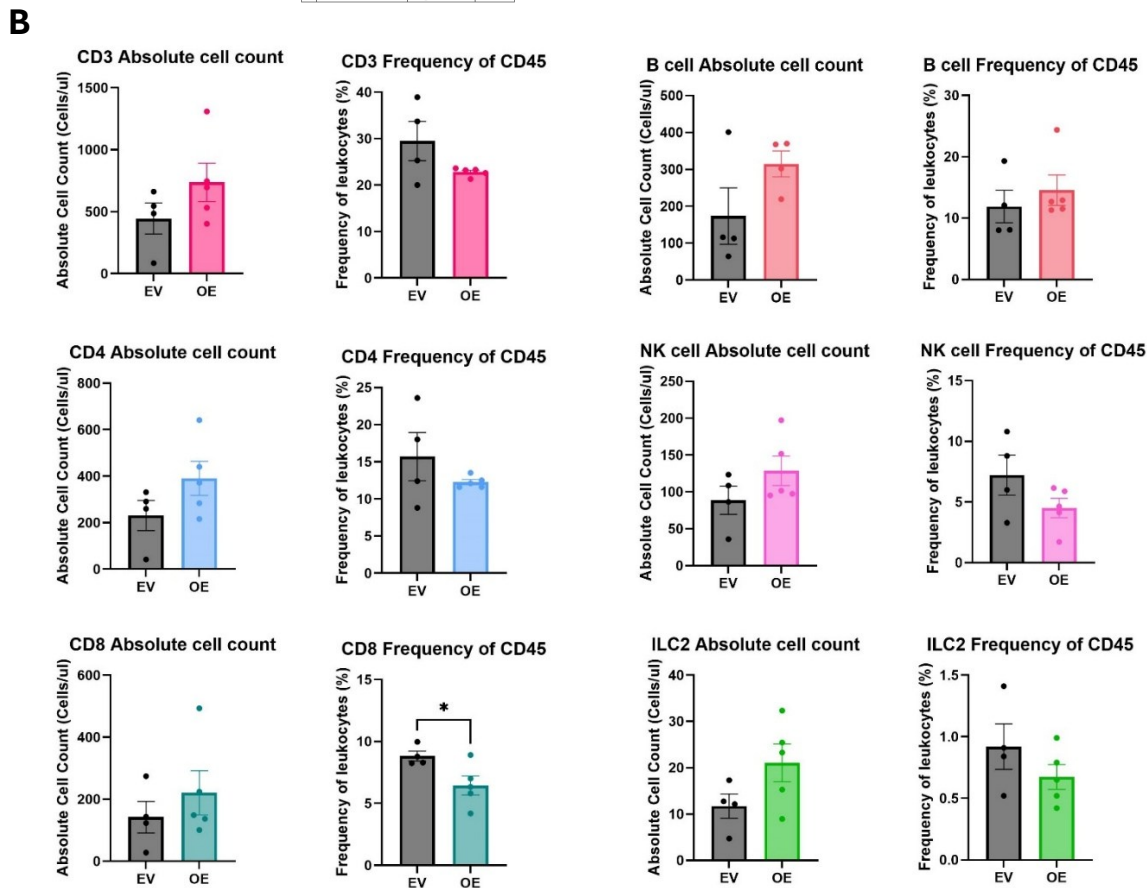
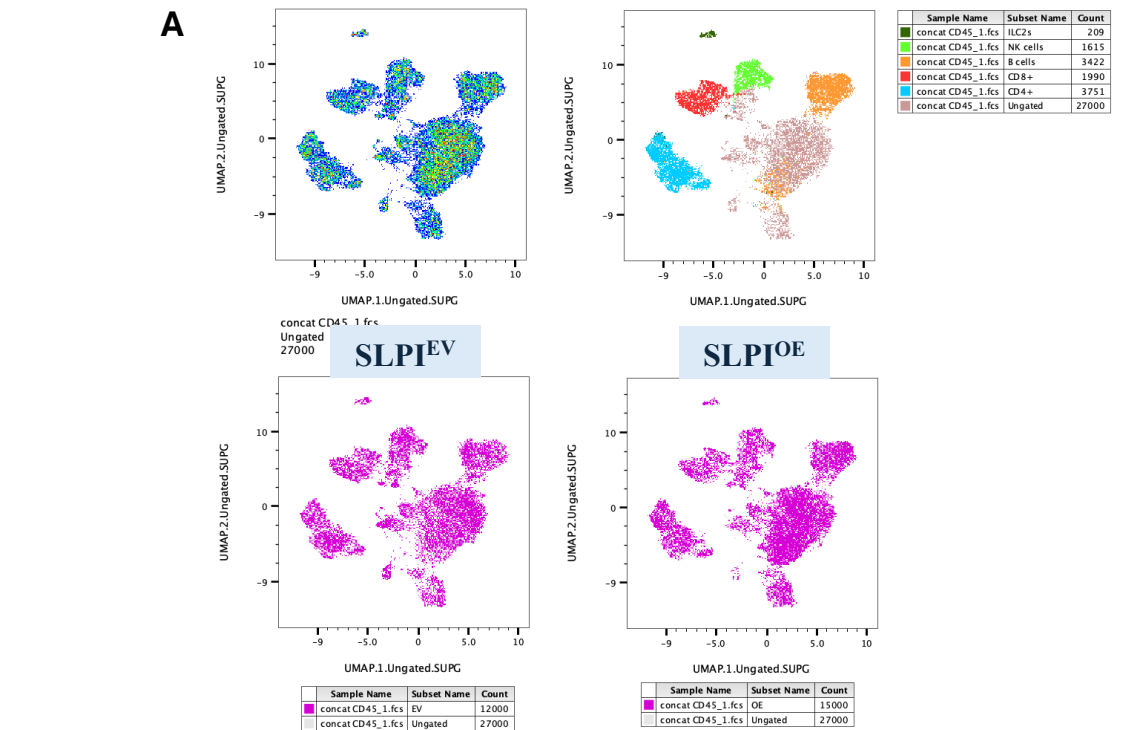
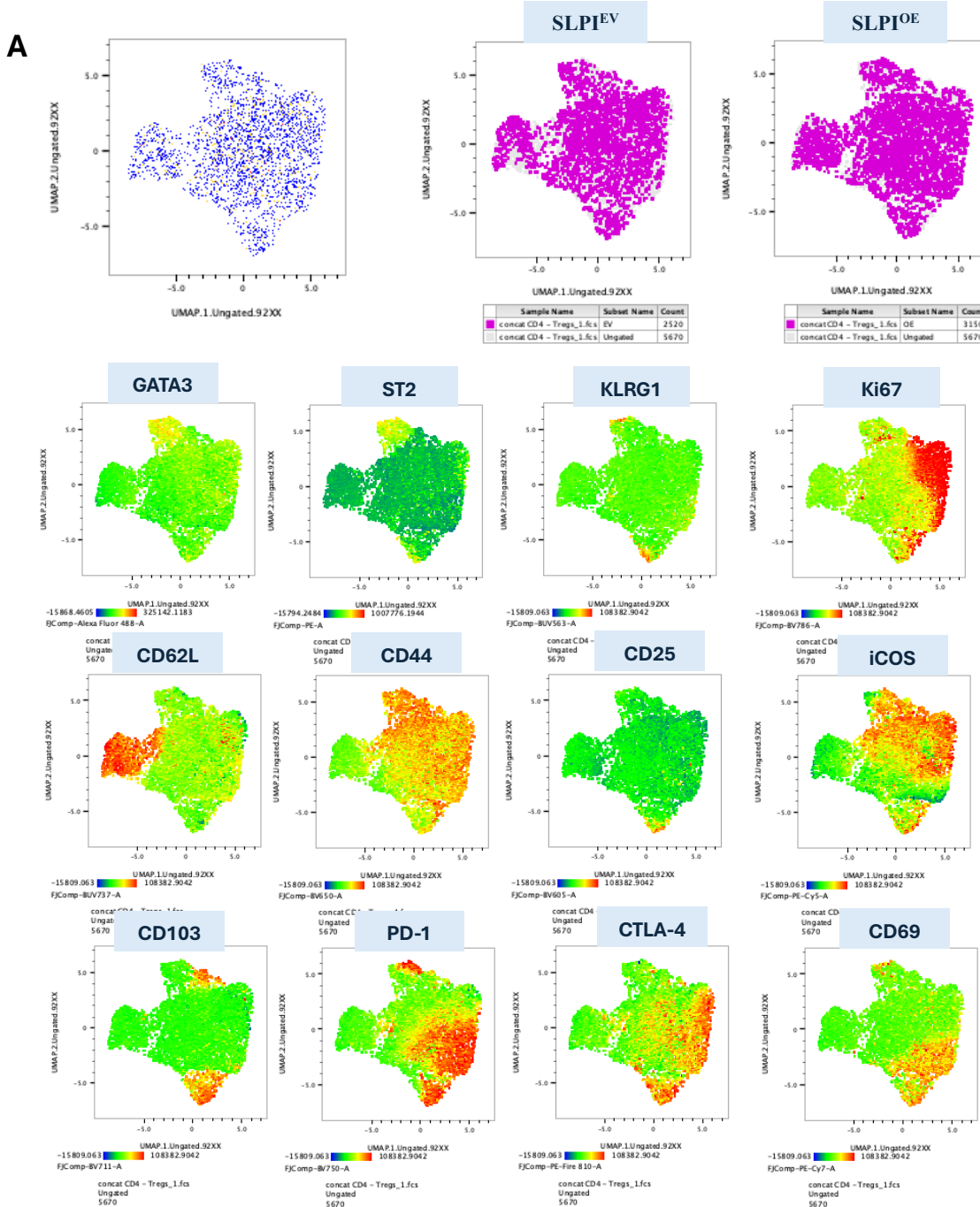


Figure 27. Distribution of lymphoid immune cells in the tumors of mice injected with ID8 *Trp53*^{-/-} SLPI-modified cells.

(A) UMAP of 27000 cells representing lymphoid populations in the tumor with distributions separated by immune cell type and by sample group, (B) Counts and frequencies of lymphoid immune cell types in the tumors. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=4-5), *p ≤ 0.05.

Tumors – CD4 population



B



C

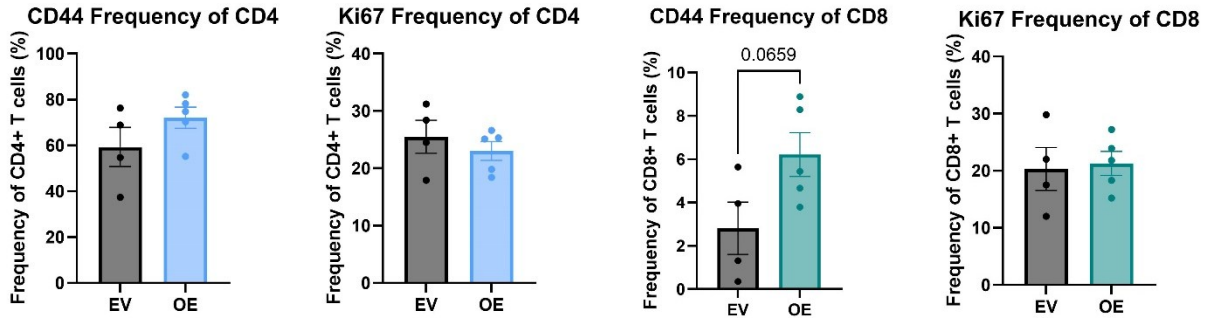
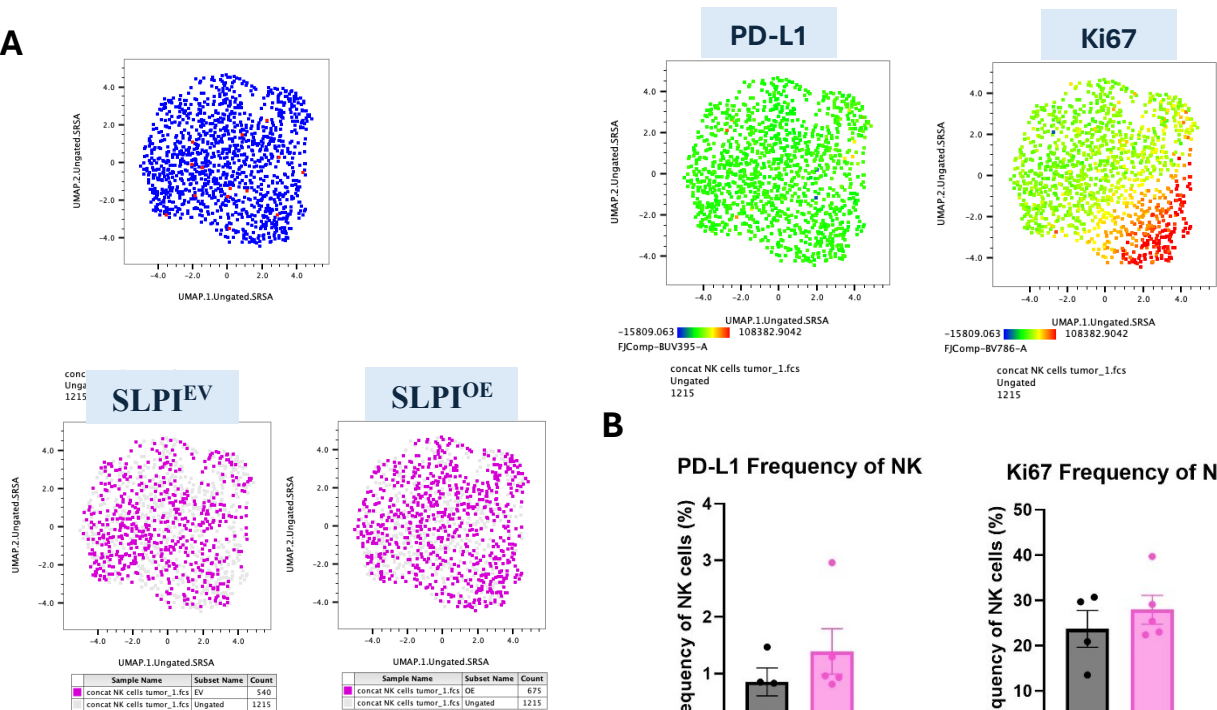


Figure 28. Characterization of the CD4+ and CD8+ T cell populations in the tumors of mice injected with SLPI-modified ID8 *Trp53*^{-/-} cells.

(A) UMAP of 5670 cells representing the CD4+ T cell population in the ID8 EV and ID8 OE tumors with spatial expression of each marker, (B) UMAP of 6291 cells representing CD8+ T cell population in the ID8 EV and ID8 OE tumors with spatial expression of each marker, (C) Frequency of CD4 and CD8 cells that are CD44 and Ki67 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=4-5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Tumor – NK cell population

A



B

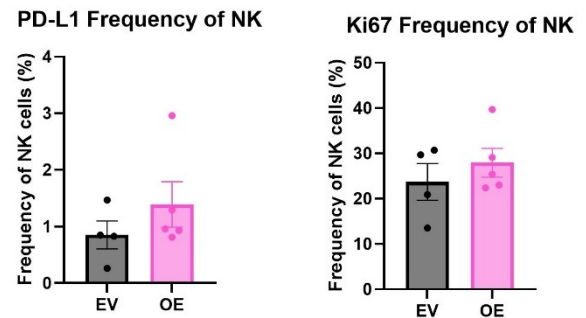
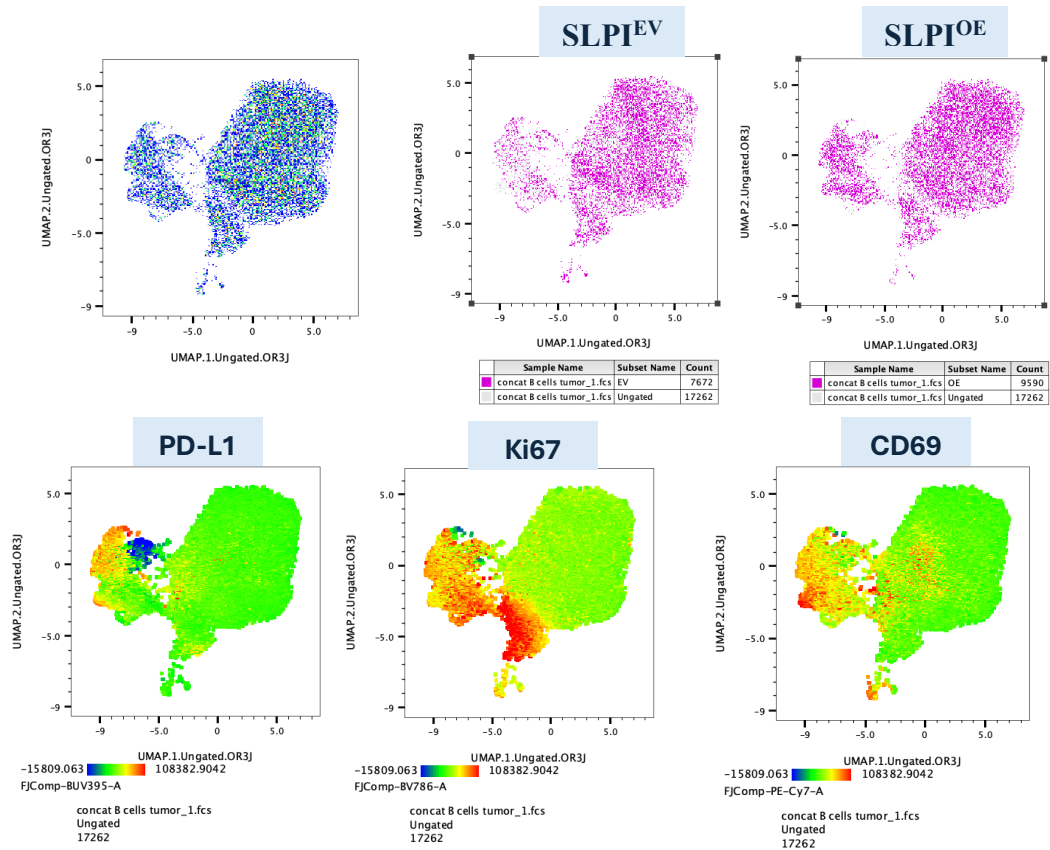


Figure 29. Characterization of the NK cell population in the tumors of mice injected with SLPI-modified ID8 Trp53^{-/-} cells.

