TLR5 Signaling Causes Dendritic Cell Dysfunction and Orchestrates the Failure of Immune Checkpoint Therapy Against Ovarian Cancer

Mitchell Thomas McGinty

Clifton, Virginia

B.S., Biology, James Madison University, 2014

M.S., Biomedical Science, Icahn School of Medicine at Mount Sinai, 2018

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Department of Microbiology, Immunology, and Cancer Biology

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Abstract

Ovarian cancer is the seventh most common and eighth leading cause of cancer death in women globally. Diagnosis is challenging due to vague symptoms and a lack of effective screening methods, leading to late-stage diagnosis and poor prognosis. Despite progress in surgical techniques and chemotherapy regimens, overall survival improvements remain modest. New approaches such as immunotherapy have shown limited success despite their effectiveness in other cancers. Patients bearing ovarian tumors infiltrated with high frequencies of T cells are associated with a greater survival probability. However, therapies to revitalize tumor-associated T cells, such as PD-L1/PD-1 and CTLA-4 blockade, are ineffective for the treatment of ovarian cancer. Thus, there is a demand to understand why immunotherapy is ineffective against ovarian cancer.

A study from a parent lab found that Toll-Like Receptor 5 (TLR5) signaling impacts inflammation, anti-tumor immunity, and the clinical outcome of ovarian cancer patients. However, the influence of TLR5 signaling on immunotherapy remained unexplored. In this dissertation, we demonstrate that in late-stage murine ovarian cancer models, TLR5 signaling, the only known ligand for which is bacterial flagellin, leads to failure of immune therapy. Mechanistically, we demonstrate that chronic TLR5 signaling on dendritic cells impairs the differentiation of functional cDC1 subsets within the tumor microenvironment (TME). Instead, chronic TLR5 signaling biases precursor cells towards myeloid-associated subsets expressing high levels of PD-L1. This culminates in impaired activation of CD8 T cells, reducing CD8 T cell function and persistence within the ovarian tumor microenvironment. Expansion of cDC1s *in situ* using FMS-related Tyrosine Kinase 3 ligand (FLT3L) in combination with PD-L1 blockade achieved significant survival benefit, but only in TLR5 KO mice, whereas no benefit was observed in the presence of TLR5 signaling. Thus, we identify a host-intrinsic mechanism leading to failure of immune therapy for ovarian cancer, demonstrating that chronic TLR5 signaling on DCs is a barrier limiting the efficacy of immune

therapy. Clinically, roughly 7.5% of the general population harbors a TLR5 SNP that diminishes TLR5 signaling and is associated with increased long-term survival for ovarian cancer patients. Therefore, patients who express the TLR5 SNP may immediately benefit from anti-PDL1 therapy, and those without the SNP, TLR5 antagonism.

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List of abbreviations:

2F8 – Ovarian cancer cell line

Abx – Antibiotics

Anti-4-1BB – Tumor necrosis factor ligand superfamily member 9

APCs – Antigen-presenting cells

BATF3 – Basic leucine zipper ATF-like transcription factor 3

CCL – C-C motif chemokine ligand

CCR – C-C motif chemokine receptor

CD – Cluster of differentiation

cDCs – Conventional dendritic cells

CDP – common DC progenitors

CFSE – Carboxyfluorescein succinimidyl ester (fluorescent cell staining dye)

CITE-seq – cellular indexing of transcriptomes and epitopes by sequencing

CLEC9a – C-type lectin domain family 9 member A

CT – Computed tomography

CTLA-4 – Cytotoxic T-lymphocyte associated protein 4

CXCL – Chemokine (C-X-C motif) ligand

DAMPs – Damage-associated molecular patterns

DCs – Dendritic cells

EGF – Epidermal growth factor

ELISA – Enzyme-linked immunosorbent assay

EMT – Epithelial-mesenchymal transition

EOC – Epithelial ovarian cancer

ER – Endoplasmic reticulum

ETV6 – ETS variant transcription factor 6

FDA – Food and Drug Administration

FLT3L – Fms-related tyrosine kinase 3 ligand

FMT – Fecal microbial transplant

FRET – Fluorescence resonance energy transfer

GI – Gastrointestinal

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HMGB1 – High mobility group box 1

HRD – Homologous recombination deficiency

ICB – Immune checkpoint blockade

ICIs – Immune checkpoint inhibitors

ID2 – Inhibitor of DNA binding 2

IDO – Indoleamine 2,3-Dioxygenase

IFN – Interferon

IKK – Inhibitor of nuclear factor-κB (IκB) kinase

IL – Interleukin

IP – Intraperitoneal

IRF – Interferon regulatory factor

IV – Intravenous

LPS – Lipopolysaccharide

M1 – Classically activated macrophage

M2 – Alternatively activated macrophage

MAPK – Mitogen-activated protein kinases

MBC – Mixed bone marrow chimera

MC38 – Murine colon adenocarcinoma cell line

MDP – monocyte/dendritic progenitor

MDSCs – Myeloid-derived suppressor cells

MHC – Major histocompatibility complex

moDCs – Monocyte-derived dendritic cells

MRI – Magnetic resonance imaging

MyD88 – Myeloid differentiation primary response protein 88

NFIL3 – Nuclear Factor, Interleukin 3 Regulated

NF-κB – Nuclear factor-kappa B

NK cells – Natural killer cells

NOS – Nitric oxide synthase

OS – Overall survival

OVA – Ovalbumin

OvCa – Ovarian Cancer

PAMPs – Pathogen-associated molecular patterns

PAP – Prostate acid phosphatase

PARP – Poly(ADP-ribose) polymerase inhibitors

PBMCs – Peripheral blood mononuclear cells

PD-1 – Programmed cell death protein 1

pDCs – Plasmacytoid dendritic cells

PD-L1 – Programmed death-ligand 1

PFS – Progression-free survival

PGE2 – Prostaglandin E2

Poly IC – Polyinosinic:polycytidylic acid

PRR – Pattern recognition receptor

ROS – Reactive oxygen species

ScRNA-seq – Single-cell RNA sequencing

SEER – Surveillance, Epidemiology, and End Results Program

SNP – Single nucleotide polymorphism

STAT – Signal transducer and activator of transcription

TAMs – Tumor-associated macrophages

tdLN – Tumor-draining lymph nodes

TGF-β1 – Transforming growth factor Beta 1

Th1 – T helper 1 cell

TILs – Tumor-infiltrating lymphocytes

TLR – Toll-like receptor

TME – Tumor microenvironment

TNF-α – Tumor necrosis factor-alpha

Treg – Regulatory T cells

VEGF – Vascular endothelial growth factor

XBP1 – X-box binding protein 1

XCR1 – X-C motif chemokine receptor 1

Chapter 1: Introduction

1.1 Ovarian Cancer Epidemiology

Ovarian cancer (OvCa) is the deadliest gynecologic malignancy in the United States, with an estimated 19,880 new cases diagnosed and 12,810 deaths in 2022[1]. Ovarian Cancer is classified into different subtypes, with epithelial cancer accounting for the majority (90%) of cases while the remaining (10%) consist of sex-cord stromal or germ cell tumors[2, 3]. Non-epithelial ovarian cancers are typically symptomatic and are found in young women at early stages. Consequently, these cancers have cure rates of 85% to 90% when treated with chemotherapy, usually at stage IA[4, 5]. In contrast, roughly 70% of women with epithelial ovarian cancer are diagnosed in advanced stages as there is no successful screening program for early discovery, and symptoms are difficult to detect[6]. Epithelial cancer survival rates are SEER stage-dependent with localized, regional, and distant stage cancer having a 93%, 75%, and 31% 5-year relative survival rate, respectively[1]. Independent of stage, epithelial ovarian cancer has a survival rate of 49% and is the leading cause of death from gynecologic cancer in the United States[7]. Epithelial ovarian cancer has two major subtypes: non-mucinous (97%) and mucinous (3%). Mucinous tumors can be further divided by histology: serous (70%), endometroid (10%), clear cell (10%), and unspecified (5%)[3, 8, 9]. The serous group can be further defined as high grade or low grade.

There is no clear cause for ovarian cancer. However, there are several risk factors associated with the disease. The most prominent association is age, with the median age of diagnosis being 63, as a longer history of ovulatory cycles and cellular divisions creates a greater opportunity for malignant neoplasms. Supporting this notion, the use of oral contraceptives that inhibit ovulation is associated with less risk for ovarian cancer[10]. Regarding genetic risk, germline mutations in BCRA1 and BRCA2, which are involved in the repair of double-strand DNA breaks, have a lifetime risk of 35–60% and 12–25%, respectively[8]. Mutations in RAD51C, RAD51D, and PALB2, which are involved in DNA repair pathways, are implicated but have weaker correlations with disease[8].

Additional risk factors for ovarian cancer include a history of endometriosis, obesity, and hormone replacement therapy[9]. Considering epithelial ovarian cancer is the most prevalent and has the worst outcomes, my dissertation is focused primarily on understanding and improving survival for this form of ovarian cancer.

1.2 Epithelial Ovarian Cancer Clinical Presentation

A significant contributing factor to ovarian cancer being diagnosed at advanced stages is a lack of apparent alarm symptoms[11]. Epithelial ovarian cancer presents with vague and nonspecific gastrointestinal, urologic, or nonacute abdominal/pelvic symptoms such as bloating, early satiety, or discomfort[12]. Because these symptoms either go unrecognized or do not manifest until advanced stages when outcomes are poor, ovarian cancer is referred to as the "silent killer[13]." Identification of an adnexal mass by pelvic examination or imaging is required for diagnosis. Further examination by computed tomography (CT) or magnetic resonance imaging (MRI) of the chest, abdomen, and pelvis is needed to identify the full extent of peritoneal dissemination[12]. Ultimately, there is a need to improve current diagnostic strategies to detect ovarian cancer. Due to the late detection of ovarian cancer in clinics because of subtle symptoms easily confused with other conditions, our mouse models are designed to replicate late-stage disease.