(A) UMAP of 1215 cells representing the NK cell population in the ID8 EV and ID8 OE tumors with spatial expression of each marker that the cells were stained with, (B) Frequency of B cells that are PD-L1, and Ki67 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=4-5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Tumors – B cell population

A



B

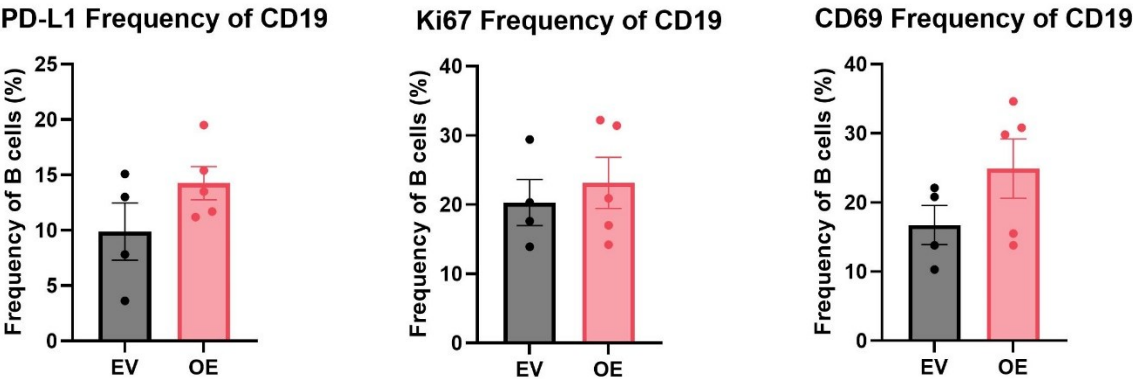
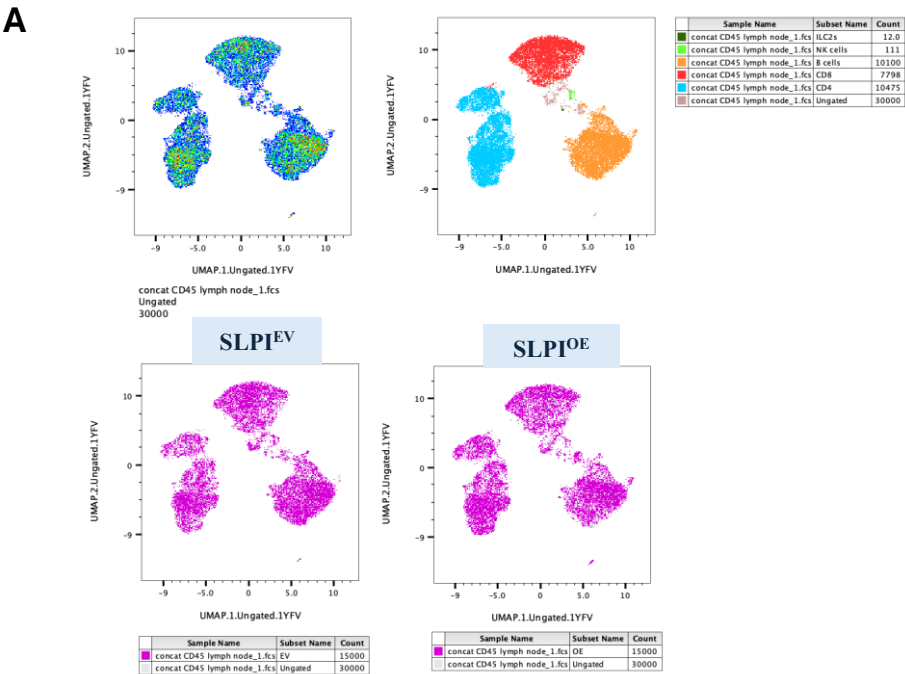


Figure 30. Characterization of the B cell population in the tumors of mice injected with SLPI-modified ID8 *Trp53*^{-/-} cells.

(A) UMAP of 17262 cells representing the B cell population in the ID8 EV and ID8 OE tumors with spatial expression of each marker that the cells were stained with, (B) Frequency of B cells that are PD-L1, Ki67, and CD69 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=4-5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Lymphoid cells in lymph nodes: In the lymph nodes, cell counts and frequencies of total leukocytes were not different for any lymphoid cell population (**Figure 31**). Additionally, there were no significant differences in any of the markers measured. In lymph nodes from mice with ID8 *Slpi*^{OE} tumors, CD4⁺ T cells were characterized by a trending increase in CD25, a late activation marker of T cells, and there was a trend in CD8⁺ T cells for an increase in CD69, an early activation marker of T cells (**Figure 32**). There is a slight visual shift in the UMAP of the B cells, but it does not correspond with significant differences in our measured markers, suggesting an uncharacterized shift (**Figure 33**).

Lymph nodes – Leukocytes (Lymphoid populations)



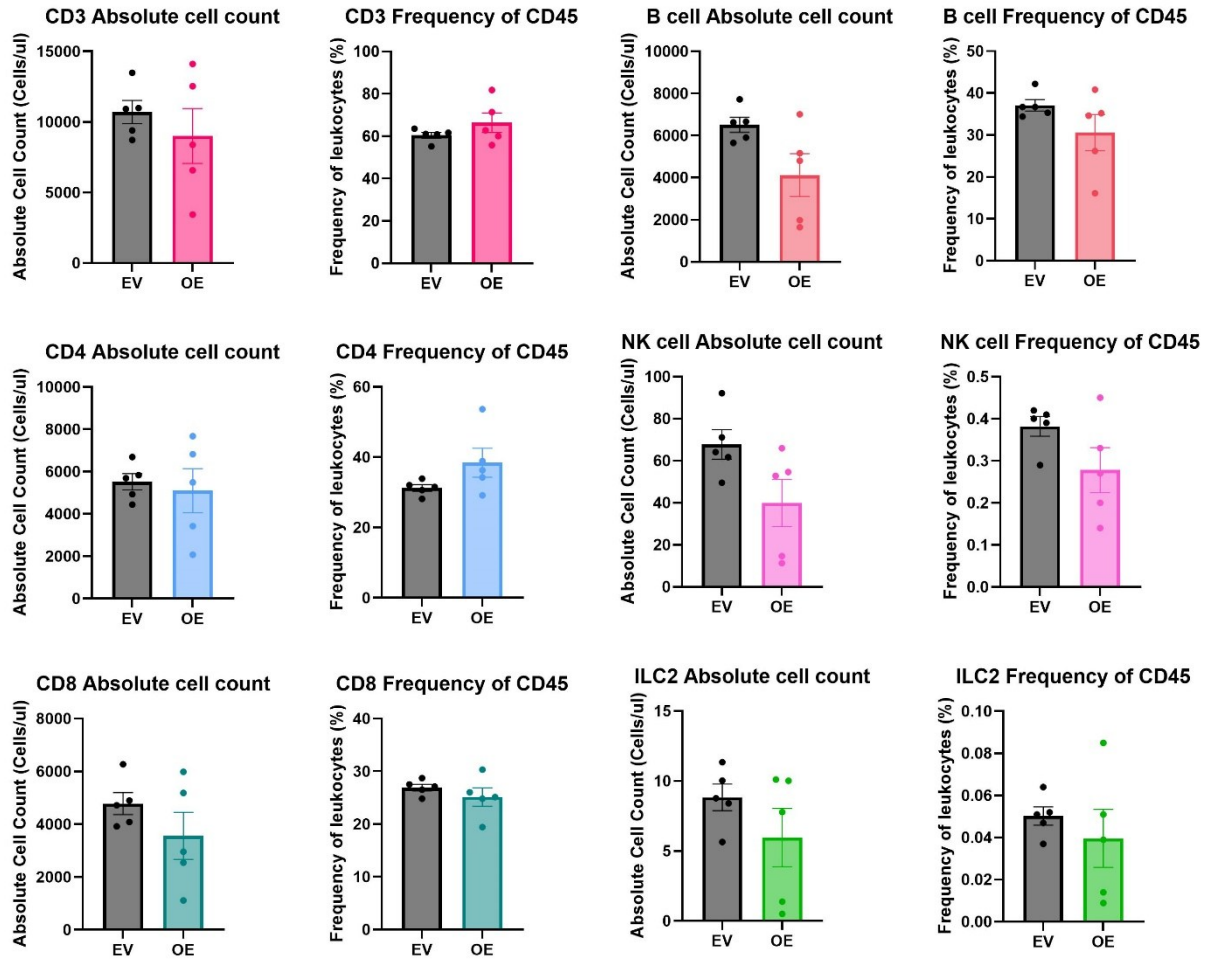
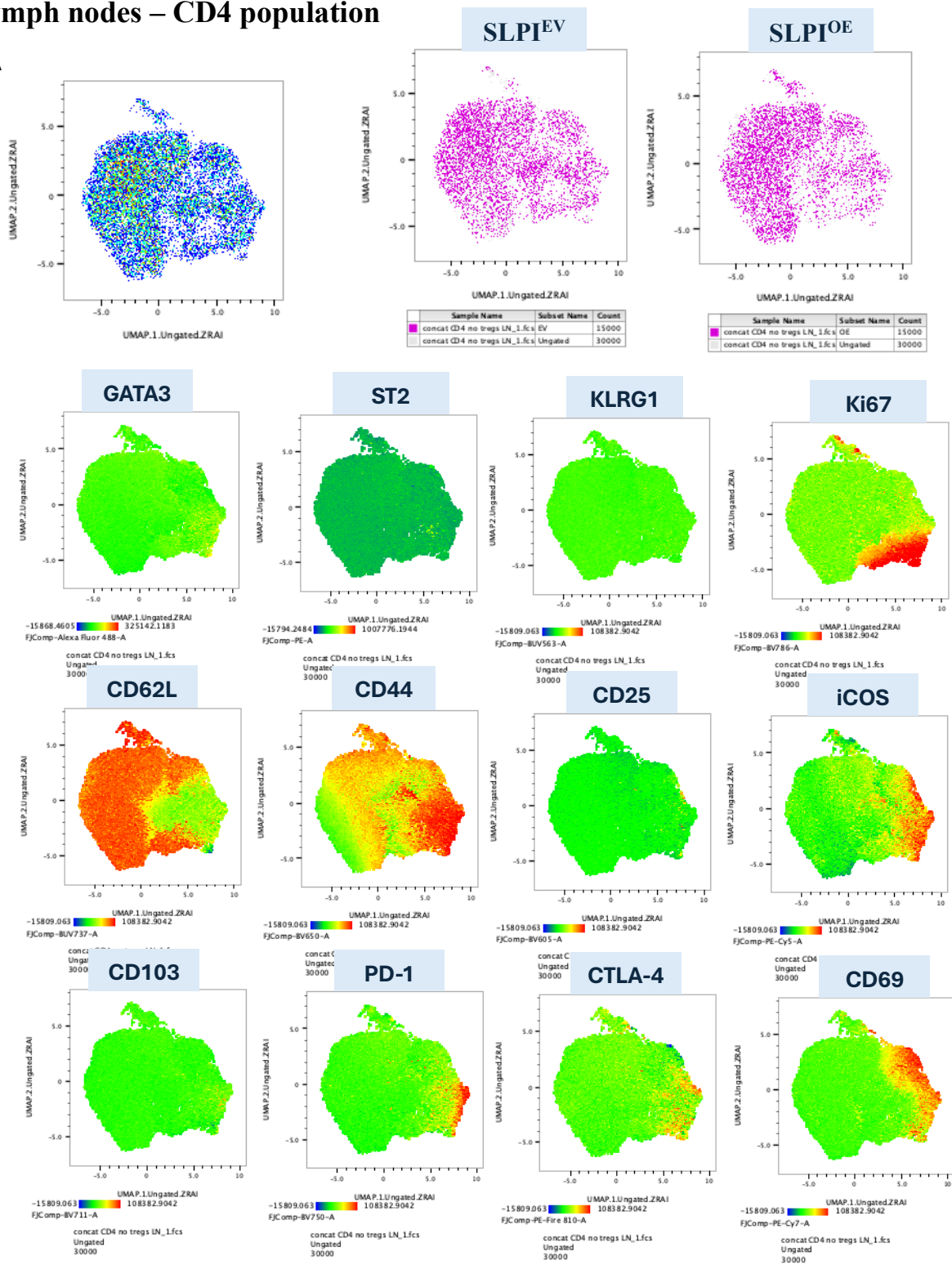
B

Figure 31. Distribution of lymphoid immune cells in the lymph nodes of mice injected with ID8 Trp53^{-/-} SLPI-modified cells.

(A) UMAP of 30000 cells representing lymphoid populations in the lymph nodes with distributions separated by immune cell type and by sample group, (B) Counts and frequencies of lymphoid immune cell types in the lymph nodes. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5).

Lymph nodes – CD4 population

A



B



C

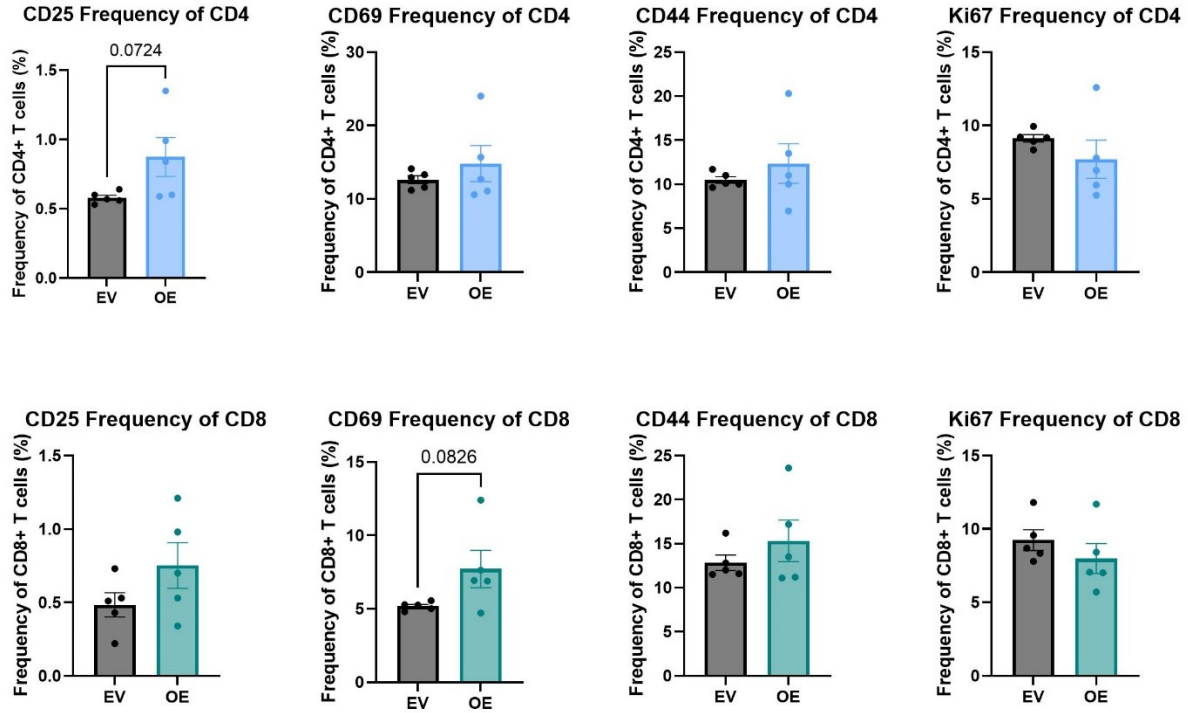


Figure 32. Characterization of the CD4+ and CD8+ T cell populations in the lymph nodes of mice injected with SLPI-modified ID8 Trp53^{-/-} cells.

(A) UMAP of 30000 cells representing CD4+ T cell population in the ID8 EV and ID8 OE lymph nodes with spatial expression of each marker that the cells were stained with, (B) UMAP of 30000 cells representing CD8+ T cell population in the ID8 EV and ID8 OE lymph nodes with spatial expression of each marker that the cells were stained with, (C) Frequency of CD4 and CD8 cells that are CD25, CD69, CD44 and Ki67 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Lymph nodes – B cell population

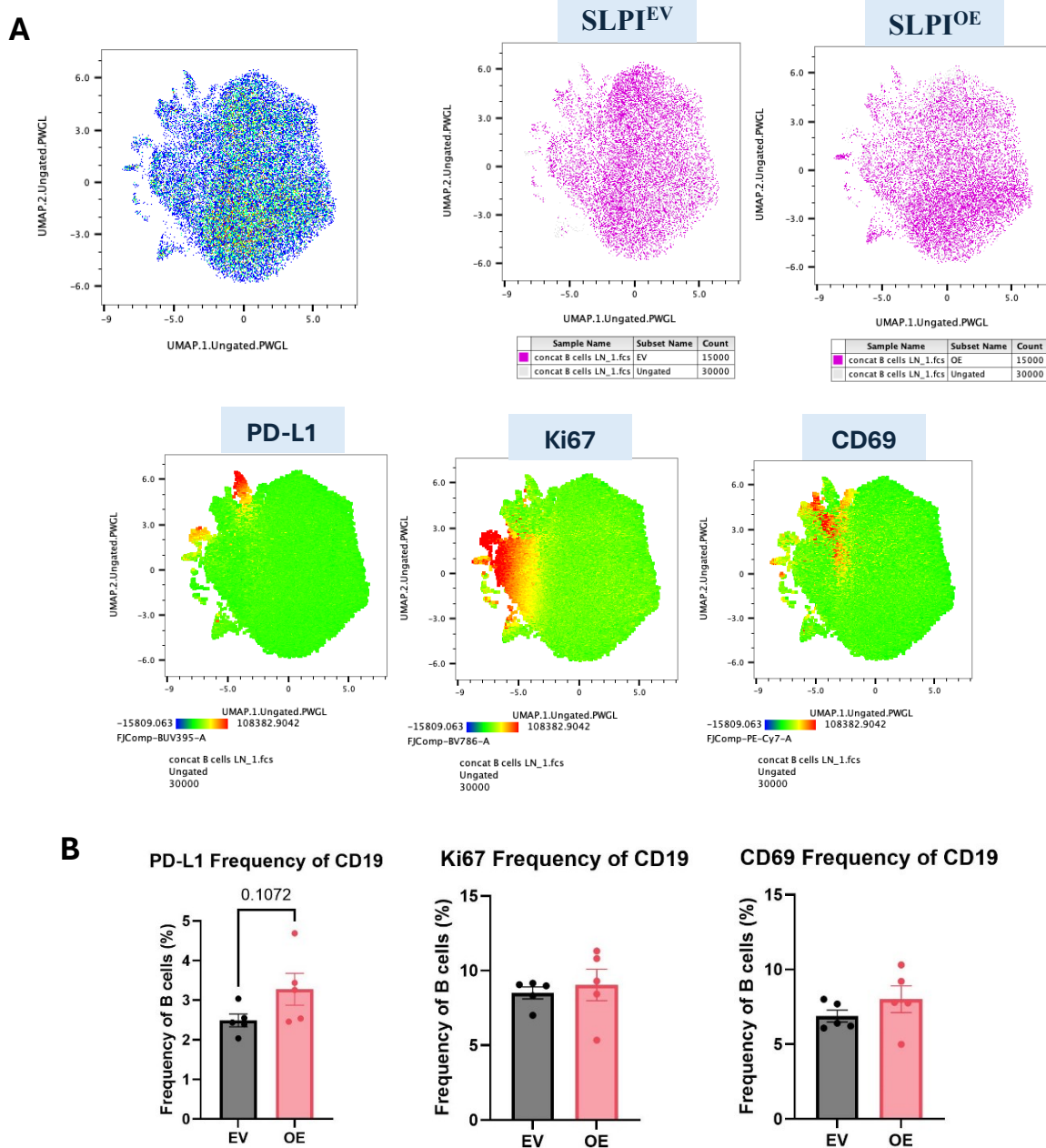


Figure 33. Characterization of the B cell population in the lymph nodes of mice injected with SLPI-modified ID8 Trp53^{-/-} cells

(A) UMAP of 30000 cells representing the B cell population in the ID8 EV and ID8 OE lymph nodes with spatial expression of each marker that the cells were stained with, (B) Frequency of B cells that are PD-L1, Ki67, and CD69 positive. EV: ID8 Trp53^{-/-} Slpi^{EV}; OE: ID8 Trp53^{-/-} Slpi^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Lymphoid cells in the peritoneal wash: As with other tissues, there were no significant differences in the cell count or frequency of total leukocytes for any of the lymphoid populations in the peritoneal wash (**Figure 34A, B**). There were, however, a few shifts in the CD4⁺ and CD8⁺ populations. CD4⁺ T cells in the ID8 *Slpi*^{OE} group had decreased expression of CD44 and of Th2 cell markers, i.e., GATA3 and ST2, the receptor for IL-33 (**Figure 35C**). CD8⁺ T cells in the ID8 *Slpi*^{OE} group had decreased Ki-67, a marker of proliferation, and CTLA-4, an inhibitory receptor on T cells (**Figure 35C**). Additionally, there was a decrease in PD-L1 expression in B cells in the ID8 *Slpi*^{OE} group (**Figure 36B**). Visually, there were dramatic differences in the B cells and NK cells when comparing both groups, but these did not correlate with the markers we measured, suggesting that there are phenotypic changes in these populations within the peritoneal cavity in ID8 *Slpi*^{OE} tumors that have yet to be characterized (**Figure 36A and 37A**).

Overall, the analysis of the lymphoid cells suggests no dramatic differences in abundance of any cell type, except for a reduction in CD8⁺ T cells in tumors that overexpressed *Slpi*, as well as some indications of increased activation of those CD8⁺ T cells (CD44, CD69, and reduced CTLA-4).

Peritoneal wash – Leukocytes (Lymphoid populations)

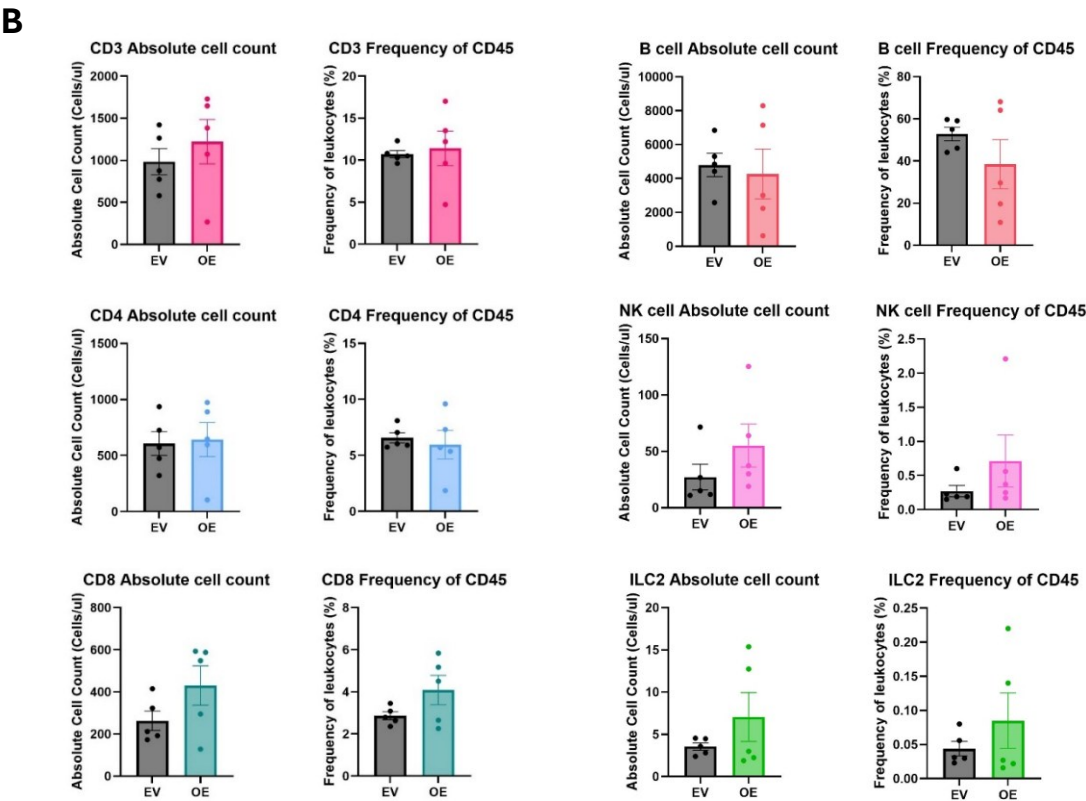
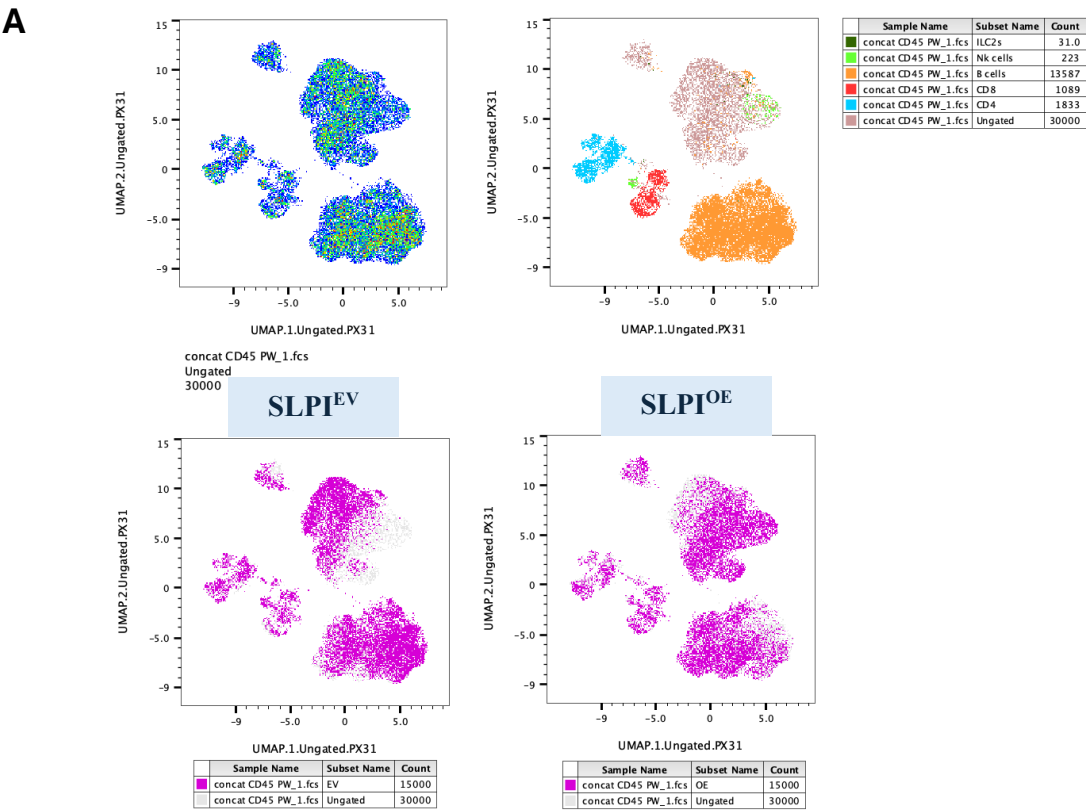
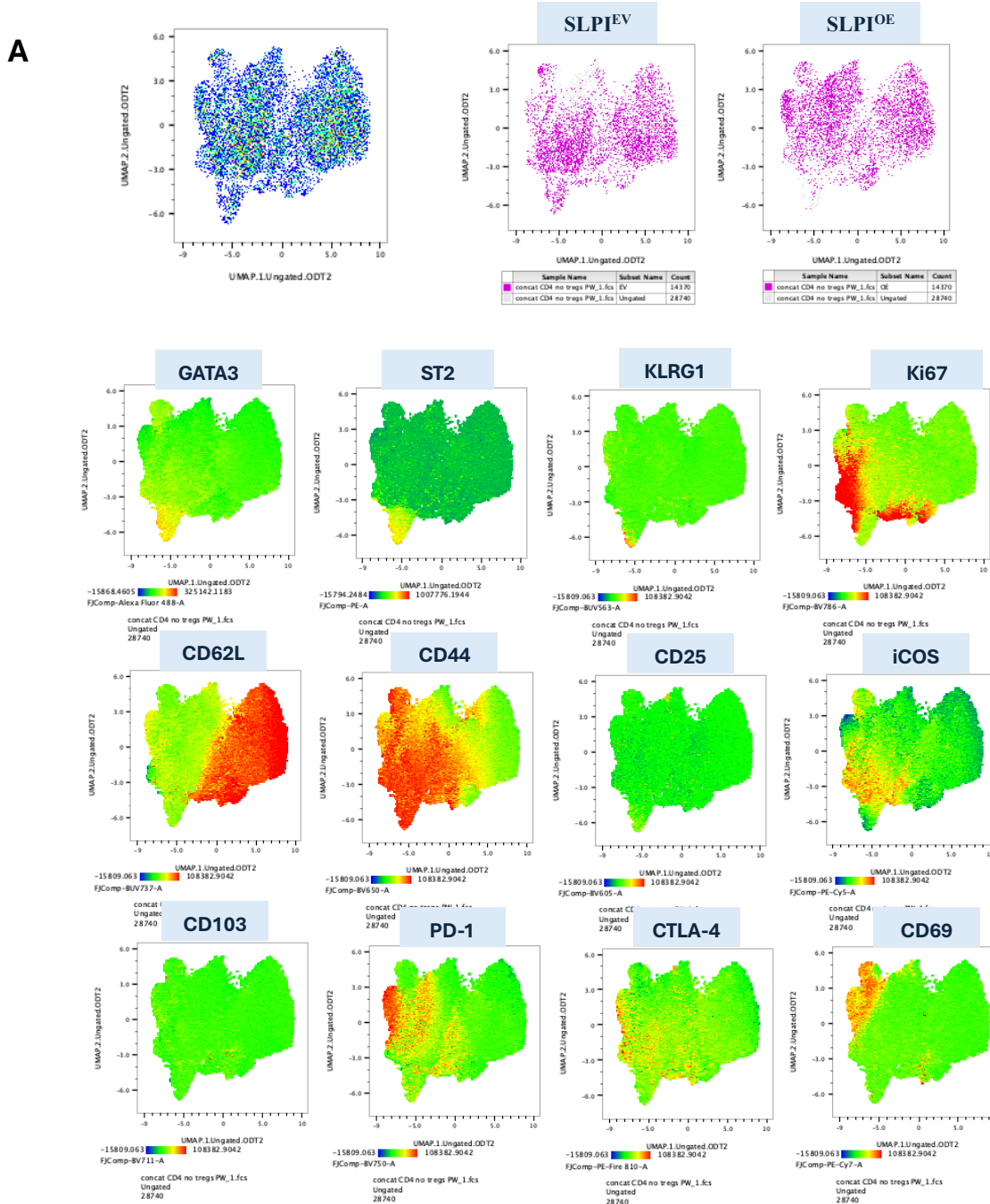


Figure 34. Distribution of lymphoid immune cells in the peritoneal wash of mice injected with ID8 Trp53^{-/-} SLPI-modified cells.