1.3 Current Treatment Strategies

The current standard for ovarian cancer disease management (stage II-IV) is cytoreduction, followed by chemotherapy with carboplatin-paclitaxel[14]. Carboplatin is a second-generation platinum agent that exerts cytotoxicity by induction of carboplatin-DNA cross-linkages, leading to DNA destruction during replication. Paclitaxel prevents mitosis by inhibiting depolymerization of microtubules by binding to the β-tubulin subunits. It has been proposed that therapeutic synergy is achieved when paclitaxel, which hinders the repair of DNA, enables the build-up of carboplatin-DNA linkages and contributes to the death of quickly dividing cancer cells[15].

The dosage of carboplatin and paclitaxel is dependent on stage, with a typical treatment schedule of IV administration every three weeks for carboplatin and paclitaxel weekly in a dose-dense manner[16]. Paclitaxel is usually dosed at 175 mg/m^2 IV over 3 hours, while carboplatin is dosed at an area under the time vs. concentration curve (AUC) of 5-6 IV over 1 hour[16]. The combination of platinum-based therapy with paclitaxel was first established in 1993 and dramatically improved outcomes. Since then, this strategy has been refined over the years with incremental improvement. More recently, there have been developments in treatment strategies that incorporate intraperitoneal (IP) delivery over intravenous (IV). It has become established that IP chemotherapy is associated with significantly improved overall survival of 81% v 71% (3-year overall survival of Stage III cytoreduced patients), compared with IV chemotherapy[17].

Despite optimization of chemotherapy dosage and treatment strategies, epithelial ovarian cancers 5-year survival rate has remained around 49% for decades[18]. This is partly due to chemotherapeutic strategies failing to manage late-stage disease. Recently, poly(ADP-ribose) polymerase (PARP) inhibitors have been included in the treatment plans for certain patients either concurrently or as maintenance therapy with chemo. This approach takes advantage of cancer cell dependency on PARP for DNA repair. Supporting this idea, PARP inhibitors show particular benefit in patients with BRCA mutations and homologous recombination deficiency (HRD)[19, 20]. Three PARP inhibitors olaparib, niraparib, and rucaparib have received regulatory approval for ovarian cancer treatment as maintenance therapy. These inhibitors significantly improve PFS; however, their impact on overall survival remains unclear[21]. Ongoing research focuses on identifying optimal patient selection criteria, overcoming resistance mechanisms, and exploring combination therapies to enhance efficacy.

Overall, current treatment approaches for ovarian carcinoma have become increasingly complex, aiming to account for the spatial and temporal heterogeneity of the disease, which consists of multiple subtypes. The molecular diversity within these subtypes leads to varying resistance levels

to standard platinum-based chemotherapy. Adding to the complexity, spatial heterogeneity within a single patient can drive progressive evolutionary divergence, accompanied by changes in clonal evolution, the tumor microenvironment (TME), immune tolerance, resistance mechanisms, and therapeutic sensitivity. Ongoing trials shown in Table 1 explore new combination strategies, such as PARP inhibitors with immune checkpoint inhibitors, with great anticipation. However, the optimal sequencing of novel therapies in ovarian cancer is yet to be determined.

1.4 Ascites

The hallmark of ovarian cancer biology is the accumulation of fluid and cells in the abdomen, known as ascites, which is comprised of tumor cells, blood cells, lymphocytes, mesothelial, fibroblasts, endothelial cells[22]. Ascites also includes acellular factors such as cytokines, metabolites, and growth factors. These cellular and acellular factors facilitate tumor cell expansion and immune evasion, enabling phenotypic changes to the ovarian TME that drive poor survival outcomes.

Ascites volume has been found to correspond with patient outcome as median survival of patients with <1800 mL ascites is 58 months while patients with >1800 mL of ascites is 28.6 months[23]. Fluid volume also correlates with the number of metastatic sites. Specifically, it has been observed that an average ascetic fluid volume of 3800ml corresponds with more than three metastatic sites[23]. Interestingly, currents of ascitic fluid can carry tumor cells through the peritoneum to distal sites and promote cell-cell adhesion via mechanical pressure[24]. In addition to the physical forces ascites exerts on the TME, cytokines and growth factors influence outcomes. Pro-inflammatory cytokines IL-6 and IL-8 are the two most abundant cytokines in ascitic fluid and are associated with worse outcomes and poor prognostic factors[22, 25]. These cytokines play a prominent role in driving a transition from acute to chronic inflammation by acting on innate and adaptive immunity. IL-6 in an acute setting promotes CD8 T cell trafficking; however, when chronically released, IL-6 interferes with the maturation of DCs necessary for

priming CD8 T cells, thus promoting immune suppression[26-28]. Furthermore, tumor-derived IL-8 promotes trafficking of neutrophils and myeloid-derived suppressor cells (MDSCs), which dampen anti-tumor responses[29]. Specifically, these MDSCs are found to secrete IL-10, which is associated with shorter progression-free survival in ovarian cancer patients[30]. Additionally, regulatory B cells that produce IL-10 and are induced by IL-6 are enriched in ovarian cancer ascites and suppress CD8+ T cell responses[31]. IL-10 inhibits T cell proliferation, hinders dendritic cell maturation, and co-stimulatory molecule expression in the TME. Together with cytokines, growth factors VEGF and EGF are present in ascites and promote angiogenesis, leading to tumor growth and metastasis[32, 33]. Specifically, VEGF levels correlate with ascites development, volume of ascites, and poor survival rates[34-36].

Ultimately, ascites plays a unique role in the ovarian TME, driving poor survival outcomes and an immunosuppressive environment. Therefore, ascites is a hurdle to developing successful therapies against ovarian cancer. Our epithelial ovarian cancer cell lines specifically drive ascites accumulation through overexpression of VEGF, which allows us to study the ovarian TME more accurately while considering the impact of ascites.

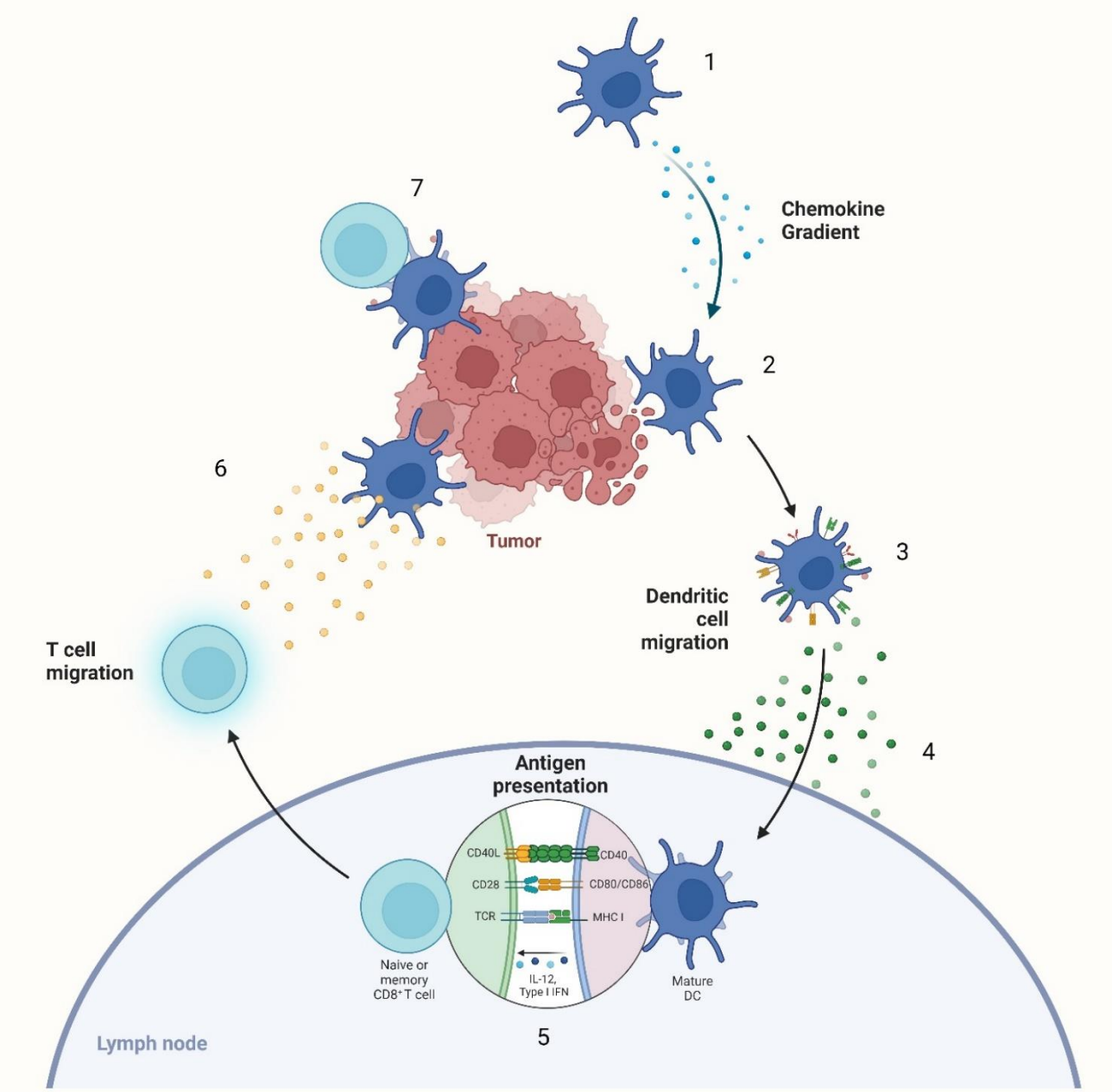

1
Chemokine Gradient
7
2
6
Tumor
3
Dendritic cell migration
T cell migration
4
Antigen presentation
CD40L
CD40
CD28
CD80/CD86
TCR
MHC I
IL-12, Type I IFN
Naive or memory CD8⁺T cell
Mature DC
Lymph node
5

Figure 1.1. Overview of Dendritic cell functional response to cancer

Dendritic cells (DCs) are specialized myeloid-derived cells that capture endogenous or exogenous antigens, process them, and present them to T lymphocytes. A simplified overview of how dendritic cells are understood to respond to cancer is as follows: (1) DCs are recruited to the TME via local production of chemokines (CCL4, CCL5, CCL20), or resident DCs are expanded via growth factors (FLT3L and GMCSF). (2) DCs proceed to capture exogenous cancer antigen from dead or dying cancer cells in the TME (3) Cytokines, DAMPs, and PAMPs from local immune cells, cancer cells, and bacteria promote the maturation of DCs to express the chemokine (CCR7) and co-stimulatory receptors (CD80, CD86, CD40) for T cell interaction. (4) Now matured, DCs follow a chemokine gradient (CCL19 and CCL21) to the tumor-draining lymph node. (5) DCs are further recruited to T-cell-rich zones within the lymph node where antigen-presentation via MHCI or MHCII and co-stimulation with naïve CD4 or CD8 T cells occurs. (6) These now antigen-experienced effector T cells are recruited to the TME via chemotactic gradients (CXCL9, CXCL10) produced from tumor-infiltrating DCs. (7) Finally, DCs sustain intratumoral T-effector response by local re-priming. Overall, DCs orchestrate T cell response to tumors by bringing them antigen, recruiting them to the TME, and sustaining them while they are there.