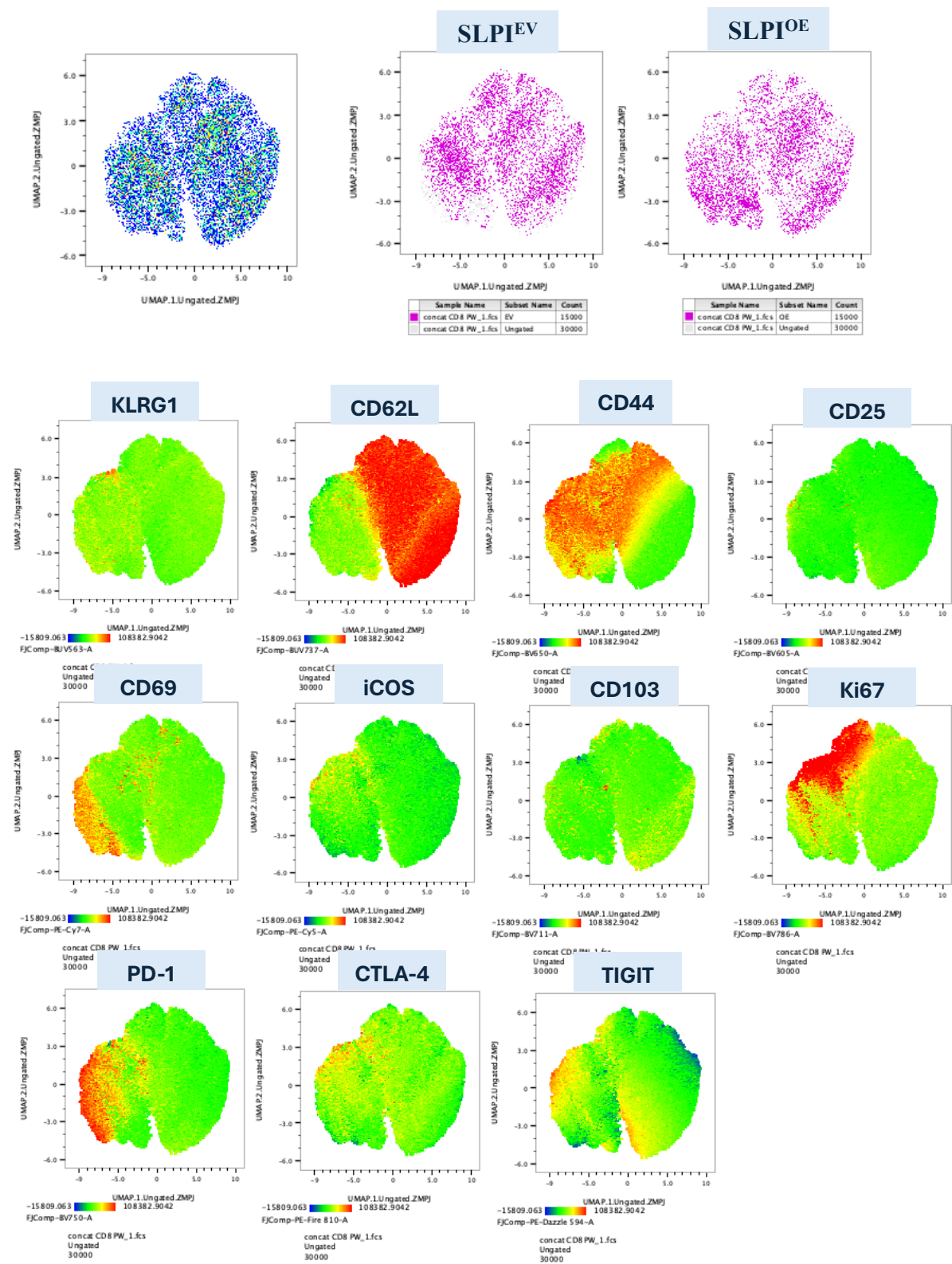
(A) UMAP of 30000 cells representing lymphoid populations in the peritoneal wash with distributions separated by immune cell type and by sample group, (B) Counts and frequencies of lymphoid immune cell types in the peritoneal wash. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5)

Peritoneal wash – CD4⁺ population



Peritoneal wash – CD8+ population

B



C

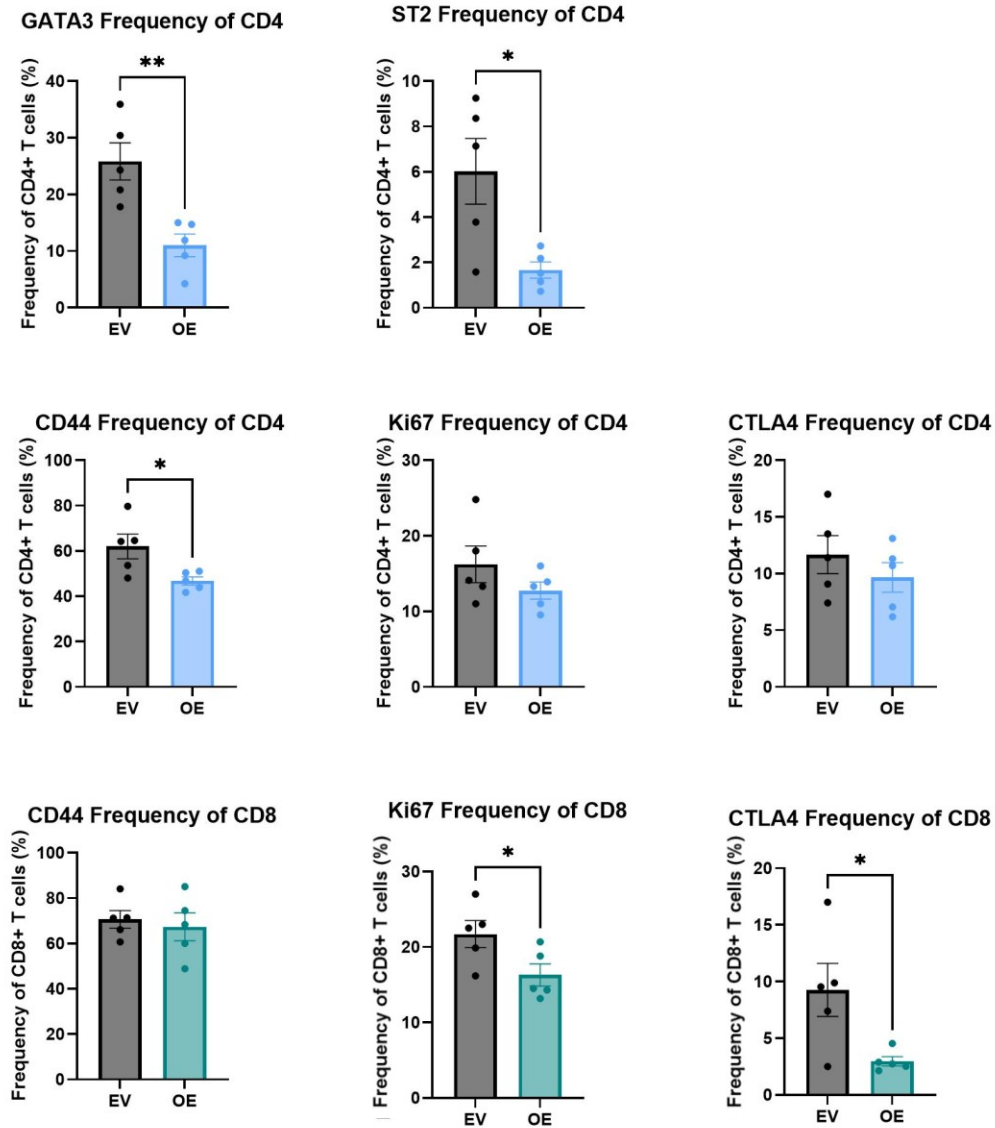


Figure 35. Characterization of the CD4+ and CD8+ T cell populations in the peritoneal wash of mice injected with SLPI-modified ID8 *Trp53*^{-/-} cells.

(A) UMAP of 28740 cells representing CD4+ T cell population in the ID8 EV and ID8 OE peritoneal washes with spatial expression of each marker that the cells were stained with, (B) UMAP of 30000 cells representing CD8+ T cell population in the ID8 EV and ID8 OE peritoneal washes with spatial expression of each marker that the cells were stained with, (C) Frequency of CD4 cells that are GATA3, ST2, CD44, Ki67 and CTLA4 positive and CD8 cells that are CD44, Ki67 and CTLA4 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Peritoneal wash – B cell population

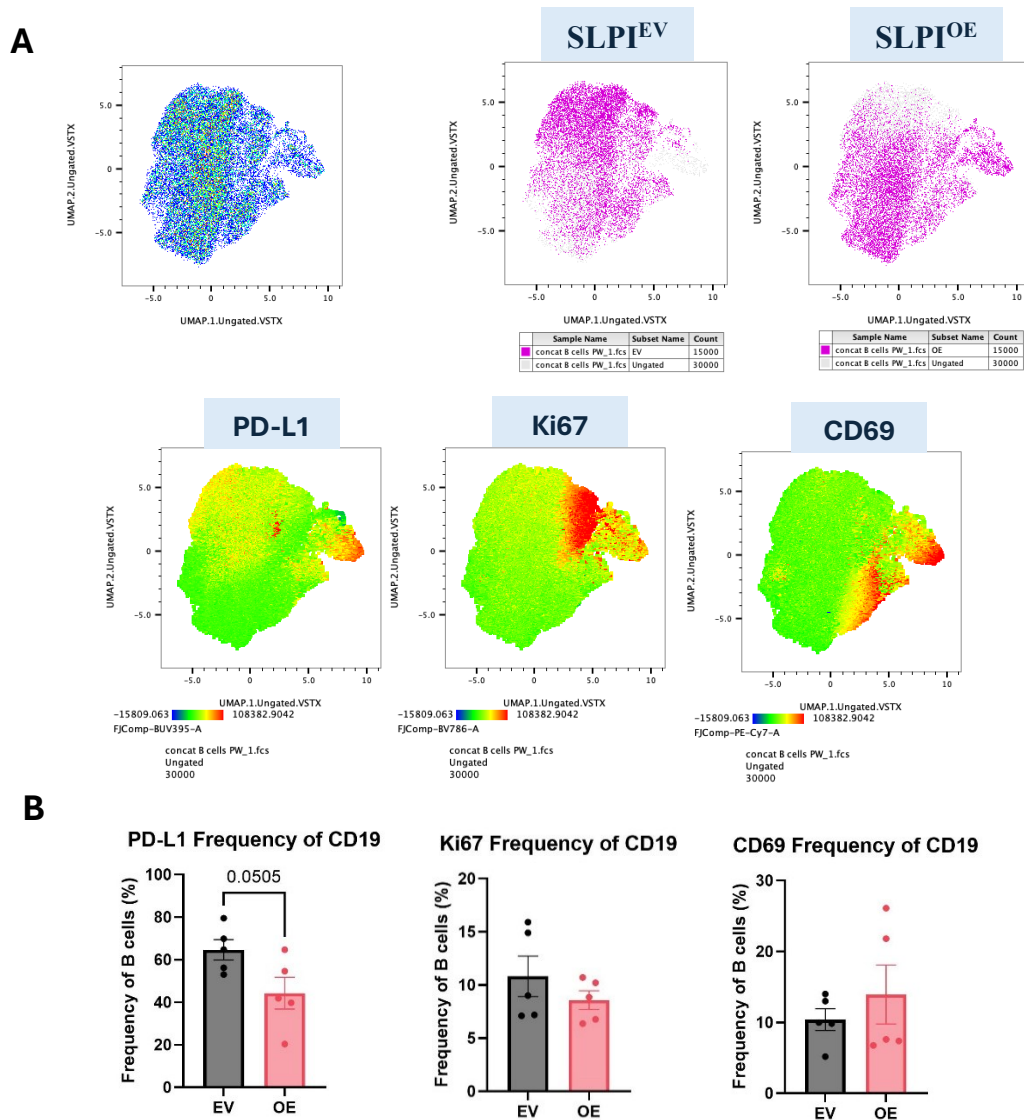


Figure 36. Characterization of the B cell populations in the peritoneal wash of mice injected with SLPI-modified ID8 Trp53^{-/-} cells.

(A) UMAP of 30000 cells representing the B cell population in the ID8 EV and ID8 OE peritoneal washes with spatial expression of each marker that the cells were stained with, (B) Frequency of B cells that are PD-L1, Ki67, and CD69 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Peritoneal wash – NK cell population

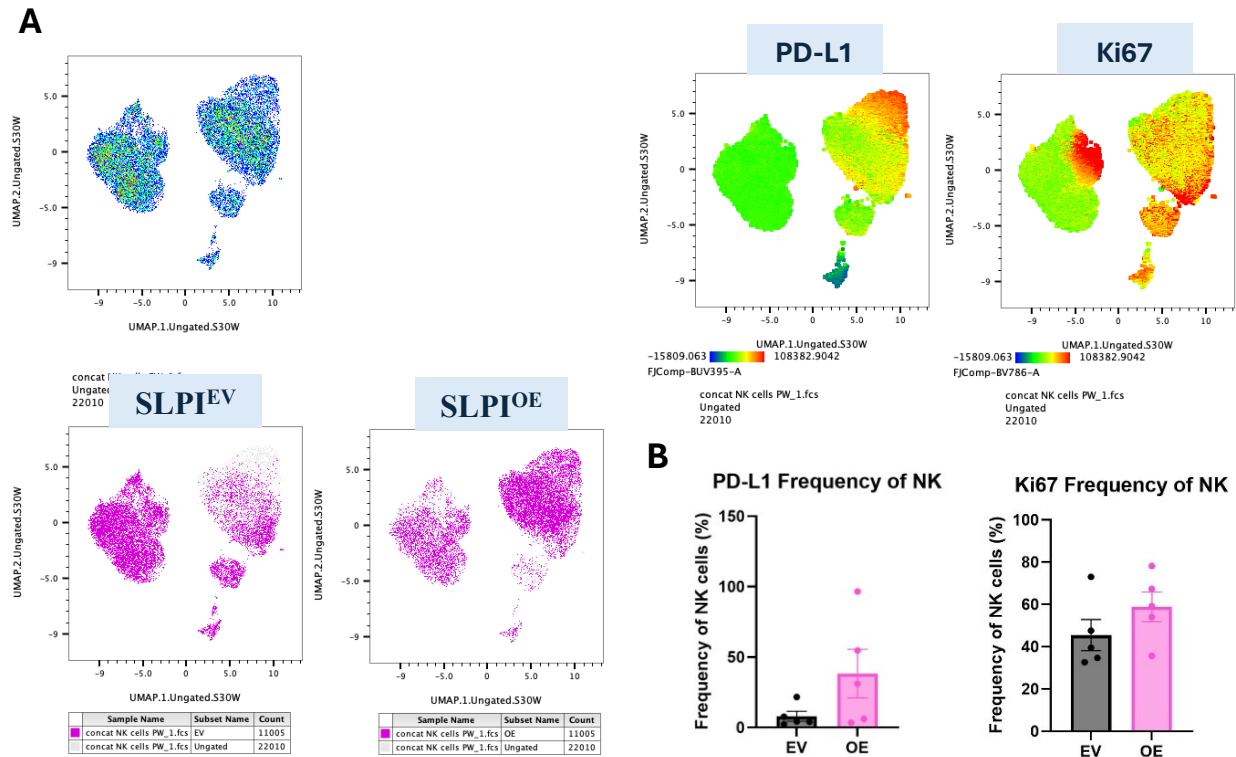


Figure 37. Characterization of the NK cell populations in the peritoneal wash of mice injected with SLPI-modified ID8 Trp53^{-/-} cells.

(A) UMAP of 22010 cells representing the B cell population in the ID8 EV and ID8 OE peritoneal wash with spatial expression of each marker that the cells were stained with, (B) Frequency of B cells that are PD-L1, and Ki67 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Myeloid cells: Much bigger shifts were observed in the myeloid population, with significant differences mostly in the tumors. Tumors with *Slpi* overexpression had significant increases in eosinophil, dendritic cell, and macrophage populations (**Figure 38**). The macrophages in the ID8 *Slpi*^{OE} tumors were characterized by increased Arginase 1, MHC II, and ST2, which potentially alludes to an M2 macrophage polarization (**Figure 39B**). The dendritic cells in the tumors expressed more CCR5, an immune cell migration marker, and the dendritic cells in the lymph nodes were characterized by increased ST2 and a trend toward increased PD-1 in the ID8 *Slpi*^{OE} group (**Figure 39B, 42B**). No other differences were found in the count, frequency, and phenotype of myeloid immune cells in the peritoneal wash and the lymph nodes (**Figure 40, 41**). Overall, this indicates a protumoral shift in the *Slpi*-overexpressing tumors, negatively impacting survival, mainly through an increased abundance of myeloid cells, a shift towards M2 macrophage polarization (more immunosuppressive), and a decrease in the proportion of CD8+ T cells.

Tumor – Leukocytes (Myeloid populations)

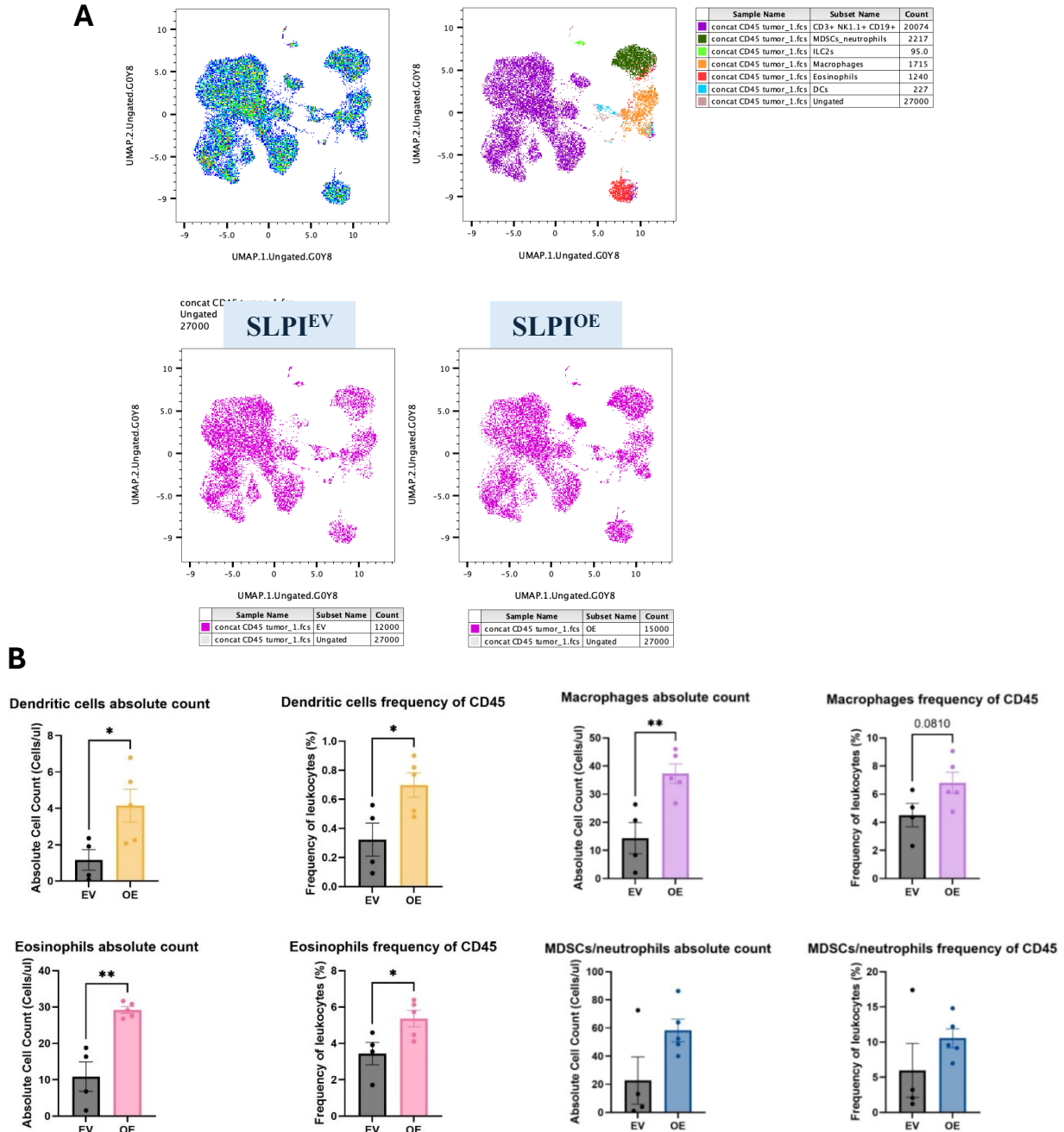
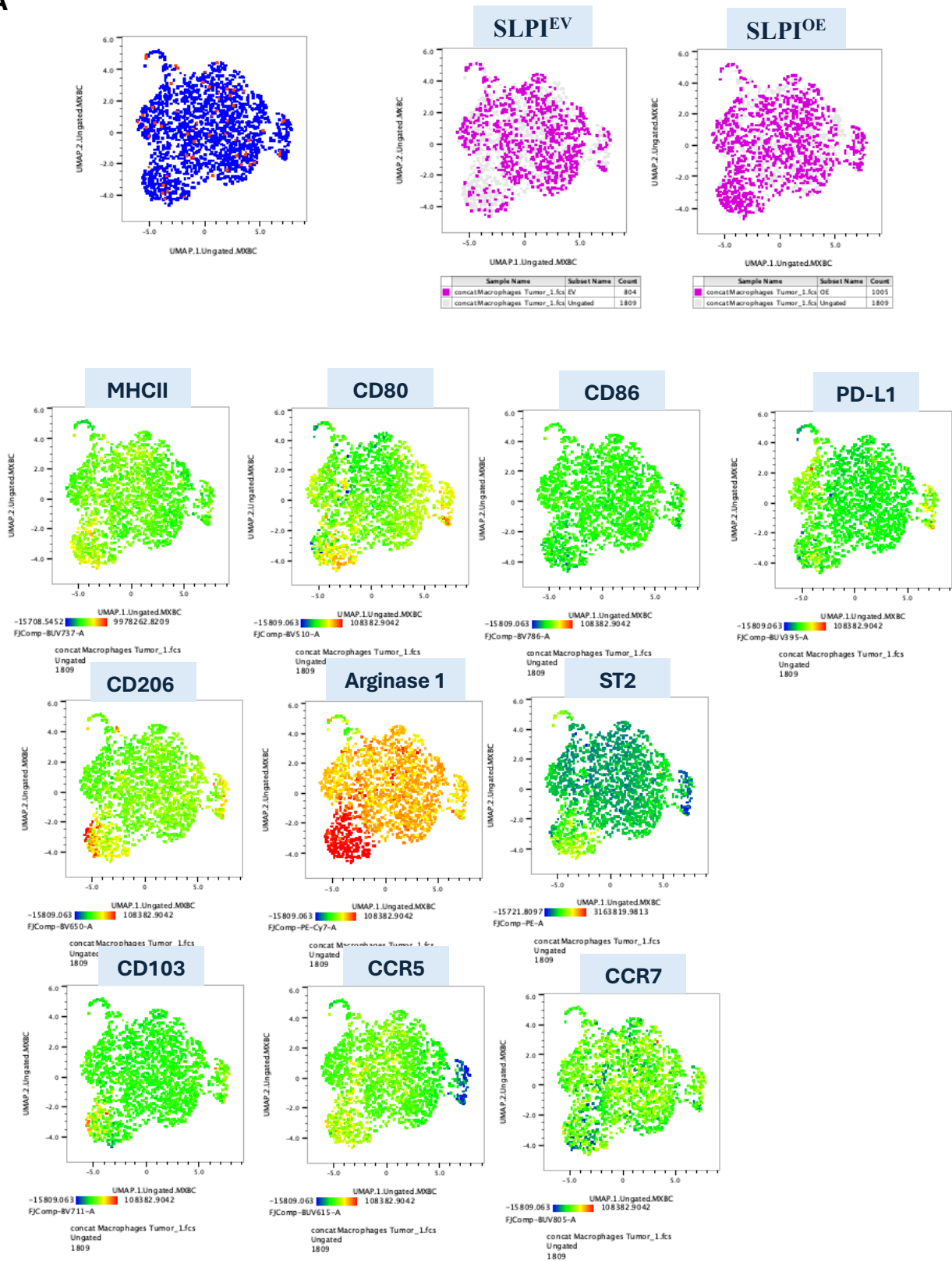


Figure 38. Distribution of myeloid immune cells in the tumors of mice injected with ID8 Trp53^{-/-} SLPI-modified cells.

(A) UMAP of 27000 cells representing myeloid populations in the tumor with distributions separated by immune cell type and by sample group, (B) Counts and frequencies of myeloid immune cell types in the tumors. EV: ID8 Trp53^{-/-} Slpi^{EV}; OE: ID8 Trp53^{-/-} Slpi^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=4-5), *p ≤ 0.05, **p ≤ 0.01.

Tumor – Macrophage and dendritic cell population

A



B

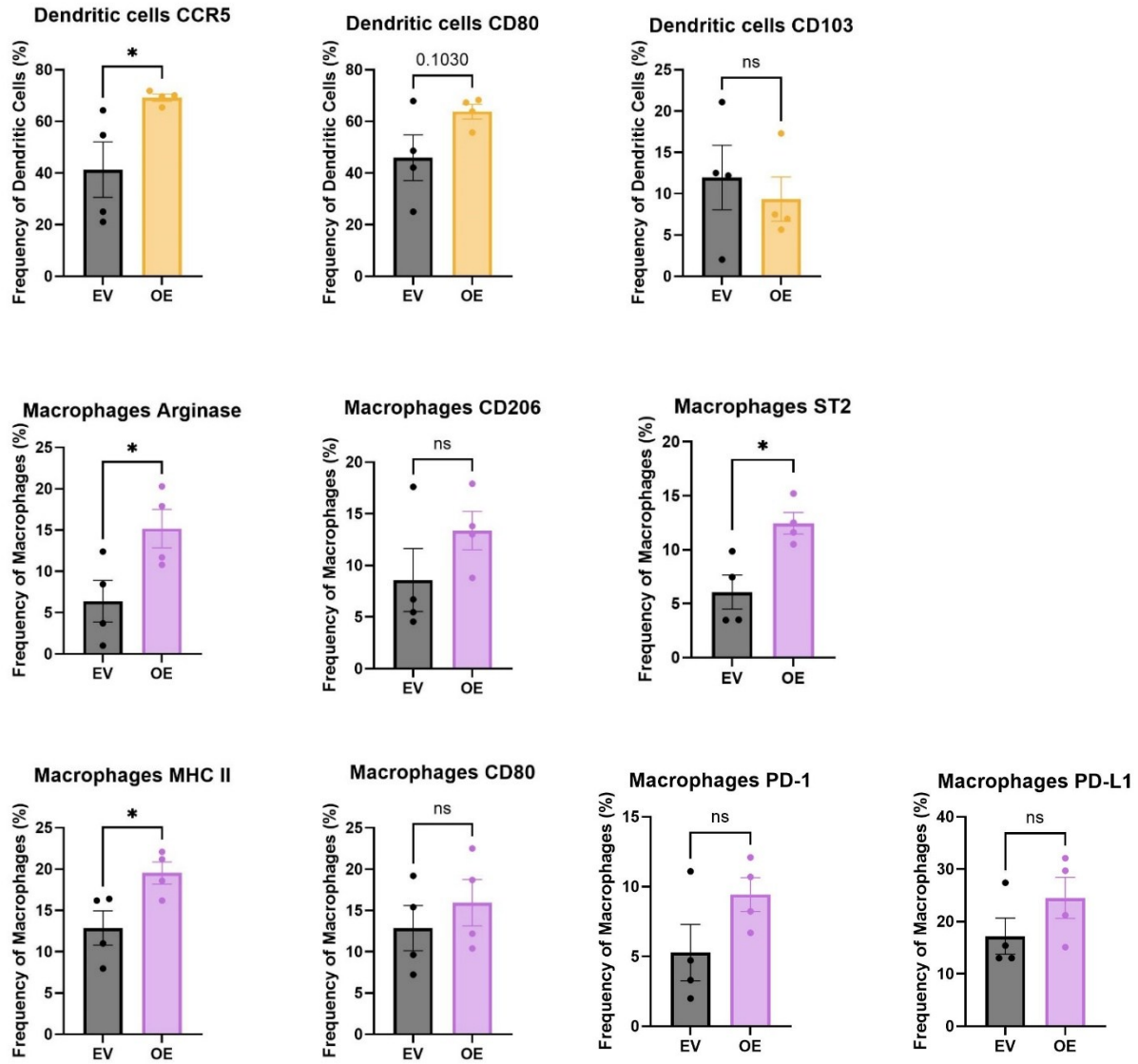


Figure 39. Characterization of the macrophage and dendritic cell populations in the tumors of mice injected with SLPI-modified ID8 Trp53^{-/-} cells.

(A) UMAP of 1809 cells representing the macrophage cell population in the ID8 EV and ID8 OE tumors with spatial expression of each marker that the cells were stained with, (B) Frequency of dendritic cells that are CCR5, CD80, and CD103 positive and macrophages that are Arginase, CD206, ST2, MHCII, CD80, PD-1, and PD-L1 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=4-5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Peritoneal wash – Leukocytes (Myeloid populations)