1.5 Heterogeneity and immune landscape of ovarian cancer

The immune cell composition within the ovarian TME includes innate immune cells such as dendritic cells, neutrophils, myeloid-derived suppressor cells, natural killer cells, tumor-associated macrophages (TAMs), and adaptive immune cells like B and T cells. Tumor-infiltrating lymphocytes (TILs) in ovarian carcinomas serve as an independent prognostic factor, with TIL-positive tumors showing significantly better progression-free survival and overall survival compared to TIL-negative ones[37]. In OvCa, the TME impairs DC function, weakening T cell-mediated antitumor immunity[38, 39]. TAMs exhibit immunomodulatory behavior influenced by signals from the microenvironment. Interferon-γ (IFNγ) promotes an M1 phenotype with tumor-killing properties, while IL-13 and IL-4 drive an M2 phenotype that secretes immunosuppressive cytokines[40]. M2 TAMs increase with disease progression and correlate with worse OvCa prognosis[41]. Recent studies have leveraged single-cell RNA sequencing (scRNA-seq) and bulk RNA-seq to identify gene signatures predictive of ovarian cancer outcomes and treatment responses. One study identified four ovarian cancer genes (SLAMF7, GNAS, TBX2-AS1, LYPD6) associated with M2 tumor-associated macrophages that predict patient prognosis[42]. Several papers reported prognostic signatures derived from tumor cells and the tumor microenvironment, including a set of EMT-associated genes (NOTCH1, SNAI2, TGFBR1, and WNT11) that can be used to predict poor outcomes in OvCa[43]. Another study identified five signature genes (IGFBP7, JCHAIN, CCDC80, VSIG4, and MS4A1) that showed enrichment in cellular immunity and immune cell interaction pathways and had elevated expression of immune checkpoint molecules, suggesting they may benefit more from immune checkpoint inhibitor therapy[44]. The development of predictive gene signatures for ovarian cancer is promising; however, different studies often find unique predictive signatures, speaking to the extent of tumor heterogeneity.

The progression of ovarian carcinomas is linked to both temporal and spatial heterogeneity in the TME. In early-stage OvCa, the microenvironment tends to be immunologically cold, with fewer

CD8+, CD4+, Tregs, and plasma cells in the epithelial compartment compared to advanced stages[45, 46]. Immune cell infiltration changes over time across different disease stages and varies spatially between distinct lesions and within tumors. These immune infiltrates may impose selective pressure over time, shaping the patterns of malignant spread and clonal diversity in OvCa[47]. One contributing factor is the peritoneal cavity, lacking physical barriers, which allows for the early spread of ovarian cancer to distant sites. Consequently, cancer and immune cells interact across different microenvironments, with disease spread in remote peritoneal locations[47]. Immune responses align with disease locations and mutations, influencing immune recognition and evasion. For instance, primary tumors tend to have a more dysfunctional T-cell response relative to metastatic tumors[48].

1.6 DCs are vulnerable to the ovarian TME

Dendritic cells are crucial in regulating immune responses against cancer. They are uniquely positioned in the tumor microenvironment to control host immunity. However, ovarian cancer can manipulate DCs to become immunosuppressive, hindering anti-tumor responses. For instance, depletion of CD11c+ DCs early in mice accelerates ovarian tumor expansion, but DC depletion at advanced stages significantly delays aggressive malignant progression[39]. Notably, the authors found tumor cell-derived PGE2 and TGF-β1 promoted DC immunosuppressive function and elevated PD-L1 expression that could be reduced with neutralizing anti-PGE2 or anti–TGF-β1[39]. Similarly, it has been observed that tumor-infiltrated DCs gradually adopt an immunosuppressive phenotype as the tumor progresses over time, represented by increased PD-1 expression[49]. These studies suggest that DCs transition from being anti- to pro-tumorigenic throughout ovarian cancer progression. It has been observed that factors such as ER stress on DCs contribute to their immunosuppressive behavior and dysfunction. One prime example of this is deleting or silencing an ER stress sensor XBP1 in tumor-associated dendritic cells, which restores their immunostimulatory capacity and extends survival[50]. These suppressed DCs contribute to tumor

immune evasion through reduced cytokine production and induction of regulatory T cells through IL-10 secretion[51].

This suppressive phenotype can be attributed to impairment of DC maturation, leading to the build-up of immature DCs in ovarian cancer patients. Factors interrupting DC maturation include immune-modulating molecules in the TME like IL-6, IL-10, and VEGF, tumor-derived soluble mediators and exosomes, activation of the oncogene STAT3 in DCs, ER stress response, and abnormal intracellular lipid accumulation[50, 52]. These factors reduce the expression of co-stimulatory molecules, secretion of pro-inflammatory cytokines, DC lymph node chemotaxis, DC differentiation, and lifespan while inducing tolerogenic phenotypes in DCs. Immature tolerogenic DCs suppress anti-tumor immunity by producing fewer pro-inflammatory cytokines and inducing immune suppressive cytokines such as IL-10. They also express enzymes like nitric oxide synthase (NOS) and Indoleamine 2,3-Dioxygenase (IDO) that negatively regulate T cell functions, suppress tumor-infiltrated lymphocyte proliferation, promote Treg differentiation, induce T cell anergy, and support tumor angiogenesis and metastasis. Increased frequencies of IDO+ DCs in tumor-draining lymph nodes have been observed in epithelial ovarian cancer (EOC) patients[53].

Despite these challenges, DC-based vaccines were tested as a potential therapeutic approach for ovarian cancer, with ongoing research focusing on optimizing their efficacy. In clinical trials, the response rates to DC vaccination in OvCa are poor, with an average response rate of only 10-15%[54]. Most clinical trials have utilized monocyte-derived DCs (moDCs) as a vaccination agent due to their superior ability to uptake antigen relative to cDCs, however this may not be the optimal approach, as moDCs have high plasticity and are vulnerable to becoming immunosuppressive within the tumor microenvironment. Several factors derived from tumors and immune cells, such as prostaglandin E2, reactive oxygen species (ROS), and IL-6, can impair the development, survival, and function of moDCs, often resulting in moDCs expressing PD-L1 and reduced co-stimulatory markers. Understanding how to shield DCs from becoming

immunosuppressive in the OvCa tumor microenvironment is crucial for maintaining anti-tumor responses and developing effective immunotherapies. Additionally, establishing what types of DCs are ideal for OvCa cancer therapy remains an ongoing question.

In conclusion, immunosuppressive signals in the ovarian cancer microenvironment cause DCs to become dysfunctional. Infusing functional DCs into the body could bypass the tumor microenvironment and directly interact with T cells in the lymph nodes, thereby compensating for the dysfunctional state of endogenous DCs. This approach forms the basis for using DC vaccines to restore the ability to present tumor antigens and elicit anti-tumor responses. However, without understanding or preventing the causes of DC dysfunction, transferred DCs are likely to adopt the suppressive behavior of endogenous DCs, highlighting a significant gap in current knowledge.

1.7 Types of DCs in the ovarian TME

cDC1s

Conventional type 1 dendritic cells (cDC1s) are a specialized subset of dendritic cells characterized by surface markers CD8a, CD103, XCR1, and CLEC9a in addition to high expression of transcription factors IRF8 and BATF3 in mice. cDC1s play a crucial role in cancer immunity by efficiently cross-presenting tumor antigens via MHC-I to CD8+ T cells, initiating potent cytotoxic T cell responses. The rejection of certain tumors, such as B16-OVA melanoma and fibrosarcoma, is lost without MHC-I expression on cDC1s[55]. Responses to immune checkpoint blockades (ICB) including anti-4-1BB (CD137), anti-PD-1, and anti-CTLA4, depend on cDC1s[56, 57]. Additionally, depletion of cDC1s prior to ICB treatment abolishes anti-tumor efficacy, while depletion after treatment onset has a moderate effect in several tumor models[58]. As a subset of DCs, cDC1s in the ovarian TME are vulnerable to becoming immunosuppressive. Consequently, cDC1s are often reduced in frequency and function in OvCa patients, especially after chemotherapy[38, 59]. The presence of cDC1s in tumors is associated with better patient survival and response to immunotherapy[60]. However, there are currently no clinical trials using

cDC1s as a vaccine against ovarian cancer, despite evidence that cDC1 vaccination strategies have shown efficacy in several mouse models of melanoma[61, 62]. This is because manufacturing high numbers of cDC1s is complex, and optimization is needed to produce high numbers of cDC1s from patient samples. Alternatively, FLT3L, a growth factor essential for cDC1 development, has demonstrated safety and dose-dependent expansion of cDCs in patients[63]. Furthermore, the administration of Flt3L in cancer patients leads to significant increases in circulating DCs and may enhance DC tumor infiltration[64, 65]. Thus, it serves as a potential avenue for expanding cDC1s *in vivo*. It is currently unclear if FLT3L will yield therapeutic efficacy against ovarian cancer. There is only one ovarian cancer trial (Phase I) examining the safety of FLT3L in combination with a CD40 antibody, pembrolizumab, or chemotherapy (KEYNOTE-A23). Strategies to boost cDC1 numbers appear limited. However new findings that NK cells recruit cDC1s to tumors via release of chemoattractant CCL5 and XCL1 introduce new therapeutic opportunities[66]. Overall, attraction and preservation of cDC1s in the TME is a viable therapeutic target that needs further investigation in ovarian cancer.

cDC2s

Conventional dendritic cells type 2 (cDC2s) play a developing role in orchestrating immune responses, particularly in tumor immunity. cDC2s are characterized by high IRF4 expression and require additional transcription factors such as RELB, ZEB2, KLF4, and NOTCH2[67]. The subset's transcriptional programs are context-dependent and heterogeneous, making defining a uniform panel of markers challenging. For example, respiratory viruses can induce the emergence of 'inflammatory cDC2s', which express the Fc receptor CD64, shared with monocyte-derived cells, and IRF8, expressed by cDC1s[68]. For the most part, cDC2s can be identified by surface markers like BDCA-1 (CD1c), SIRPα (CD172a), CLEC10A (CD301b or MGL), CD11b, CD11c, CD5, and MHC-II with some tissue type variability[69]. Although these markers are considered cDC2-specific,

they often overlap with other myeloid cells, making it difficult to attribute specific functions to cDC2s.