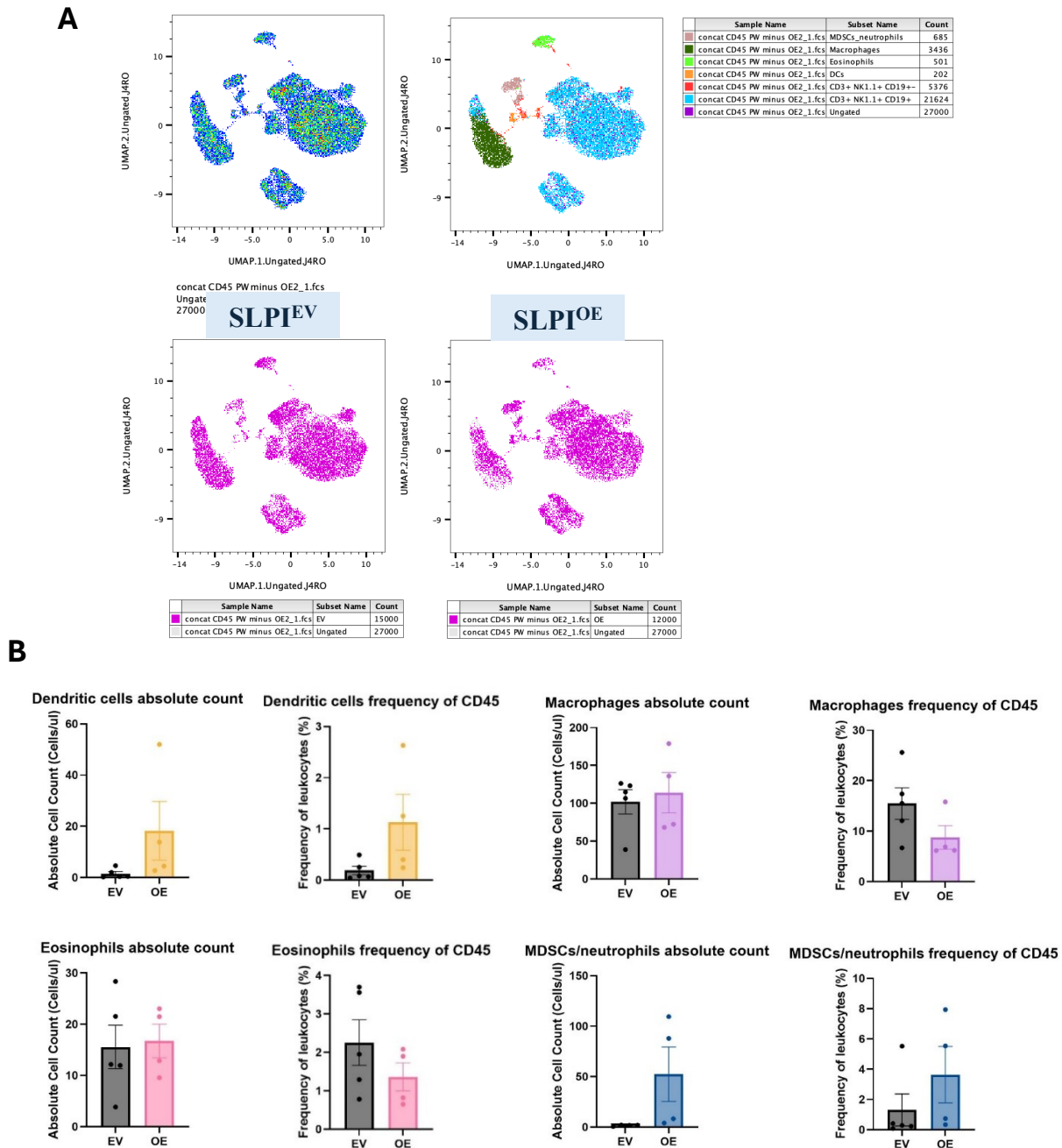
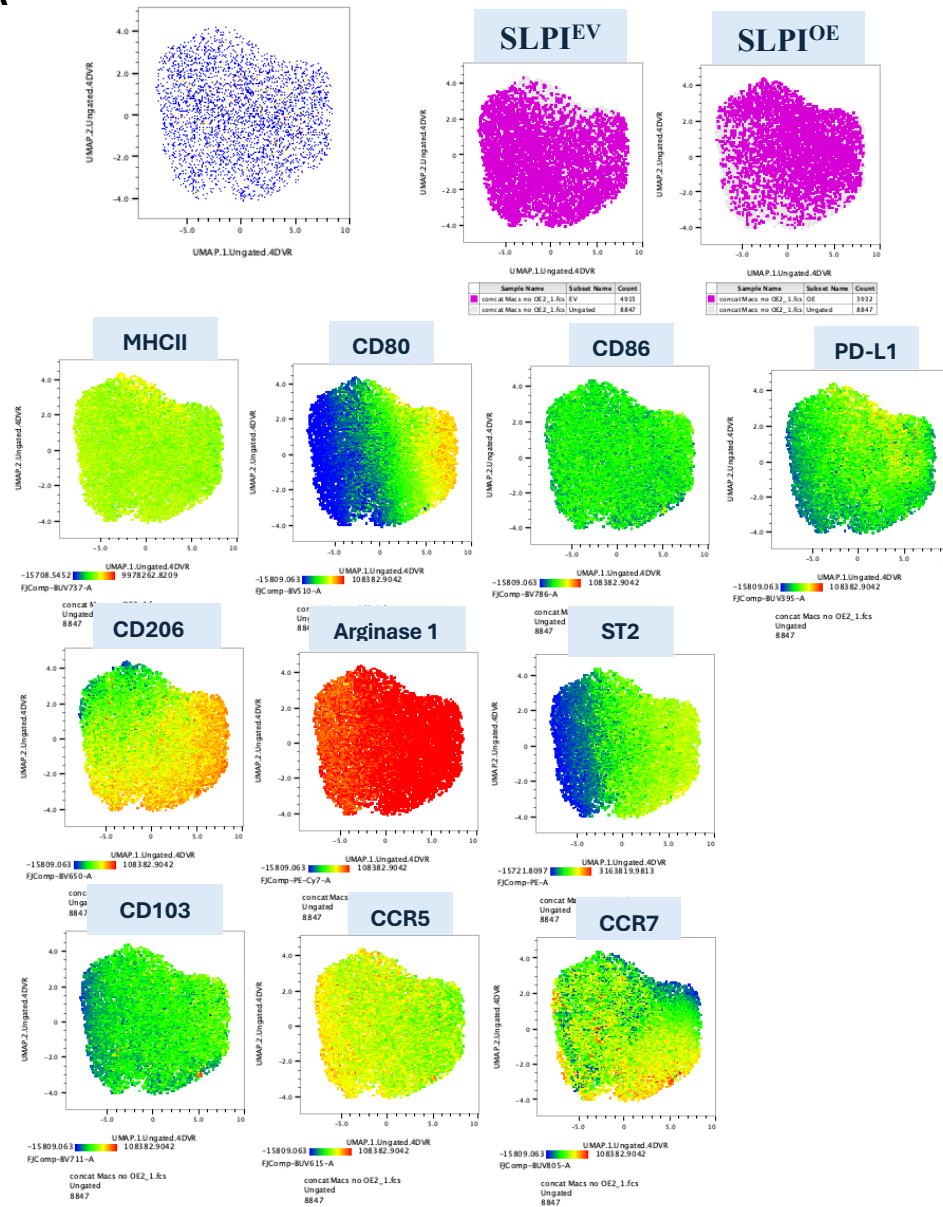


Figure 40. Distribution of myeloid immune cells in the peritoneal wash of mice injected with ID8 *Trp53*^{-/-} SLPI-modified cells.

(A) UMAP of 27000 cells representing myeloid populations in the peritoneal washes with distributions separated by immune cell type and by sample group, (B) Counts and frequencies of myeloid immune cell types in the peritoneal washes. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5).

Peritoneal wash – Macrophage population

A



B

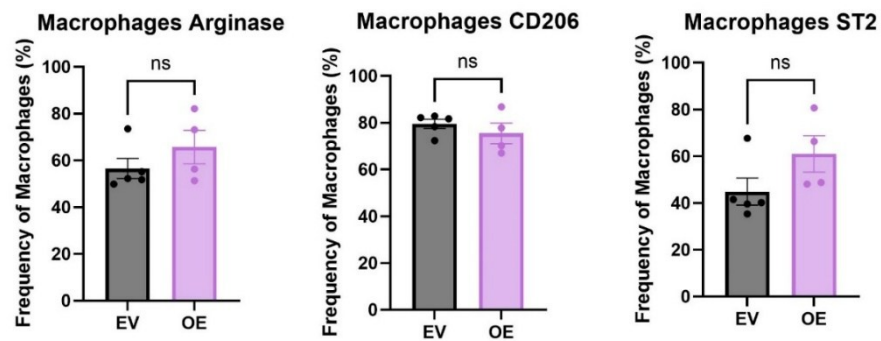


Figure 41. Characterization of the macrophage populations in the peritoneal wash of mice injected with SLPI-modified ID8 Trp53^{-/-} cells.

(A) UMAP of 8847 cells representing the macrophage population in the ID8 EV and ID8 OE peritoneal washes with spatial expression of each marker that the cells were stained with, (B) Frequency of macrophages that are Arginase, CD206, and ST2 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5). The UMAP spectrum goes from blue (low expression) to red (high expression).

Lymph nodes – Dendritic cell population

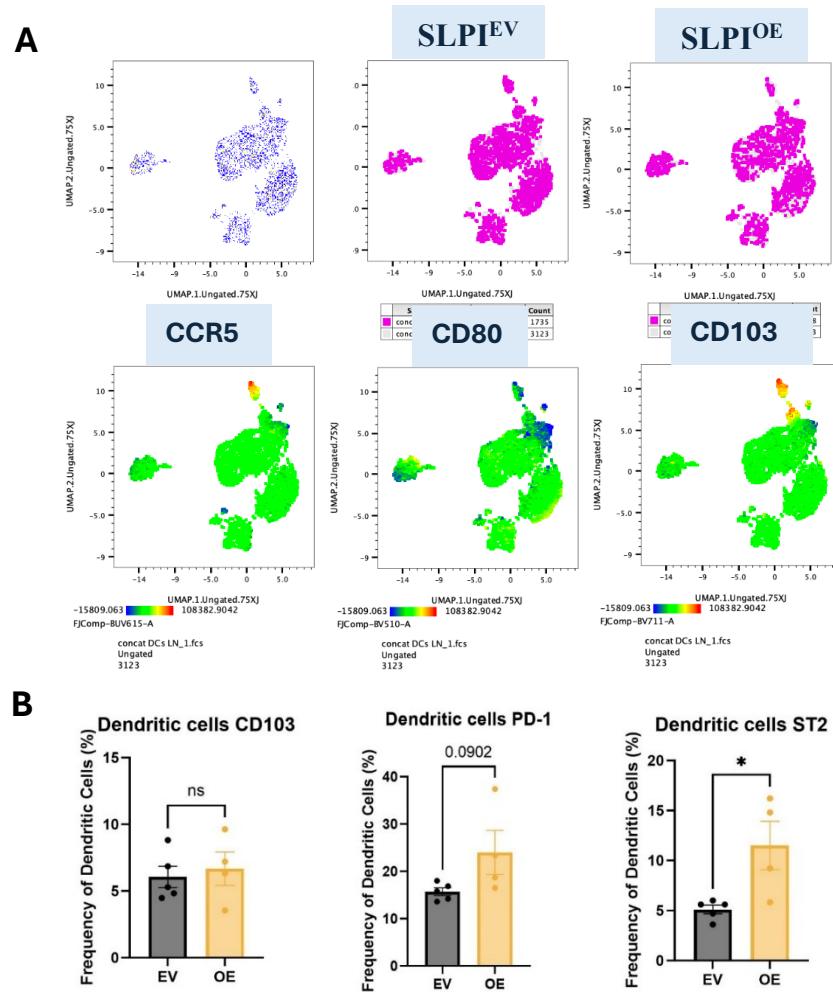


Figure 42. Characterization of the dendritic cell populations in the lymph nodes of mice injected with SLPI-modified ID8 Trp53^{-/-} cells.

(A) UMAP of 3123 cells representing the dendritic cell population in the ID8 EV and ID8 OE lymph nodes with spatial expression of each marker that the cells were stained with, (B) Frequency of dendritic cells that are CD103, PD-1, and ST2 positive. EV: ID8 *Trp53*^{-/-} *Slpi*^{EV}; OE: ID8 *Trp53*^{-/-} *Slpi*^{OE}. The mean is shown +/- SEM. Statistical analysis was done using an unpaired t-test (n=5). The UMAP spectrum goes from blue (low expression) to red (high expression).

3.5.3. Characterization of bone marrow-derived macrophages following treatment with recombinant SLPI

Given the observation of increased abundance of M2-polarized macrophages in tumors that overexpress *Slpi*, we then aimed to test whether this is a direct effect of SLPI on macrophages. Bone marrow cells were extracted from three healthy mice and differentiated into M0 macrophages. M0 macrophages were then treated with an M1 polarizing factor, IFN- γ , or with an M2 polarizing factor, IL-4. Macrophages treated with IFN- γ and M0 macrophages were either treated with rSLPI for 48 hours or left untreated. The cells were then collected and analyzed by flow cytometry using various markers of M1 or M2 polarization: Arginase 1 (M2), CD206 (M2), MHC II (M1), iNOS (M1), CD80 (M1), and CD86 (M1), the proliferation marker Ki67, and the immune checkpoint marker PD-L1 (M1-leaning).

The results show that SLPI selectively increases the expression of both M1 and M2 markers in both M0 and IFN- γ -treated macrophages (**Figure 43**). M0 macrophages treated with SLPI modestly increased expression of Arginase 1, and more dramatically increased the expression and/or frequency of expression of iNOS, CD86, and PD-L1. This collectively suggests that SLPI shifts M0 macrophages towards an M1 (tumor-suppressive) phenotype.

The effects of SLPI on IFN- γ -treated macrophages (M1 + SLPI) were more extensive. Specifically, SLPI increased both frequency and expression levels of Arginase 1 and CD86, as well as the MFI of CD206, iNOS, and PD-L1, and the frequency of CD80, while decreasing frequency and expression of MHCII in M1 macrophages (**Figure 43**). Interestingly, SLPI causes a decrease in the proliferation of macrophages, as seen by a decrease in Ki67 (**Figure 43**).

Some markers in this study do not seem representative of the polarization phenotype since they do not increase with their respective polarization phenotype control; for example, CD206, a marker of M2 polarization, was not more highly expressed in the M2 macrophages compared to the M1 group. This also applies to CD80, an M1 marker that was not significantly higher in the M1 macrophages compared to M2.

The upregulation of both M1 and M2 markers makes it difficult to conclude that SLPI has any specific effect on macrophage polarization that might contribute to the more rapid tumor growth seen in vivo. Further, the decrease in macrophage proliferation in vitro does not align with the increased abundance of tumor macrophages shown in **Figure 38B**, which may suggest that there is greater infiltration of macrophages into the tumor. Overall, the results suggest that SLPI activates macrophages in a manner that is not confined to M1 or M2 polarization, and its actions on macrophages may not be sufficient to explain the faster growth of SLPI-overexpressing tumors.

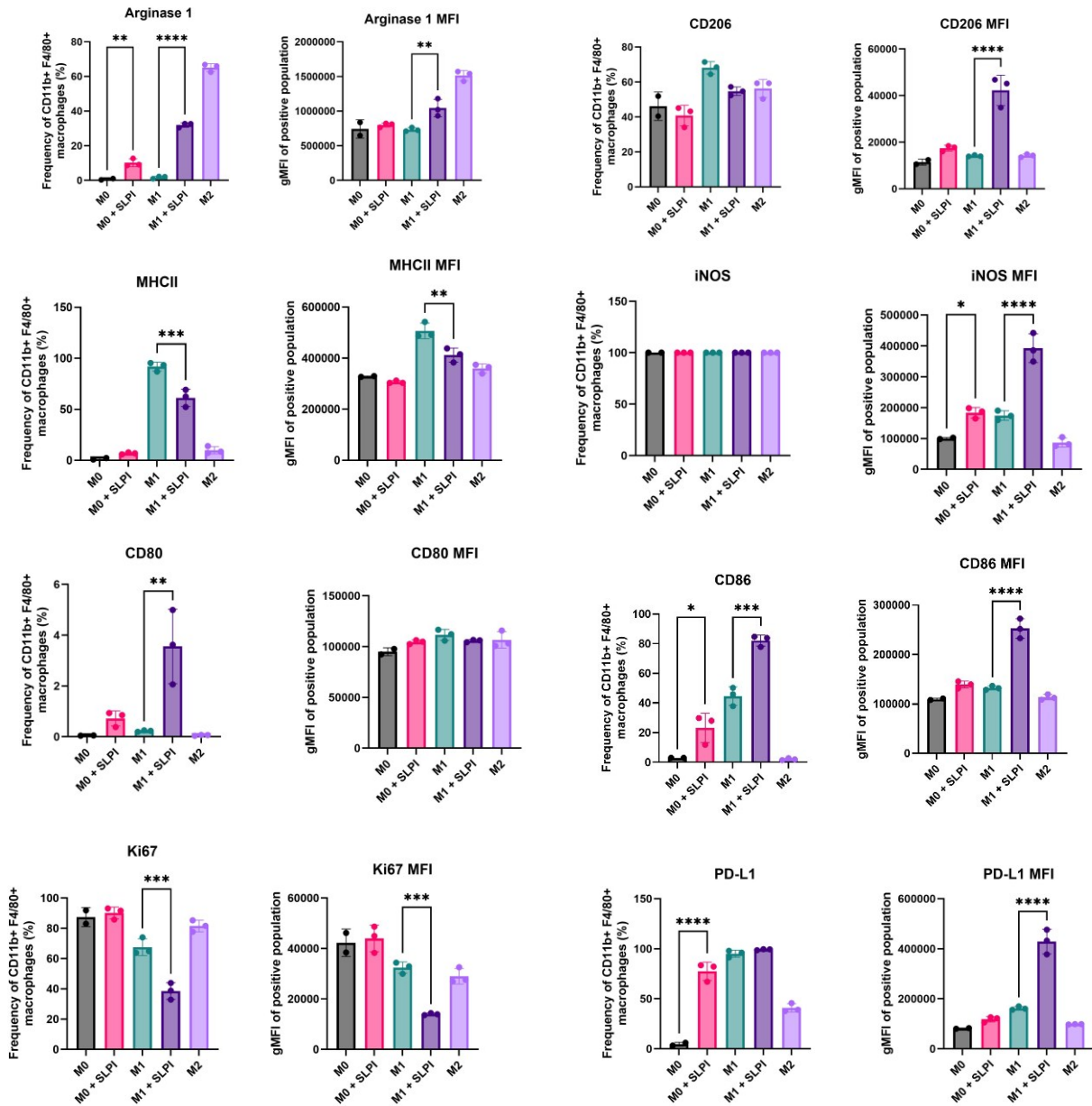


Figure 43. Levels of various markers characterizing phenotypes of M0 and M1 macrophages treated with SLPI.

The frequency (%) and the geometric mean fluorescent intensity (gMFI) for various markers are presented following polarization and treatment with rSLPI. The mean is shown +/- SEM.

Statistical analysis was done using a one-way ANOVA (n=2-3). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Chapter 4: Discussion

This investigation into the contributions of SLPI to ovarian cancer progression and chemoresistance revealed several novel findings. First, SLPI does not appear to be associated with the ability of ovarian cancer cells to respond to chemotherapy. Second, cancer progression occurs faster with overexpression of *Slpi*, but the tumors have fewer metastases, whereas tumors lacking *Slpi* have more metastases. Exploring the potential autocrine actions, we found that SLPI did not modify the proliferation rate of the cell lines, but loss of *Slpi* increased their ability to form spheroids in suspension with slightly decreased resistance to anoikis. This was associated with an increase in expression of genes associated with collagen production, and a decrease in expression of cell junction genes. Since SLPI is a secreted factor, we also explored its potential to affect immune cells in the tumors, revealing that tumors with overexpression of *Slpi* have increased levels of macrophages, dendritic cells, and eosinophils, and exhibited phenotypic shifts in the macrophages that were more positive for Arg-1, ST2, and MHCII. Within the peritoneal cavity, we observed uncharacterized shifts in the B cell and NK cell populations. Macrophages treated with rSLPI in vitro showed upregulation of both M1 and M2 macrophage polarization markers.

4.1. SLPI expression is regulated by different signaling pathways

Due to its homeostatic function, SLPI is upregulated by a variety of factors, most of them being inflammatory signals that activate SLPI to counteract and regulate the inflammatory response⁵⁷. Accordingly, there are various binding sites that are found in the promoter region of the mouse *Slpi* gene associated with inflammatory regulators, including seven interferon- γ response elements (γ -IRE), three nuclear factor (NF)-IL-6-binding sites, one NF- κ B binding site, one H-APF-1 binding site, two AP-2 binding sites, one AP-1 binding site, one cyclic adenosine

monophosphate-responsive element (CRE), one HNF-5 binding site, one c-Myb binding site, one GATA-1 binding site, and one Ets-1 binding site¹⁰³. Additionally, the following factors have been shown to upregulate the expression of *SLPI* mRNA in epithelial cells in various tissues and contexts: interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), pattern recognition receptor ligands, insulin-like growth factor 1 (IGF-1), transforming growth factor alpha (TGF- α), progesterone, and corticosteroid⁵⁴. However, pathways that regulate SLPI expression in the context of cancer are not well established.

The results of our experiments suggest that *Slpi* is upregulated by the MEK pathway in murine ovarian cancer cell lines since *Slpi* expression almost completely diminishes in the presence of MEK inhibitors. We originally conjectured that KRAS was the upstream factor responsible for SLPI upregulation through MEK. We showed that SLPI expression is associated with MEK expression in a mouse cell line with a KRAS mutation (MPK) and one with no known mutations (STOSE). However, STOSE cells have elevated pERK levels and could have amplification of KRAS signaling without a mutation⁷⁹. Further studies are necessary to pinpoint whether KRAS is an initiator of SLPI upregulation and to investigate if amplification of other downstream KRAS signaling factors can be responsible for initiating SLPI upregulation in our cell lines. It has also been shown that BRAF, the kinase directly upstream of MEK, is responsible for upregulation of SLPI in the context of BRAF^{V600E}-mutant papillary thyroid cancer⁸⁶. From what is known to date, it is not apparent that ERK can bind directly to the promoter region of *SLPI* to activate it; however, there is evidence of ERK activating various transcription factors that may bind to the promoter region of *SLPI*. For example, it has been hypothesized that, in the context of cancer, ETS proteins upregulate targets of RAS/ERK through binding to ETS/AP-1 binding sites¹⁰⁴, both of which are found in the promoter region of *SLPI*. While the MEK inhibitor used in

this study was specific, ERK has countless downstream effectors that could be responsible for SLPI regulation; therefore, the direct regulator of SLPI in this context is unknown.

Contrary to what we observed in the mouse cell lines, there is an increase in *SLPI* following MEK inhibition in human ovarian cancer cell lines, showing that MEK inhibits SLPI expression in this context. In fact, the human *SLPI* gene and the murine *Slpi* gene are 68% homologous, while their amino acid sequences are 60% homologous¹⁰³. Therefore, if there is variability in their nucleotide/amino acid sequence, it is not unlikely that their promoter regions also differ from each other, which could lead to the activation of *SLPI* through different factors or pathways. With SLPI being a homeostatic, immunoregulatory protein, many of the factors that it targets are also regulators of SLPI, creating cyclical pathways or negative feedback loops. While our data suggest that *Slpi* is upregulated by MEK, multiple cancer studies have described SLPI as an upstream factor to the AKT and ERK pathways^{68,69,105}, hinting at a potential feedback loop. We found that *Slpi* expression seems to increase when AKT is inhibited, suggesting that AKT might suppress SLPI, but found no difference in pAKT levels between our SLPI-modified cell lines and their control, suggesting that SLPI does not regulate downstream pAKT in our models.

4.2. SLPI has minimal influence on chemoresistance with the exception of the KPCA.B model

We observed no difference in chemoresistance with or without *Slpi* in our murine cell lines except for the KPCA.B cell line, which was more sensitive to paclitaxel with overexpression of *Slpi*. The KPCA.B cell line has mutations that are not found in the other models we use, i.e., a *Ccne1* overexpression and an *Akt2* overexpression. We hypothesize that the unique observed effect of SLPI on chemosensitivity in the KPCA.B model is due to one or both of these mutations. However, this is contradictory to what is shown in the literature, which is that *Ccne1* and *Akt* are

thought to increase resistance to paclitaxel^{106,107}. This knowledge, in combination with our results, suggests that there is an unknown interaction between SLPI and *Ccne1*, *Akt*, or both that is conferring SLPI-overexpressing cells with increased chemosensitivity.

4.3. SLPI is negatively correlated with metastatic weight

Spheroids are aggregates of cells that self-assemble when they cannot attach to a surface in vitro. These cells aggregate due to long-chain ECM fibers and cadherins that strengthen cell-to-cell adhesion of neighboring cells¹⁰⁸. Spheroids are formed of an external layer that is in contact with oxygen and nutrient-rich media and is therefore proliferative, a relatively quiescent middle layer, and a necrotic center due to lack of oxygen and nutrients when the spheroids attain a certain size¹⁰⁹. Spheroid formation can also be used to measure cancer stem cell capacity¹¹⁰. Our in vitro investigations showed that MPK cells with an *Slpi* knockout form many smaller aggregates, suggesting that they can efficiently form abundant spheroids. However, these cells have slightly lower cell viability in suspension and are not able to form spheroids in semi-solid media conditions where they cannot move freely and bump into each other. In contrast, MPK Control cells do not prioritize formation of many spheroids but are able to survive better in suspension and can grow more efficiently with less cell-to-cell contact. This indicates that the inverse correlation between size and number in standard suspension cultures is not purely due to cellular aggregation, but also due to anoikis resistance and proliferation. This may be explained by the upregulation of collagen transcripts in MPK *Slpi*^{-/-} cells, which confers them the ability to initially form many spheroids. Their reduced viability in suspension and reduced ability to grow with minimal cell-to-cell contact may be explained by the downregulation of certain desmosomal plaque-associated genes, which are important in forming robust cell-to-cell adhesion necessary for proper spheroid development and growth. The presence of numerous spheroids in the MPK *Slpi*^{-/-} group leads to more

metastatic seeding events in vivo, which correlates with the observed increased weight of metastases in this group. ID8 cells were unable to form spheroids in vitro, making this correlation difficult to confirm in this model. Importantly, there was a decrease in survival in vivo in ID8 tumors that overexpress *Slpi*, meaning that disease progression is happening faster in this group. Since metastases weight was only quantified and compared during the survival study, where ID8 *Slpi*^{OE} tumors were collected sooner since their endpoint occurred earlier, the reduced metastases weight may reflect this earlier collection. Since the MPK model showed no difference in survival and an increase in metastases weight in the MPK *Slpi*^{-/-} group, consistent with lower metastases weight in the ID8 *Slpi*^{OE} group, we can hypothesize that SLPI has a real impact on the ability of the cells to metastasize.

In alignment with these observations, a previous student in the Vanderhyden lab, Maryam Echaibi, completed a survival study where the SLPI-modified cell lines and their controls were injected intraperitoneally (IP), that is, within the peritoneal cavity. This method removes the requirement for cancer cells to develop the capacity to transition from their orthotopic site to metastatic sites and is more representative of how cells act in metastatic disease. In that study, injection of MPK *Slpi*^{-/-} cells resulted in shortened survival compared to the MPK Control group, while ID8 *Slpi*^{OE} tumors resulted in prolonged survival (unpublished results). These results are consistent with our observation that *Slpi* expression is correlated with lower metastatic burden and explain why increased *Slpi* is associated with prolonged survival when cells are injected IP.

A study by Symowicz et al. proposed a mechanism linking integrins to submesothelial collagen and to impaired E-cadherin function¹¹. They first determined that dysfunction of E-cadherin, contributing to metastatic progression in many cancers, does not happen at the gene level in ovarian cancer and instead happens through post-translational modification of E-cadherin,

creating soluble E-cadherin (sE-cad), which was found at high levels in ascites from patients with ovarian cancer. When aggregates of metastatic cells bind to the collagen-rich ECM of the mesothelium, integrins that bind to collagen, such as $\alpha_2\beta_1$ and $\alpha_3\beta_1$, cluster. This clustering was determined to be the cause of shedding of E-cadherin to create sE-cad. This process was dependent on cleavage of the ectodomain by matrix metalloproteinase-9 (MMP-9). Lastly, adherens junctions in cancer cells were disrupted following incubation with recombinant human sE-cad, suggesting that sE-cad has a role in tumor dissemination. RNA-seq analysis of our MPK cell line showed that the MPK *Slpi*^{-/-} cell line had increased expression of *Mmp-9* (data not shown, $p = 0.0408$), of *Cdh2* (**Figure 24**, $p = 0.0002$), coding for N-cadherin, which is the mesenchymal equivalent of E-cadherin, and of many collagen genes, notably *Colla2* (**Figure 22**), forming part of type 1 collagen, to which ovarian cancer cells preferentially adhere¹¹². These gene expression changes suggest that a similar mechanism may explain how spheroids formed in the MPK model system.

4.4. SLPI's effect on immune modulation

Type 2 immune responses are initiated in response to extracellular pathogens, mostly helminth infections, and are characterized by activation of eosinophils, stimulation of antibody production, elevated mucus production by epithelial cells of mucosal linings in the respiratory and digestive tract, and increased smooth muscle contraction, and are activated by T helper 2 (T_H2) CD4 cells^{113,114}. The opposing phenotype of CD4 T helper cells is the T helper 1 (T_H1) phenotype, which leads to a type 1 immune response. Type 1 immune responses are initiated in response to large intracellular pathogens, such as bacteria and viruses, are characterized by IFN- γ -stimulated inflammation, and act primarily through phagocytosis and oxidative burst^{113,114}. Type 1 responses are associated with tissue damage as a byproduct of inflammation and production of antimicrobial factors by neutrophils and monocytes. In contrast, type 2 immunity is geared towards reducing

inflammation to avoid excessive tissue damage¹¹³. Similarities can be drawn between the function and mechanisms of type 2 immunity and the role of SLPI, as SLPI has well-established anti-inflammatory, tissue-protective functions⁵⁷ and is known to inhibit destructive neutrophil products, namely the serine protease neutrophil elastase, and neutrophil extracellular traps (NETs)^{115,116} as well as matrix metalloproteinases (MMPs) produced by monocytes¹¹⁷. Classically, type 1 and type 2 immunity are accompanied by polarization of macrophages into two diverging phenotypes, M1 and M2, respectively. M1 macrophages are typically pro-inflammatory and are associated with anti-tumor properties. M2 macrophages are more immunosuppressive. Thus, M1 and M2 binary phenotypes present extreme states that are not representative of the nuance and spectrum of polarization that is present in macrophage populations. Many macrophage subsets that remain to be classified express different combinations of M1 and M2 markers. They are also not permanently differentiated into those phenotypes and can shift from one phenotype to another depending on environmental signals received from other cells, tissues, and pathogens, such as cytokines, growth factors, and conditions such as inflammation, injury, and hypoxia¹¹⁸. When examining the results of our TME analysis as a whole, we observed many parallels with type 2 immunity and the response that occurs with the overexpression of *Slpi*. Analysis of the TME showed major changes in the myeloid populations, notably the macrophages, the dendritic cells, and the eosinophils.

Effect of SLPI on macrophages: Analysis of the TME revealed an increase in the macrophage population in the tumors with *Slpi* overexpression. This population had increased expression of Arg-1, MHC-II, and ST2. While this phenotype may seem contradictory, as Arginase-1 is typically a marker of M2 macrophages while MHCII is a marker of classical M1 macrophages, there have been reports identifying a macrophage population positive for both of these markers, which was termed as alternatively activated macrophages or M2a^{119,120}. This

phenotype is generally induced by IL-4, IL-13, or glucocorticoids, and they secrete IL-10 and IL-1 receptor antagonist (IL-1RA)^{119,121}. They have been shown to increase expression of MHC class II molecules without making them necessarily more competent at antigen presentation, since they have been shown to inhibit T cell proliferation in certain contexts¹¹⁹. They are also involved in the promotion of wound healing and angiogenesis and have been associated with fungal and helminth infections^{121,122}. The M2a macrophages have been shown to promote tumor growth by induction of angiogenesis, and cancer cell migration and invasion through secretion of VEGF and CCL18 in the context of breast cancer¹²³. M2a macrophages also increased tumor growth and cellular invasion of lung cancer cells¹²⁴. It was later determined that the IL-4/STAT6 pathway was crucial in conferring the pro-tumoral effects of M2a macrophages in lung cancer¹²³. A study by Guerrieri et al. showed that monocytes treated with SLPI induced secretion of IL-4, IL-6, and IL-10. IL-4 reduced PBMC proliferation, and supernatant from SLPI-treated monocytes decreased proliferation of monocyte-excluded PBMCs⁵⁵. This suggests that SLPI induces an M2a-like differentiation, promoting secretion of cytokines that suppress T cell responses and promote tumor growth.

It has been well documented that SLPI and macrophages interact and that macrophages can express SLPI¹²⁵. SLPI carries out its effect in macrophages through inhibition of NF- κ B by various mechanisms. Taggart et al. showed that the monocytic cell line U937 could uptake SLPI and inhibit NF- κ B activation¹²⁶. They first showed that, following treatment with SLPI, SLPI was easily transported to the cytoplasm and nucleus and hypothesized that it could bind to negatively charged regions of DNA since SLPI is highly cationic. Therefore, they investigated SLPI's ability to bind to consensus transcription factor binding sites and found that SLPI had a preference for binding to NF- κ B and AP-1 sites but not GATA-1, Sp-1, or CREB consensus oligonucleotides. Due to its

transactivation domain (TAD), the subunit p65 (RelA) can dimerize with other subunits to activate NF- κ B transcriptional activity in the nucleus and is sequestered in the cytoplasm by the I κ B inhibitory molecules at rest¹²⁷. Taggart et al. determined that SLPI could competitively bind to NF- κ B DNA-binding sites in the presence of p65 and found that the intensity of p65–NF- κ B DNA complexes decreased with increasing concentrations of SLPI. They found that SLPI could bind to IL-8 and TNF- α promoters that have many NF- κ B binding sites and prevented p65 from binding to these regions. This translated into a decrease in IL-8 and TNF- α production in macrophage-differentiated U937 cells following exposure to LPS. In summary, SLPI can specifically bind to NF- κ B sites to prevent p65 binding and context-dependent NF- κ B activation in monocytes, e.g., by LPS.

While it has been shown by multiple studies that SLPI can bind directly to NF- κ B to prevent its activation, other studies have shown that SLPI can also inhibit or dampen NF- κ B activation through regulation of its inhibitory proteins, I κ B proteins. When NF- κ B signaling is triggered, there is first activation of the I κ B kinase (IKK) complex, which phosphorylates I κ B α or I κ B β , leading to their ubiquitination and marking them for degradation by the proteasome, which ultimately releases NF- κ B subunits, allowing them to move to the nucleus to activate NF- κ B transcription¹²⁸. A previous study by Taggart et al. showed that SLPI can inhibit degradation of the NF- κ B inhibitory proteins, IRAK, I κ B α , and I κ B β in the U937 monocytic cell line in the context of exposure to LPS¹²⁹. This mechanism was associated with SLPI's antiprotease activity. When cells were treated with SLPI and LPS, IRAK, I κ B α , and I κ B β protein levels did not decrease in comparison to LPS alone. They determined that SLPI's effects were not due to a reduction of LPS-induced phosphorylation of I κ B α , ubiquitination of I κ B α , or 20S-associated proteasomal peptidase activity. Therefore, SLPI's effect is due to regulation of an unidentified step preventing I κ B α

degradation that is contingent on its antiprotease activity¹²⁹. In the context of lung inflammation, Lentsch et al. found that treatment with SLPI increased protein levels of I κ B β but had no effect on I κ B α protein levels in both normal and inflamed lungs¹³⁰. It was hypothesized that SLPI is acting on endothelial cells and not alveolar macrophages in this context. This presents evidence that SLPI has capacity for tissue- and cell-type-dependent regulation of NF- κ B.