In cancer, the role of cDC2s is less clear due to their heterogeneity and functional diversity. However, they are largely understood to drive CD4 T cell responses. Tumor-infiltrating cDC2s drive CD4 T cell responses by capturing antigens and migrating to tumor-draining lymph nodes (tdLN) to prime naïve CD4+ T-cells[70]. Studies have shown that a higher cDC2:Treg ratio is predictive of CD4+ T-cell infiltration in the tumor microenvironment (TME), with higher ratios associated with robust CD4+ T-cell infiltration[70]. Recent findings suggest that cDC1s are also involved in early CD4+ T-cell priming, challenging the conventional understanding of their exclusive role in CD8+ T-cell activation[55]. Future research should focus on the interplay between cDC1 and cDC2 subsets and their regulation of T-cell infiltration and treatment response in cancer.

Experimental mouse models have shown that cDC2s can be pro-tumorigenic. CD11b+ dendritic cells (DCs) infiltrating B16 melanoma exhibited reduced capacities for antigen uptake, presentation, and migration to tumor-draining lymph nodes compared to normal skin DCs[71]. Additionally, cDC2s promoted the growth of MC38 tumor cells and inhibited Th1 and TNF-α–producing cell infiltration into the tumor, suggesting that cDC2s can attenuate antitumor immunity by limiting antitumor CD4+ effector T cell responses[72]. In humans, cDC2s co-expressing CD1c and CD14 are enriched in individuals with advanced cancer[69]. cDC2s, along with Tregs and exhausted T cells, were found in higher numbers in lung cancer tissue compared to normal lung tissue, implying that human cDC2s may induce immunosuppression and correlate with poor prognosis[69].

Ultimately, cDC2s can alter the tumor immune landscape through T cells to be tumor-protective or tumor-repressive. However, it is unclear what dictates this response across various cancers.

The answer to this question lies in understanding cDC2 heterogeneity. The cDC2 compartment needs to be deconvoluted to organize multiple sub-populations and discern different potential functionalities in different cancer contexts.

pDCs

Plasmacytoid dendritic cells (pDCs) are specialized immune cells that play a crucial role in antiviral immunity through their rapid and massive production of type I interferons (IFNs) in response to viral nucleic acids. pDCs detect pathogens primarily through Toll-like receptors 7 and 9, initiating innate immune responses and linking them to adaptive immunity[73]. In mice, plasmacytoid dendritic cells (pDCs) express a distinct combination of markers, including SIGLEC-H, PDCA1, CD11c, B220, and Ly6C/G[74, 75]. They also express Ly49Q in the bone marrow, which helps define their developmental stages and precursor subsets[74].

While pDCs can potentially activate anti-tumor responses through type I interferon production, tumor-associated pDCs often exhibit immunosuppressive functions[51, 76]. pDCs infiltrating ovarian tumors are associated with poor prognosis and early relapse[51]. Furthermore, it is observed they contribute to immune tolerance by inducing regulatory T cells, particularly ICOS+ Foxp3+ Tregs, which suppress effector T cell functions[77]. Tumor-associated pDCs show reduced responsiveness to TLR stimulation and decreased production of pro-inflammatory cytokines, less IFN-α, TNF-α, IL-6, macrophage inflammatory protein-1β, and RANTES[51, 76]. Additionally, pDCs induce immunosuppressive CD8+ regulatory T cells and promote angiogenesis through TNF-α and IL-8 production[78]. Overall, pDCs tend to be pro-tumorigenic in the ovarian TME, and are a potential target for therapeutic inhibition.

moDCs

Monocyte-derived dendritic cells (MoDCs) originate from monocytes in peripheral blood. MoDCs are commonly induced by GM-CSF and IL-4, in *in vitro* bone marrow cultures. They are

distinguished from other cell types by CD11c, CD1a, CD14, CD83, CD80, CD86, and CD40, like conventional DCs[79]. Consequently, there is difficulty distinguishing them from cDC2s by surface markers alone. Single-cell RNA sequencing has revealed heterogeneity within MoDCs, identifying subtypes resembling cDC2s but with distinct transcriptional features[80]. Unlike cDCs, MoDCs require IRF4 but not BATF3 for differentiation and cross-priming capacity[81].

In the context of cancer, MoDCs can present antigens and activate CD8+ T cells, potentially enhancing anti-tumor responses in the short-term[82]. However, MoDCs from late-stage cancer patients often exhibit dysfunctional phenotypes and present less antigen[83]. For example, these cells are found to preferentially induce regulatory T cells in breast cancer patients, contributing to immune evasion[84]. Like other DC subsets, the tumor microenvironment can polarize MoDCs into an immunosuppressive state where they express less costimulatory molecules, reduced antigen presentation, and abnormal motility[85, 86]. Monocyte-derived dendritic cells from ovarian cancer patients have a lower capacity to stimulate lymphocyte proliferation compared to healthy controls as measured by allogeneic mixed leukocyte reaction[87]. Additionally, exposing Mo-DCs to peritoneal fluid from ovarian cancer patients can promote Treg differentiation from naive CD4+ lymphocytes[88], suggesting the ovarian TME contributes to the polarization of MoDCs to become immunosuppressive.

The plasticity of MoDCs suggests potential for therapeutic manipulation. Studies have demonstrated that MoDCs can also be activated through Toll-like receptors to produce pro-inflammatory cytokines and stimulate T-cell responses[89]. Potentially serving as a strategy to reverse polarization in the TME. MoDCs remain a crucial target for cancer immunotherapy strategies, with ongoing research exploring ways to enhance their immunostimulatory functions and overcome tumor-induced suppression.

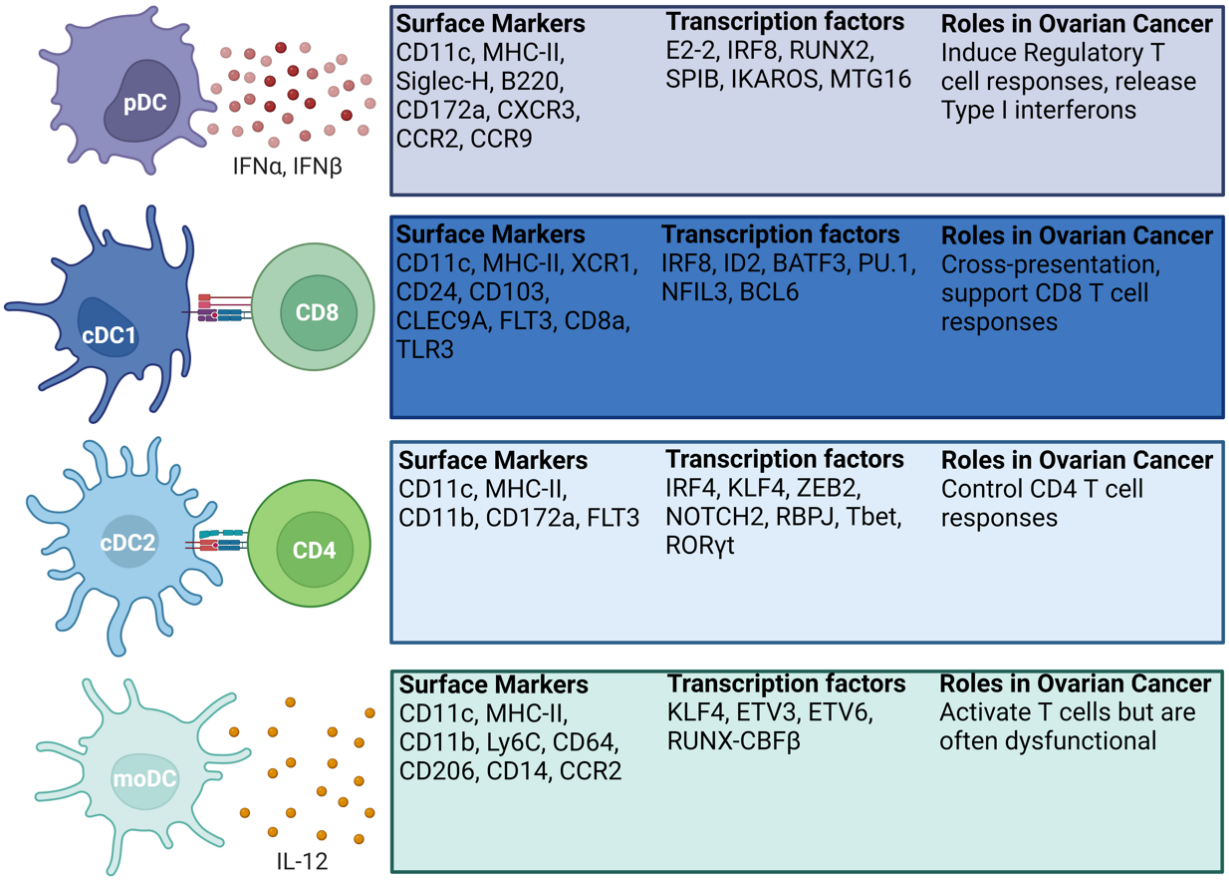


Figure 1.2. Characterization of Ovarian Cancer DCs

Commonly expressed surface markers and transcription factors used to identify subsets of dendritic cells that are present in the ovarian tumor microenvironment. General roles of these populations are also described as the field currently understands them.

1.8 The role of T cells in Ovarian cancer

T lymphocytes are essential for immune surveillance and adaptive immunity, efficiently patrolling the body to detect and respond to infections and cancer. The presence of CD8 T cells across ovarian cancer tissues predicts ovarian cancer survival outcomes. Intratumoral CD3+ and CD8+ T cells correlate with better progression-free and overall survival rates in advanced epithelial ovarian carcinoma[37]. Additionally, intraepithelial CD8+ T cells are an independent prognostic factor, particularly in serous ovarian cancer, and are associated with BRCA1 loss[90]. This correlation is not limited to solid tumors. High densities of tumor-infiltrating CD8+ effector memory T cells in ascites are associated with improved overall survival in OvCa patients[91]. Mechanistically, evidence suggests that CD8 T cells can induce complete regression of advanced ovarian cancers through an IL-2/IL-15-dependent mechanism[92], albeit in the context of an OT-1 model. Additionally, engineered CD8+ T cells targeting tumor antigens like mesothelin can effectively kill ovarian cancer cells and prolong survival in preclinical models[93]. Like DCs, the tumor microenvironment can impair CD8+ T cell function and induce regulatory CD8+ T cells, potentially limiting antitumor immunity[94].

Regarding CD4 T cells, some studies suggest they can enhance anti-tumor responses by recruiting and activating dendritic cells that prime CD8+ T cells[95], specifically by secreting high levels of CCL5 to recruit DCs and licensing them via CD40. Others indicate that specific CD4+ subsets, particularly CD4+CD25+Foxp3+ Tregs, which correlate with poor outcomes, may suppress anti-tumor immune responses[96]. The ratio of CD8+ to CD4+ T cells, rather than absolute numbers, appears to better predict patient survival than CD8 numbers alone[96]. On the other hand, adoptive transfer of tumor-reactive CD4+ T cells has shown promise in a pilot study of four patients, potentially by modulating endogenous cytokine levels and CD8+ T cell populations[97]. Overall, tumor-infiltrating lymphocytes' composition and functional properties, including various T cell subsets, significantly impact ovarian cancer outcomes[98, 99]. Consequentially, a significant

effort has been made to boost their presence and function in the TME, which will be covered in the next section.

1.9 The status of checkpoint therapy as an Ovarian cancer treatment

Immune checkpoints are surface receptors on immune cells that control the activation or suppression of the immune response. Immune checkpoint inhibitors (ICIs) are usually antibodies or small molecule inhibitors that enhance antitumor immunity by blocking the cell surface receptors of T lymphocytes, which play a vital role in the treatment of various cancers. Several immune checkpoints are expressed on OvCa infiltrating T cells, including PD-1, CTLA-4, TIM-3, LAG-3, and other co-inhibitory receptors. However, only checkpoint blockade therapies targeting PD-1/PD-L1 and CTLA-4 have been investigated for treating ovarian cancer in clinical trials[100, 101]. There is abundant data to suggest that targeting PD-L1 should be an effective therapeutic strategy. For example, OvCa patients with high PD-L1 expression have significantly lower 5-year survival rates and are more likely to have ascites compared to those with low PD-L1 expression[102]. There is also an inverse correlation between the number of intra-tumoral CD8+ T cells and PD-L1 expression[103]. Furthermore, preclinical studies demonstrated increased survival and T-cell infiltration in ovarian cancer mouse models treated with anti-PD-L1 antibodies. A study by Mony et al. showed that anti-PD-L1 treatment increased T cell infiltration and survival in MUC1 humanized mice bearing 2F8 ovarian tumors[104]. Additionally, Grabosch et al. found that cisplatin treatment enhanced tumor immunogenicity and T-cell responses when combined with anti-PD-L1 therapy[105]. These findings suggest that anti-PD-L1 therapies could restore the anti-tumoral function of T cells in OvCa. However, A meta-analysis reported a 9% overall response rate for single PD-1/PD-L1 inhibitors, only increasing to 36% when combined with chemotherapy in OvCa patients[106]. Even in the KEYNOTE-028 trial, pembrolizumab demonstrated an objective response rate of only 11.5% in PD-L1-positive advanced ovarian cancer patients[107]. The clinical efficacy of anti-PD-L1 in ovarian cancer has been modest compared to other malignancies. Melanoma is a

prime example, where the response rate ranges from 40-60%[108]. Ovarian cancer remains one of the few cancers in which no FDA-approved immune therapies exist to improve the standard of care. The resistance mechanisms to these therapies are not fully understood but may be due to a complex immunosuppressive environment in OvCa garnered by ascites and proximity to the gut. Ongoing research focuses on refining biomarkers and developing combination strategies to enhance the efficacy of PD-1/PD-L1 blockade in ovarian cancer.

1.10 The Influence of Antibiotics on Ovarian Cancer

The microbiome is recognized as a significant factor in determining OvCa outcomes. Specific bacterial signatures have been identified in ovarian cancers, suggesting a unique oncobiome relative to healthy tissues[109]. These studies imply that targeting the microbiome with antibiotics may have therapeutic impact. Retrospective clinical analysis of patients with ovarian cancer indicates antibiotic use during chemotherapy is associated with poor overall survival[110]. Supporting this observation, treatment of ovarian cancer models with a broad-spectrum antibiotic cocktail (vancomycin, neomycin sulfate, metronidazole, ampicillin) changed the gut microbiome and increased tumor growth and development of cisplatin resistance[111], supporting that a healthy microbial diversity is necessary for effective chemotherapy. Although few OvCa patients have been treated with checkpoint therapies, a retrospective cohort study of 101 women with recurrent gynecologic cancers (including 26 patients with OvCa) found antibiotic treatment prior to immunotherapy was associated with a significantly lower response rate, PFS, and OS[112]. These studies indicate that changes to the microbiome via antibiotics may have influenced ovarian cancer immunotherapy clinical trial outcomes, potentially by shifting the microbiome diversity and, in turn, the baseline level of pattern recognition receptor (PRR) stimulus the microbiome provides to the immune system. In addition, FMT studies in mouse models of OvCa reveal that antibiotic-induced disruption of the gut microbiome promotes tumor growth, enhances cisplatin resistance, and increases cancer stem cell populations[111]. Overall, modulating the microbiome with antibiotics

in OvCa patients is detrimental to outcomes. Despite numerous studies pointing to a relationship between the microbiome and ovarian cancer, the mechanisms underlying this relationship have not yet been fully elucidated.

1.11 The influence of Microbiome on checkpoint therapy

Microbiota play a crucial role in programming immune responses from birth, fine-tuning the balance between inflammation, infection, and tolerance of antigens[113]. Recent studies have highlighted gut microbiota's significant influence on immunotherapy's efficacy in cancer treatment. One prime example is the negative association of antibiotics (within 30 days of ICIs) with clinical activity (progression-free and overall survival) of immune checkpoint inhibitors in patients with advanced renal cell and non-small-cell lung cancer[114]. Moreover, higher gut microbiome diversity and abundance of specific bacteria, such as *Akkermansia muciniphila* and *Bifidobacterium*, in Melanoma patients are associated with more significant response to anti-PD-1 therapy[115-117]. Additionally, the efficacy of CTLA-4 blockade depends on the composition of the gut microbiota, specifically the presence of certain *Bacteroides* species such as *B. fragilis* and *B. thetaiotaomicron*, which promote anti-tumor immune responses against melanoma[118]. The gut microbiome's influence is often clearly presented in the results of fecal microbial transplant (FMT) experiments and clinical trials. Germ-free mice receiving FMT from PD-1 blockade-responsive patients restores anti-tumor immunity against melanoma models[115]. Conversely, germ-free mice treated by FMT from non-responsive patients reduce sensitivity to PD-1 blockade[115]. Clinically, a melanoma trial evaluating FMT and pembrolizumab in melanoma patients primarily resistant to PD-1 inhibitors showed clinical benefit in 6 of 15 patients[119]. Patients who responded to the combination therapy showed an increased abundance of microbes associated with response to anti-PD-1 therapy, increased activation of CD8+ T cells, and decreased frequency of myeloid cells expressing IL-8[119]. The increased responsiveness to checkpoint therapy post-FMT is thought to be due to the correction of dysbiosis, an imbalance in the gut microbiota.

Tuning of microbial populations may improve treatment efficacy. One strategy to restore balance is using pre or probiotics to support or add live micro-organisms to the body. A clear example of the potential of probiotics was recently demonstrated in this first prospective study, where live bacterial *C. butyricum* (CBM588) was combined with dual anti-PD-1 and anti-CTLA-4 therapy to treat advanced kidney cancer patients. The response rate and median progression-free survival improved among patients receiving dual checkpoint inhibition and probiotic vs. immunotherapy alone, revealing that probiotics can boost the efficacy of immunotherapy against cancer[120]. Additionally, enriching beneficial bacteria with pre-biotics like inulin and mucin can improve anti-tumor immunity and tumor growth inhibition in mouse models of colon cancer and melanoma[121]. Although this finding was outside the context of checkpoint therapy, increases in tumor-infiltrating CD4 and CD8 T cells in the pre-biotic fed groups suggest immunotherapy should be examined in a future study. Additionally, gut microbiota composition is being explored as a biomarker for predicting immunotherapy response in various cancer types. Specifically, gut bacteria capable of short-chain fatty acid production, including *Eubacterium, Lactobacillus*, and *Streptococcus*, were positively associated with anti-PD-1/PD-L1 response across different GI cancer types[122].