Effect of SLPI on eosinophils: Eosinophils are not only crucial in the unfolding of type 2 immunity, but they are also involved in allergy-associated inflammatory diseases, and can interact with mast cells and plasma cells to regulate tissue remodeling, metabolism, and innate and adaptive immunity¹³¹. Osiecka et al. described that certain allergy-associated inflammatory diseases, eosinophilic granulomatosis with polyangiitis (EGPA) and atopic dermatitis (AD), are characterized by high levels of eosinophils and SLPI, hypothesizing that they may be regulating each other. They determined that eosinophils, neutrophils, and low-density granulocytes from granulocytes and peripheral blood mononuclear cells (PBMCs) of healthy human donors were positive for SLPI. Although SLPI did not increase the lifespan of eosinophils, recombinant SLPI treatment of eosinophils from the peripheral blood of healthy donors increased the percentage of migrating eosinophils, chemotaxis-specific migration, as well as a higher chemotactic response to eotaxin-1 (CCL11), a chemokine that specifically regulates eosinophil migration¹³¹. The mechanism by which SLPI increased the migration and chemotactic ability of eosinophils was not established in this study. One of the hallmark differences we observed in the TME of tumors with *Slpi* overexpression was an increase in the abundance of eosinophils. While the presence of tumor-associated tissue eosinophilia in most cancers is generally more favorable for overall survival, the data for gynecological cancers are quite divided¹³². In cervical cancers, some studies reported that the presence of eosinophils correlates with better survival, while others claimed the opposite¹³². In

endometrial cancers, increased eosinophil-to-lymphocyte and/or neutrophil ratio was linked to worse survival¹³². This has not yet been investigated in ovarian cancer.

Effect of SLPI in dendritic cells: Previous studies involving SLPI and dendritic cells (DC) have shown that DCs express SLPI in a location-dependent manner, as it is mostly expressed in DCs from mucosal sites¹³³. Other studies have shown that SLPI reduces the activation of DCs. In a study by Samsom et al., baseline levels of NF- κ B p65 and levels after low-dose treatment of LPS were higher in *SLPI*^{-/-} bone-marrow-derived DCs (BM-DCs) compared to WT BM-DCs, suggesting lower activation of DCs in the presence of endogenous SLPI¹³⁴. However, at higher doses of LPS, *SLPI*^{-/-} DCs had decreased NF- κ B p65 levels and were also associated with increased T cell proliferation compared to WT DCs, suggesting that the presence of SLPI can reduce T cell activation. In vivo, *SLPI*^{-/-} mice had increased expression levels of CD40, CD86, and MHC class II on CD11c⁺ cells and secreted higher levels of inflammatory cytokines, suggesting that SLPI in DCs dampens the activation of DCs and immune responses. The results from this study showing that SLPI reduces NF- κ B activation in DCs and DCs' effect on T cells are in line with our hypothesis that SLPI is creating an immunosuppressive environment through its anti-inflammatory function. It is not yet known if exogenous SLPI can enter DCs to recapitulate the effects seen in the study in a different context. Studies have shown that SLPI can dampen DC activation but not necessarily proliferation or migration. Therefore, our observed increase in dendritic cell counts and frequency in the ID8 *Slpi*^{OE} tumors is in agreement with the literature. However, it remains unclear whether the increase in DCs in the tumor is due to SLPI itself or is a response to changes in other immune cells. In our results, while DCs in the tumors of ID8 *Slpi*^{OE} mice had elevated levels of CCR5, which could facilitate migration of immune cells, the DCs in

the lymph nodes had increased ST2 and a trend to increase PD-1, indicating promotion of a type 2 immune response in these DCs.

Effect of SLPI on B cells: Since we did not expect SLPI to have an effect on B cells, we did not stain the cells with enough markers to fully characterize the B cell population in the tumor, lymph node, and peritoneal wash. According to our UMAP representation, there is a dramatic shift in the B cell phenotype in the lymph nodes and the peritoneal wash of the ID8 *Slpi*^{OE} group. Previously published evidence indicates that SLPI can suppress activation of B cells and class switching^{135,136}. Xu et al. established a mechanism following microbial infection where epithelial cells induced class switching in B cells, which was then restrained by SLPI. Activated B cells can perform a process called immunoglobulin class-switch recombination (CSR), which involves a switch in the isotype of immunoglobulins, specifically a switch from IgM and IgD to IgG, IgA, or IgE to modify effector function¹³⁵. Their proposed mechanism begins with microbial products binding to Toll-like receptors (TLR), which leads to B cell-activating factor (BAFF) and IL-10 release by epithelial cells. This induces B cell activation and their expression of an enzyme indicating active CSR, activation-induced cytidine deaminase (AID). The stimulated B cells undergoing CSR secrete polyreactive IgG and IgA to overcome microbial infection. This process was enriched by thymic stroma lymphopoietin (TSLP) released by epithelial cells, which induces dendritic cells to also produce BAFF. SLPI is able to enter the cytoplasm and nucleus of epithelial cells and B cells and regulates this response. It is released by epithelial cells at a later time point than B cell-stimulating factors, indicating that SLPI acts as a negative feedback protein preventing overreactive immunoglobulins. SLPI inhibits NF- κ B proteins more specifically, preventing translocation of p50, p65, and c-Rel to the nucleus and their binding to B cell DNA after exposure to activating factors, which induce CRS by activation of the I_H promoter. Treatment with SLPI did

not negatively affect survival and proliferation of B cells and IgM production, indicating that SLPI is specifically modulating the class-switching ability of B cells¹³⁵. Bringing this back to our in vivo TME model where SLPI is overexpressed, we hypothesize that SLPI is inhibiting class switching in B cells, explaining the uncharacterized shift in our B cell UMAPs, and leading to attenuated tumor killing. Studies have shown that intratumoral tertiary lymphoid structures have anti-tumoral function through production of B cell class-switched antibodies and are correlated with better survival outcomes in ovarian cancer patients¹³⁷. Therefore, B cell class switching is important for anti-tumor immunity and is potentially inhibited by *Slpi* overexpression in our model, explaining the decrease in survival. Additionally, the B cells in the peritoneal wash of ID8 *Slpi*^{OE} mice had lower levels of PD-L1, which has been associated with the formation of long-lived plasma cells (a successor of B cells that produce high amounts of antibodies)¹³⁸, further suggesting that SLPI is dampening B cell responses.

Effect of SLPI in T cells: Our TME analysis showed very modest changes with *Slpi* overexpression in the T cell populations. No changes were seen in the count and frequency of total leukocytes in the tumors, peritoneal wash, and lymph node between the two groups of mice except for a significant decrease in cytotoxic CD8⁺ T cells in the tumors of the ID8 *Slpi*^{OE} mice. There was a slight increase in the CD44⁺ population of CD8⁺ T cells with SLPI in the tumors, but no other changes in CD8 and CD4 T cells. In the lymph nodes, there was a trending increase of CD25 in CD4 cells and a trend for increased CD69 in CD8 T cells with SLPI, but no other changes. Lastly, in the peritoneal wash, fewer CD4⁺ T cells were GATA3⁺, ST2⁺, and CD44⁺, indicating a naïve state and a shift away from T_H2 polarization. CD8⁺ T cells were less Ki67⁺ and CTLA4⁺, indicating less proliferation but potentially more activation. There is little evidence in the literature describing SLPI interactions with T cells. One study by Py et al. showed that SLPI interacts with

the membrane proteins phospholipid scramblase 1 (PLSCR1) and PLSCR4, in the context of human immunodeficiency virus (HIV-1) infection¹³⁹. The CD4 receptor is one of the receptors that is used by HIV-1 to enter the cells it targets, which are T lymphocytes and macrophages¹³⁹. It was previously shown that SLPI interacts with a cell membrane receptor other than CD4 to prevent HIV-1 entry, hypothesized to be the phospholipid scramblase 1 (PLSCR1), since the phospholipid binding protein annexin II, with which SLPI has been shown to interact, does not appear to be expressed on T cells^{139,140}. Py et al. confirmed the anti-HIV-1 action of SLPI on T-lymphocytes and SLPI's inhibition of the transfer of HIV-1 from dendritic cells to T-lymphocytes. They established that SLPI can bind to both PLSCR1 and PLSCR4, transmembrane proteins that can transport phospholipids between the inner and outer lipid bilayer of a cell membrane, and these receptors can interact with the CD4 receptor on T-lymphocytes. The domain of PLSCR1 that interacts with both SLPI and CD4 is the same, the cytoplasmic domain, suggesting competitive binding of SLPI to PLSCR1 to prevent CD4 binding to PLSCR1¹³⁹.

Evidence showing an SLPI-mediated effect on T cells is most likely due to a non-direct interaction and is happening via phenotype changes in other cells. For example, Guerrieri et al. showed SLPI induced effects on T cell proliferation through modulation of the monocyte phenotype⁵⁵. SLPI treatment reduced proliferation of monocyte-depleted PBMCs (CD14- PBMCs) only in the presence of monocytes. They then showed that monocytes treated with SLPI increased secretion of IL-4, IL-6, and IL-10. To validate that SLPI treatment of monocytes affected T cells, CD4 and CD8 cells were isolated, stimulated, and treated with the supernatant of SLPI-treated monocytes, which led to a decrease in proliferation of CD4+ cells, while CD8+ cell proliferation was unaffected. The supernatant also dampened expression of T-bet and reduced secretion of IFN- γ in CD14- PBMCs, indicating reduced T_H1 differentiation. The supernatant did not contain SLPI, so

these effects were due to cytokines released from monocytes following SLPI treatment⁵⁵. Therefore, SLPI has generally no direct effect on T cells, but the presence of SLPI in more complex immune environments can modulate T cell responses indirectly.

While NF- κ B activation is known to induce T_H1 cell differentiation¹²⁸, there is no evidence that SLPI is able to enter T cells to inhibit this pathway, potentially explaining why we see very modest changes in the T cell population in our TME experiment. Additionally, we had originally hypothesized that SLPI could interact with CD4 receptors on T cells due to outcomes from the analysis of the single-cell RNA sequencing data of human ovarian cancer by Maryam Echaibi. Investigation of ligand-receptor interactions involved in the communication between cancer cells and immune cells in tumors revealed that the most abundant interaction was between SLPI and the CD4 receptor on monocytes. While CD4 is expressed on monocytes in humans, it is not expressed on mouse monocytes¹⁴¹, leading us to hypothesize that SLPI would mostly interact with T cells in mice. However, there is no reported evidence of a direct interaction between SLPI and the CD4 receptor in the literature. In fact, there is little research on what membrane proteins SLPI binds to and how it enters or engages with its target cells. One study attempting to establish a receptor for SLPI identified Annexin II¹⁴². Ma et al. discovered annexin II as a membrane-binding protein for SLPI on macrophages and determined that it is important in carrying out SLPI's inhibitory effect during HIV-infection¹⁴². Annexin II is generally more highly expressed on monocytes and myeloid dendritic cells compared to T cells and eosinophils¹⁴³, potentially explaining why SLPI has a significant effect on those cell types in tumors. Others have hypothesized that SLPI's arginine-rich structure, making it cationic, allows it to bind to the mostly negatively charged membrane and potentially be internalized, or that it has its own protein transduction domain¹²⁶. However, further

investigations are necessary to determine the mode of entry of SLPI in cell types other than monocytes, and if and how it is internalized.

Comparison of macrophages in vitro vs. in vivo experimental outcomes: When we attempted to isolate the effect of SLPI on macrophage phenotype in vitro, we found discrepancies when compared to the in vivo TME results. While Arginase-1 expression increased with SLPI treatment, which is consistent with the in vivo results, rSLPI decreased the expression of MHCII in M1-polarizing macrophages, contrary to what was observed in vivo. We also saw increases in M1 markers in vitro, such as iNOS (MFI), CD80, and CD86, that did not change in vivo.

Additionally, rSLPI treatment of M1-polarizing macrophages reduced Ki67 levels in vitro, which could imply that the increase in macrophages in the tumor in the in vivo model is a consequence of migration and not necessarily an increase in proliferative ability. An important observation is that many of the significant and more dramatic changes were seen in M1-polarizing macrophages co-treated with rSLPI, and not in M0 macrophages treated with rSLPI. We can hypothesize that IFN- γ , the treatment used to polarize M0 macrophages to an M1 phenotype, may interact with SLPI. These molecules have opposing functions, as SLPI is an anti-inflammatory molecule while IFN- γ is generally proinflammatory. While we still observed modest changes in the M0 + SLPI group, this may explain why markers of both M1 and M2 were highly affected in the M1 + SLPI group.

Effect of SLPI on cell adhesion molecules affecting immune cell interactions: The decrease in IgSF CAM in MPK cells that express *Slpi* could further support that tumors with high SLPI expression have less capacity for adhesion to immune cells and therefore have higher capacity for immune evasion and immunosuppression. This analysis was done only with the MPK

model, and additional models, protein, and functional experiments need to be completed to solidify this interpretation.

4.5. SLPI as an angiogenic factor

Though we have not investigated many angles that could explain the increased aggressiveness in the ID8 *Slpi*^{OE} group, there are studies that suggest alternative mechanisms of SLPI action. While there is contradictory evidence of SLPI's effect on angiogenesis in different contexts^{144,145}, studies have shown that SLPI induces vasculogenic mimicry to supply the tumor with nutrients and oxygen from the blood^{146,147}. Angiogenesis is the process of developing and branching new blood vessels from pre-existing ones¹⁴⁸. It is utilized in tumor growth by secretion of proangiogenic factors to supply the tumor with nutrients and oxygen as it grows¹⁴⁹. Vasculogenic mimicry is a process of blood supply in tumors that is independent of angiogenesis. This was first described in melanomas, where tumors establish a circulatory network through creation of channels lined by ECM and tumor cells without the need for endothelial cells¹⁵⁰. One study by Chueajedton et al. determined that SLPI was upregulated in human cholangiocarcinoma (CCA) tissues, is associated with advanced CCA stages, is negatively correlated with overall survival, and that overexpression of SLPI increases tumorigenicity in their models¹⁵¹. By applying conditioned media from control and SLPI-overexpressing cells to endothelial cells, they found no difference in proliferation, migration, or tube formation and therefore concluded that SLPI does not affect angiogenesis. However, SLPI-overexpressing cells formed more tubular structures than control cells and were associated with increased expression of VEGFA and VE-cadherin and reduced expression of N-cadherin. These molecular changes are representative of tumor cells that can perform vascular mimicry^{151,152}. Interestingly, RNA-seq analysis of our murine SLPI-modified

cell lines showed that MPK *Slpi*^{-/-} cells have increased expression of *Mmp-2* and *Twist-1* (data not shown), which are known to be involved in vascular mimicry¹⁵².

Limitations

Certain limitations concerning this study need to be considered. First, many of our results were not tested in human models, which limits translation of our findings because we cannot account for inherent differences such as CD4⁺ receptor expression on immune cells. Additionally, SLPI expression was modified in in vitro human models through knockdown only, not allowing us to observe the effects of *SLPI* overexpression or complete knockout in human cell lines. Second, the RNA sequencing analysis was only robust in one *Slpi*-modified cell line pair, which presents a limited view of SLPI's effect in cancer cells since *Slpi*-specific expression patterns cannot be evaluated for consistency across different cell lines. Additionally, these results were used to explain patterns seen in our spheroid assays, while RNA was extracted from adherent cell lines, and these only represent gene expression levels, not protein or functional readouts. Third, we only measured total metastasis weights in our survival studies. To better translate our in vitro spheroid findings to our in vivo findings, the size, number, and location of metastases formed in vivo will need to be measured. Lastly, very few markers characterizing B cells and NK cells were used in the lymphoid panel of the flow cytometry analysis, but a drastic phenotypic shift was observed in the populations in the peritoneal wash, which remains to be more fully characterized.

Conclusion

Taken together, we have established that *Slpi* is upregulated by the MEK pathway in mouse ovarian cancer cell lines, but it has no effect on cell proliferation in vitro and has generally no effect on chemosensitivity. We showed that MPK *Slpi*^{-/-} cells had increased metastases consistent with their ability to form more numerous spheroids in suspension cultures. Regarding SLPI's role

in the TME, we conjecture that SLPI is secreted by tumor cells, which send protective and immunosuppressive signals to the immune environment, manifested by increased migration of macrophages, eosinophils, and DCs to the tumor by initiating a type 2-like immune response. Notably, SLPI acts through modulation of the macrophage phenotype by inducing an M2a-reminiscent phenotype. Mechanistically, SLPI's effect on the immune composition and phenotype of the TME is most likely mediated by SLPI's ability to inhibit NF- κ B signaling. There is evidence that SLPI inhibits downstream NF- κ B in a variety of contexts, specifically in macrophages, where it inhibits M1 polarization^{125,153}. Going forward, SLPI should be investigated as a therapeutic target in the context of restoring anti-tumor immunity, which is lost with SLPI.

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