The immunological mechanisms by which gut microbiota influence cancer therapies are poorly understood. Some studies suggest a CD4+ T-cell-mediated mechanism, where *Akkermansia muciniphila* promotes IL-12 release increasing the recruitment of $CCR9^{+}CXCR3^{+}CD4^{+}$ T lymphocytes into mouse tumors[115]. While other studies highlight a CD8+ T-cell-mediated mechanism, as certain gut microbes like *Bifidobacterium* may increase CD8+ T cells in tumors[123]. Further studies suggest that cells upstream of T cells coordinate an immunological response. For example, feeding mice *Bifidobacterium* has been observed to restore defective processing and presentation of tumor antigens by DCs, re-establish infiltration of melanomas by T cells, and reduce malignant growth[123]. It was speculated that *Bifidobacterium*-derived signals modulate the activation of DCs in steady state. However, it was unclear what components of the bacteria drove

these changes. The next section will discuss specific bacterial components, their analogous pattern recognition receptors, and their potential role in ovarian cancer.

1.12 Toll-like receptor 5 (TLR5) Background

The innate immune system defends against infections by recognizing microbial pathogens through PRRs, which detect pathogen-associated molecular patterns (PAMPs). Among these PRRs, Toll-like receptor 5 (TLR5) plays a key role by specifically recognizing flagellin, the structural protein of bacterial flagella, and triggering immune responses. Consequently, TLR5 is crucial in mediating interactions between the gut microbiome and host immune responses. Furthermore, TLR5 is constitutively expressed by immune cells, such as neutrophils, monocytes, and dendritic cells, as well as respiratory and intestinal epithelial cells[124]. TLR5, for instance, detects the bacterium *Legionella pneumophila* in the airway epithelium, triggering IL-8 release to clear the pathogen[125]. The effective clearance of *L. pneumophila* from the airways relies on Flagellin-TLR5-MyD88-dependent signaling in respiratory epithelial cells. In fact, a common stop codon polymorphism in the TLR5 gene (TLR5392STOP or R392X) is associated with increased susceptibility to Legionnaires' disease and is expressed in roughly 7% of the population[125, 126]. The *TLR5*^{R392X} polymorphism has also been linked to susceptibility to bronchopulmonary dysplasia in preterm infants[127] and may protect against Crohn's disease in Jewish populations by reducing adaptive immune responses to flagellin[128]. There are 12 additional known missense SNPs in the TLR5 gene. However, it is unknown if they have clinical relevance[129].

The TLR5 signaling pathway is activated when bacterial flagellin binds to the extracellular domain of TLR5, forming a 2:2 tail-to-tail signaling complex[130]. This interaction involves a leucine-rich sequence in TLR5[131] and recruitment of the adaptor protein MyD88 to form a complex with IRAK4[132]. This leads to activation of the MAPK pathway and IKK kinases which phosphorylates NF-κB inhibitory protein IκBα causing NF-κB translocation into the nucleus to induce pro-inflammatory gene expression[133, 134]. TLR5 signaling can lead to transcriptional activation of at

least 500 genes[135] which are involved in various physiological processes, including intestinal epithelial cell responses to commensal bacteria[132] and tumor growth modulation[136]. Recently, the TLR5 pathway has emerged as a potential therapeutic target for various conditions, including cancer and autoimmune diseases[137]. The impact of TLR5 activation and blockade will be explored in the next section.

1.13 TLR5 agonism and antagonism

The most thoroughly studied TLR5 agonist is entolimod (CBLB502), a recombinant flagellin derivative developed by Cleveland Biolabs designed as a specific agonist of TLR5. The most notable finding regarding entolimod is its ability to protect mice and rhesus monkeys from lethal total-body irradiation by reducing apoptosis in radiosensitive tissues[138]. Additionally, in a murine model of acute ischemic renal failure, entolimod protected against renal dysfunction and inflammation, including decreased leukocyte infiltration, proinflammatory cytokine production, and tubular injury[139]. Furthermore, it reduces the toxicity of chemotherapy drugs like 5-fluorouracil via IL-6 induction while maintaining their antitumor efficacy[140]. Interestingly, on top of entolimod's immunoprotective and toxicity-negating properties, it has been shown to have anticancer effects, particularly in the context of liver cancers. In a preclinical study, entolimod was observed to activate NF-κB, AP-1, and STAT3 pathways in hepatocytes, triggering a cascade of immune responses involving NK cells, dendritic cells, and CD8+ T cells[141]. Clinically, a phase I trial demonstrated entolimod's safety in patients with advanced cancers at a dosage of 30 ug/day[142]. Although entolimod induced the secretion of IL-6, IL-8, and IL-10, no tumor responses were observed. A randomized phase 2 study (NCT02715882) involving entolimod in colorectal cancer patients began in Russia in 2016, but the trial's status is unclear[143]. Currently, no active clinical studies are investigating entolimod as an anticancer agent. TLR5 agonism has seen the most promise as a vaccine adjuvant. VAX125, flagellin fused to an influenza HA1 antigen, was found to be safe and induced a greater than 10-fold increase in viral antibody levels and nearly complete

seroprotection in subjects over 65 years old[144]. Additionally as a vaccine adjuvant, entolimod significantly increased antibody titers against diphtheria and tetanus compared to the tetanus-diphtheria vaccine alone[145]. These findings suggest that TLR5 agonists are encouraging vaccine adjuvants warranting further investigation in various disease models.

Currently, no clinical trials investigate TLR5 antagonists, so most research is still focused on developing TLR5 inhibitors. Pyrimidine triazole thioether derivatives, such as TH1020, have shown potential as TLR5 antagonists by disrupting the TLR5-flagellin interaction[146]. However, their bioavailability when administered *in vivo* remains unclear. Alternatively, the only other strategy to inhibit TLR5 signaling is by anti-TLR5 antibody blockade. Most findings related to TLR5 inhibition involve ablating TLR5 in genetic KO models or in the context of TLR5 polymorphisms that cause changes to the receptor structure, such as the $TLR5^{R392X}$ SNP. The implication of these studies and their relationship to ovarian cancer will be covered in the next section.

1.14 Toll-like Receptors and Ovarian Cancer

Bacterial dissemination and translocation often occur in cancers within the peritoneal cavity due to chronic inflammation enabling a breakdown in gut barrier integrity, a process which is exacerbated by chemotherapy[147]. Bacteria and their components are frequently acted upon locally by APCs or surrounding epithelial cells before entering the systemic circulation. These bacterial components can impact tumor progression by stimulating PRRs in ovarian tumor cells. It has been established that TLR2, TLR3, TLR4, and TLR5 are strongly expressed on the surface epithelium of normal human ovaries and human ovarian cancer cell lines[148]. TLR4, a receptor for Lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria, and oxidized phospholipid, is the best-studied PRR in OvCa. LPS is found to increase tumor cell production of IL-6 and IL-12, inhibiting CD8 T cells thus facilitating immune evasion by cancer cells[149]. These findings also translate to ovarian cancer as it has been reported by several labs that TLR4 is overexpressed in many ovarian epithelial tumors, and its high levels are associated

with enhanced tumor progression and a greater likelihood of developing resistance to Paclitaxel chemotherapy[150-152]. Additionally, in OvCa patients with metastatic disease, elevated levels of hypo-methylated DNA, a TLR9 ligand, were observed, further highlighting the detrimental impact of TLR9 signaling[153]. TLR9 signaling in ovarian and breast cancer cells has been linked to increased disease aggressiveness and poor clinical outcomes, with higher TLR9 expression correlating with more severe tumor grade, greater migratory capabilities, and poorer differentiation[153]. It is hypothesized that NF-κB signaling is primarily responsible for the enhanced aggressiveness of OvCa as it is constitutively activated in numerous cancer types and downstream of TLR-ID8 signaling[154]. The *TLR5*^{R392X} polymorphism, which reduces TLR5 signaling, is associated with decreased survival in luminal breast cancer but increased survival in ovarian cancer, highlighting the cancer-type-specific impact of TLR5 signaling[155]. This difference is attributed to IL-6 responsiveness, where TLR5 inhibition improves survival in ovarian cancer by reducing IL-6-dependent recruitment of myeloid-derived suppressor cells, while in breast cancer, it worsens outcomes by increasing IL-17 production[155].

Despite the negative impact of TLR stimulation on OvCa outcomes, immune activation by TLRs has shown some therapeutic potential. TLR agonists, particularly those stimulating TLR3, have shown promise in enhancing immune responses against OvCa. In 2009, it was reported that TLR3 activation with poly-inosinic-cytidylic acid (poly I:C) on DCs enhanced antigen processing and presentation in combination with CD40 co-stimulation resulting in a boost in T cell antitumor activity and rejection of ovarian carcinomas in mice[156]. Furthermore, the use of TLR3 agonist and poly IC derivative poly-ICLC demonstrated consistent, antigen-specific antibody, CD8 and CD4 T cell response with synthetic overlapping long peptides from a human tumor self-antigen in a phase I trial[157]. Despite the immunological boost, using TLR agonists in OvCa treatment therapeutic applications remains murky. For instance, adding a TLR4 agonist to dendritic cell immunotherapy (DCs loaded with tumor lysate) did not improve survival in an ID8 orthotopic mouse model despite

an increase in NK cells and decrease in Tregs[158]. Currently there are no FDA-approved therapies for OvCa targeting TLRs and seldom clinical trials.

Research on TLR activity in OvCa suggests that inhibiting certain TLRs in cancer cells or stimulating them in immune cells could offer therapeutic benefits. While TLR stimulation in immune cells can induce an anti-cancer effect, activation in tumor cells often leads to immunosuppression, promoting tumor growth and angiogenesis, making TLR targeting a complex and double-edged sword. Future strategies should focus on immune cell-specific targeting of TLR pathways, or novel drug delivery mechanisms for more precise therapeutic delivery.

1.15 TLR5 and DCs

TLR5 is highly expressed on mucosal DCs and CD11c+CD11b+ lamina propria DCs[159, 160]. TLR5 expression corresponds to the cell's critical role in maintaining intestinal immune homeostasis and host defense against bacterial infection. Ligation of TLR5 by flagellin on lamina propria DCs induces downstream signaling via MyD88 and subsequent NFκB activation, leading to the induction of Th17 T cell responses to help control bacteria[161]. Consequently, TLR5 deficiency has been shown to induce spontaneous colitis in mice[162] and change the intestinal microbiota composition[163]. This study suggests that TLR5 DCs are important in maintaining the homeostasis of the microbiome. TLR5 signaling on human DCs (derived from PBMCs cultured with GM-CSF and IL-4) stimulated by flagellin for 24 hours induces maturation characterized by expression of CD83, CD80, CD86, MHC class II, and CCR7[164]. Despite these studies, it is unclear how DC phenotype changes in response to chronic TLR5 signaling, and whether chronic exposure within the TME recapitulates a similar response to what occurs at the gut mucosa. However, there is a gap in this knowledge as few studies exist that have compared the differentiation and function TLR5+ compared to TLR5-deficient DCs in any tumor microenvironment, including colon cancer.

1.16 Conclusions and Thesis Rationale

Epithelial ovarian cancer is the most prevalent subtype and has the worst outcomes of any manifestation of OvCa. This can be primarily attributed to the late detection of ovarian cancer due to its insidious onset with hidden pathology. Until early detection strategies are discovered, therapies are needed that can treat advanced disease. The current standard for ovarian cancer disease management (stage II-IV) is cytoreduction, followed by chemotherapy with carboplatin-paclitaxel, which has changed little in the last 25 years[14]. Thus, there is an unmet need for new therapies to treat this cancer. Additionally, ovarian cancer is characterized by a high degree of inter and intratumor heterogeneity between and within patients, which poses therapeutic challenges because this disease cannot be considered as a single entity. Thus, a better understanding of the cause of tumor and patient divergence is needed.

One defining characteristic of advanced OvCa is ascites, which drives poor survival outcomes and an immunosuppressive environment[22-25]. This environment causes DCs to become dysfunctional initiating a cascade of poor anti-tumor responses conducted by T cells[26-28], which may account for the clinical failure of checkpoint therapy against OvCa. However, it is unclear what causes DC dysfunction in the ovarian TME highlighting a significant gap in current knowledge. One potential source of DC dysfunction is bacteria. It has been demonstrated modulating the microbiome with antibiotics in OvCa patients is detrimental to OvCa outcomes[110-112]. Furthermore, bacterial-derived components are present within the ovarian TME[109] and are known to be immunomodulatory through the TLR pathway[148]. In particular, TLR5 signaling driven by flagellin, has been demonstrated to modulate DC function[161, 164]. However, it is unknown how chronic stimulus with TLR5 signaling will impact DCs within a tumor microenvironment, assuming that gut leakage is a chronic feature of peritoneal cancers. Recently, the TLR5 pathway has emerged as a potential therapeutic target for various conditions, including cancer and autoimmune diseases[137]. Studies of TLR5 agonists have shown they are immunoprotective in the

context of radiation and acute ischemic renal failure but an excellent adjuvant regarding vaccines, highlighting the duality of TLR5 signaling[138, 139, 144]. Few studies explore TLR5 inhibition, particularly in conditions where TLR5 signaling may overwhelm an immune response already engaging cancer cells.

Prior to this study, it was observed that both mice and humans that were less sensitive to TLR5 signaling (TLR5 KO and *TLR5*^{R392X} patients respectively) exhibited greater survival when bearing ovarian cancer[155]. Additionally, it was found that ovarian cancer patients survived longer with more significant T cell infiltration[37]. Taking advantage of these observations, we find treating TLR5 KO ovarian tumor-bearing mice with checkpoint inhibitors, which relinquish T cells from inhibition, increases mouse survival robustly and, in some cases, indefinitely. These results were surprising as checkpoint inhibition is historically not efficacious clinically or in mouse models of ovarian cancer. This dissertation addresses two unanswered questions in the field: (1) What cell types in the ovarian tumor microenvironment express TLR5? TLR5 expression has only been rigorously assessed in proximity to the gut and never in the cancer setting. (2) What TLR5-expressing cell type is most responsible for preventing the efficacy of checkpoint therapy against ovarian cancer? The cause of checkpoint therapy failure is unknown for ovarian cancer.

Chapter 2: TLR5 signaling causes dendritic cell dysfunction and orchestrates the failure of immune checkpoint therapy against ovarian cancer

2.1 Forward

This work is in press at *Cancer Immunology Research*, accepted _____, with me as first author. The text and figures were adapted from the publication for Chapter 2.

Below is the full citation.

Mitchell T. McGinty[1], Sree H. Kolli[1], Audrey M. Putelo[1], Tzu-Yu Feng[1], Madison R. Dietl[1], Cara N. Hatzinger[1], Simona Bajgai[1], Mika K. Poblete[1], Francesca N. Azar[1], Melanie R. Rutkowski[1*]

1 Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, VA, USA

* Corresponding Author

2.2 ABSTRACT

Ovarian cancer accounts for more deaths than any other cancer of the female reproductive system. Patients bearing ovarian tumors infiltrated with high frequencies of T cells associate with a greater survival probability. However, therapies to revitalize tumor-associated T cells, such as PD-L1/PD-1 blockade, are ineffective for the treatment of ovarian cancer. We demonstrate that in models of late-stage murine ovarian cancer that Toll-Like Receptor 5 (TLR5) signaling, the only known ligand for which is bacterial flagellin, leads to failure of immune therapy. Mechanistically, we demonstrate both *in vivo* and *in vitro* that chronic TLR5 signaling on CD11c+ cells impairs the differentiation of functional IL-12-producing XCR1+ CD103+ cDC1 subsets within the tumor microenvironment (TME). Instead, chronic TLR5 signaling biases precursor cells towards myeloid-associated subsets expressing high levels of PD-L1. This culminates in impaired activation of CD8 T cells, reducing CD8 T cell function and persistence within the ovarian tumor microenvironment. Expansion of cDC1s *in situ* using FMS-related Tyrosine Kinase 3 ligand (FLT3L) in combination with PD-L1 blockade achieved significant survival benefit, but only in TLR5 KO mice, whereas no benefit was observed in the presence of TLR5 signaling. Thus, we identify a host-intrinsic mechanism leading to failure of immune therapy for ovarian cancer, demonstrating that chronic TLR5 signaling on DCs is a barrier limiting the efficacy of immune therapy.

2.3 SIGNIFICANCE

This study uncovers that chronic TLR5 signaling enhances the accumulation of suppressive PD-L1 expressing myeloid cells in the tumor environment at the expense of functionally mature conventional type I dendritic cells (cDC1). TLR5 signaling disrupts a critical DC-CD8 T cell axis, culminating in failure of PD-L1 blockade or *in vivo* agonism of FLT3 to expand cDC1. Blocking TLR5 signaling in combination with anti-PD-L1 and FLT3L presents a novel therapeutic strategy against ovarian cancer.

2.4 INTRODUCTION

Ovarian cancer is the deadliest gynecologic malignancy in the United States, with an estimated 19,880 new cases diagnosed and 12,810 deaths in 2022[1]. The current standard for disease management is intensive surgical staging and cytoreduction, followed by chemotherapy with carboplatin–paclitaxel[165]. Patients often respond initially to therapy; however, roughly 85% exhibit recurrence[166]. Thus, there is an urgent need for therapies that actively adapt to combat recurrence. The presence of tumor-infiltrating T cells correlates with increased survival and delayed recurrence in advanced ovarian cancer patients[37]. Despite this, strategies such as blockade of T cell inhibitory receptors to harness/reinvigorate T cell activity against ovarian tumors are largely ineffective. These shortcomings emphasize the need for complementary therapies that overcome resistance to checkpoint therapy, enhance anti-tumor T-cell responses, and improve patient outcomes.

Dendritic cells (DCs) are potent antigen-presenting immune cells that orchestrate robust and durable anti-tumor T cell responses in multiple cancer types. For ovarian cancer, DCs have a fundamental role in dictating patient outcomes[167, 168]. However, within the ovarian tumor microenvironment, DCs phenotypically diverge from promoting immunosurveillance during early stages of tumor growth to inhibiting T-cell function during advanced disease[39, 169, 170]. Reversing the suppressive function of tumor-associated DCs through blockade of inhibitory receptors[170-172] or utilizing DC-based vaccines[173-175] have recently been demonstrated to improve progression-free survival, supporting the critical importance of DCs for improving survival in patients with ovarian cancer. Despite these advances, DCs are unable to sustain anti-tumor T cell function for ovarian cancer. Defining the host-intrinsic and tumor-associated factors that modulate the phenotype and function of DCs in ovarian cancer patients is therefore crucial for the development of effective immunotherapeutic strategies.

Although much is known about how tumor cells hijack myeloid cells to potentiate tumor growth and/or metastasis, little is known as to how chronic exposure to the microbiome influences the phenotype of myeloid cells or the cellular makeup of the ovarian tumor microenvironment (TME). We demonstrated that TLR5 recognition of flagellated host commensal bacteria enhances the growth of ovarian tumors by enabling the accumulation of suppressive myeloid cells in the ovarian TME, culminating in sustained T cell dysfunction[126]. The only known ligand for TLR5 is flagellin, the structural element of bacterial flagella. In homeostatic conditions, acute engagement of TLR5 in dendritic cells leads to secretion of proinflammatory cytokines, including IL-6 and IL-1β[176], which aid the induction of adaptive immunity. On the other hand, there is a growing body of evidence that chronic TLR engagement can tip the balance towards a TME that favors tumor progression due to excessive inflammation[177, 178]. Flagellated bacteria are found within TME of patients with ovarian cancer due to breakdown of gut integrity facilitated by tumor-promoting inflammation and chemotherapy[109, 179]. Therefore, it is possible that chronic TLR5 signaling and inflammatory cytokines in the ovarian TME impair DC function, facilitating tumor growth.

This study investigated the effects of TLR5 signaling on dendritic cell function and anti-tumor immunity, and the consequence of this signaling pathway on efficacy of immune therapy. We demonstrate that TLR5 signaling impairs the accumulation of cross-presenting XCR1+ CD103+ conventional type 1 dendritic cells (cDC1s) into the TME. cDC1s are central for activation and/or priming of tumor-reactive CD8 T cells[180-184] and control of tumor growth[183, 185, 186]. In the absence of TLR5 signaling, durable and long-term survival of mice bearing aggressive ovarian tumors is achieved using PD-L1 blockade (anti-PD-L1). In addition, we show potential for DCs, expanded in situ, as a therapy for ovarian cancer in combination with anti-PD-L1 in the absence of TLR5 signaling.

2.5 RESULTS

In the absence of TLR5 signaling, PD-L1 blockade achieves a significant survival benefit for ovarian cancer.

Blockade of tumor- or myeloid-associated PD-L1 from interacting with the T cell inhibitory receptor PD-1 has shown promise in ovarian cancer patients as a single therapy[187-189] and combined with other therapies[190]. Unfortunately, few patients respond, and of those that do, many ultimately experience recurrent disease. Because TLR5 signaling in ovarian cancer reduces anti-tumor T cell function[126], we examined the efficacy of checkpoint therapy in the absence of TLR5 signaling. TLR5 KO or wild-type mice were injected orthotopically with the ovarian epithelial cell line ID8-*Defb29/Vegf-A*[191, 192]. ID8-*Defb29/Vegf-A* is an aggressive ovarian tumor cell line derived from the parental ID8 tumor model[193] that recapitulates stage III/IV ovarian cancer. Mice were treated as depicted in **Figure 2.1A**. Untreated TLR5 KO mice had a slight, although significant, survival benefit compared to wild-type mice (**Figure 2.1B**, red dotted versus block dotted line), confirming our previous findings using the less aggressive parental ID8 cell line[126]. Although PD-L1 blockade modestly enhanced survival in wild-type mice (solid black line), 56% (51 total) of TLR5 KO mice given PD-L1 blockade did not display any evidence of disease, resulting in long-term survival greater than 100 days (**Figure 2.1B**, solid red line). We next examined the durability of protection in surviving TLR5 KO mice. Twenty-seven of the TLR5 KO mice surviving greater than 100 days were re-injected with ID8-*Defb29/Vegf-A* tumors. Compared to naïve TLR5 KO tumor-bearing mice, a majority of surviving TLR5 KO mice from **Figure 2.1B** continued to survive after re-injection of the ID8-*Defb29/Vegf-A* cell line, despite not receiving additional anti-PD-L1 therapy (**Figure 2.1C**). Of the 27 rechallenged mice, 21 mice survived without evidence of tumor outgrowth, while many of the mice that did eventually succumb survived greater than 100 days before developing evidence of disease. These findings were recapitulated using another ovarian tumor cell line. PD-L1 blockade promoted significant survival and protection during initial tumor

rechallenge in TLR5 KO mice bearing the UPK10 ovarian epithelial cancer cell line (**Figure 2.1D and E**). Taken together, these results indicate that in the absence of TLR5 signaling, anti-PD-L1 therapy achieves a robust and durable response for ovarian cancer.

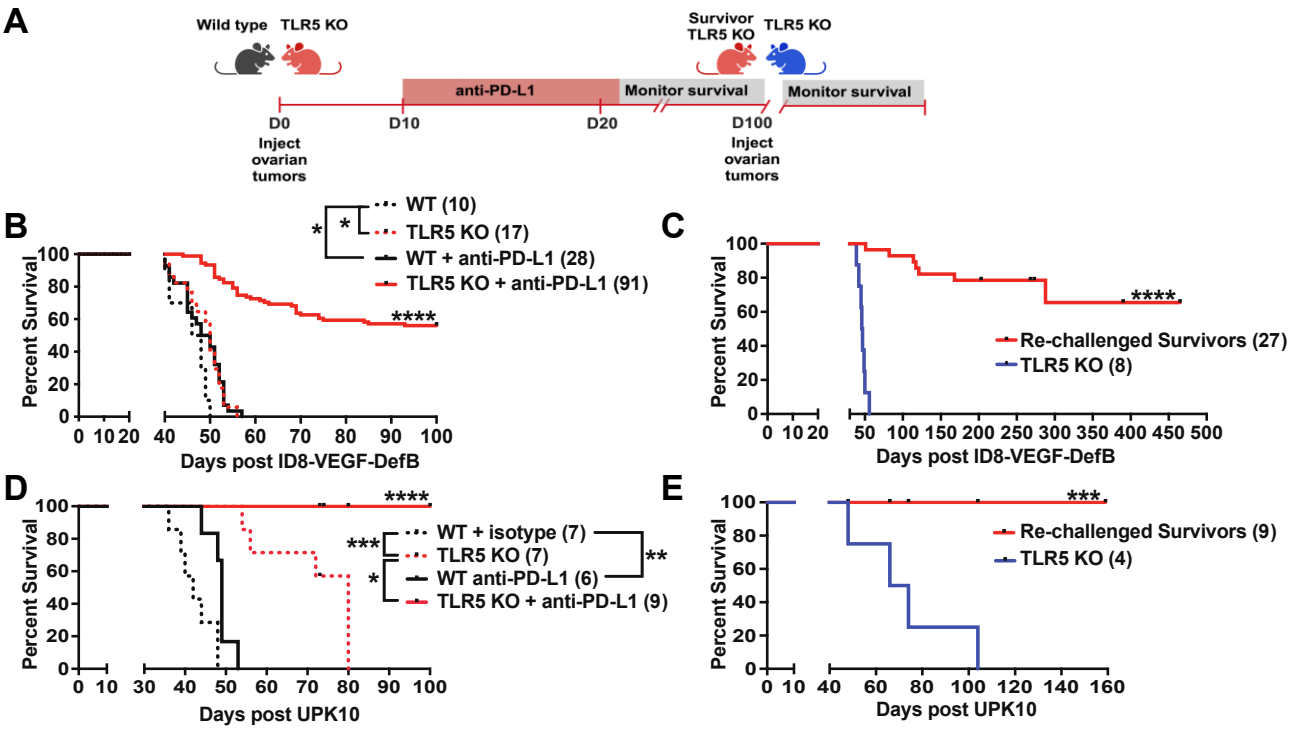

A
Wild type
TLR5 KO
Survivor TLR5 KO
TLR5 KO
anti-PD-L1
Monitor survival
Monitor survival
D0
Inject ovarian tumors
D10
D20
D100
Inject ovarian tumors
B
WT (10)
TLR5 KO (17)
WT + anti-PD-L1 (28)
TLR5 KO + anti-PD-L1 (91)
Percent Survival
Days post ID8-VEGF-DefB
C
Re-challenged Survivors (27)
TLR5 KO (8)
Percent Survival
Days post ID8-VEGF-DefB
D
WT + isotype (7)
TLR5 KO (7)
WT anti-PD-L1 (6)
TLR5 KO + anti-PD-L1 (9)
Percent Survival
Days post UPK10
E
Re-challenged Survivors (9)
TLR5 KO (4)
Percent Survival
Days post UPK10

Figure 2.1. TLR5 signaling impairs efficacy of PD-L1 blockade for ovarian cancer.

(**A**) Treatment schema. On day 0, tumors were initiated in wild-type (WT) or TLR5 KO mice. Anti-PD-L1 was initiated on day 10 for a total of 4 injections. After 100 days, surviving or naïve TLR5 KO mice were injected with the same ovarian tumor cell line as established in surviving mice on day 0. (**B**) Survival of mice bearing ID8-*Defb29/Vegf-A* ovarian tumors after treatment with anti-PD-L1. (**C**) Survival of surviving TLR5 KO mice from **B** re-injected after 100 days or naïve TLR5 KO mice bearing ID8-*Defb29/Vegf-A* tumors. (**D**) Survival of mice bearing UPK10 ovarian tumors after treatment with anti-PD-L1. (**E**) Survival of surviving TLR5 KO mice from **D**, re-injected after 100 days or naïve TLR5 KO mice bearing UPK10 tumors. Log-rank test was used to compare survival proportions. Numbers in parentheses indicate numbers of mice within each group. (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$).

During tumor outgrowth, TLR5+ dendritic and myeloid cells expressing PD-L1 accumulate within the ovarian tumor microenvironment.

To define the immunological basis of extended survival in TLR5 KO mice after treatment with PD-L1 blockade, we first sought to understand the connection between TLR5 signaling and the cellular evolution of the ovarian TME. We used TLR5 reporter mice to comprehensively define the phenotypic and functional attributes of TLR5-expressing immune subsets within the ovarian TME, tumor-draining lymph nodes, and spleen.

TLR5 reporter mice have a knock-in *IRES-tdTomato* sequence placed at the 3' end of the *Tlr5* gene[194], enabling flow cytometric analysis of TLR5-expressing cells. In mice with advanced tumor burden (day 25), assessment of TLR5-expressing immune populations within the ovarian TME revealed negligible expression of TLR5 on tumor-associated T cells (**Figure 2.2A**). On the other hand, most tumor-infiltrating cells co-expressing CD11c+ and MHCII+, putative dendritic cells, in addition to a large proportion of CD11b+ myeloid cells, were found to express TLR5 at this advanced temporal point (**Figure 2.2A**). Given that the TLR5-expressing cell subsets were either myeloid-derived or putative dendritic cells, and that TLR5 signaling associates with the accumulation and/or polarization of myeloid subsets[126, 195], we next interrogated the association between TLR5 expression, accumulation, and functional modulation of myeloid cells within the ovarian TME and in response to degree of tumor burden.

TLR5-expressing and non-expressing cell subsets were analyzed from tumor nodules, tumor-draining lymph nodes, and spleens of TLR5 reporter mice with low (day 10) to progressively increased tumor burden (days 20 and 30). Changes in immune composition were compared to peritoneal wash exudates, mediastinal lymph nodes, and spleens of non-tumor-bearing tdTomato mice. The goal was to define how the frequency, phenotype, and function of TLR5-expressing or TLR5-negative cell subsets change throughout the course of ovarian tumor progression. Using the gating strategy outlined in **Figure 2.3**, we observed expansion in the frequencies of TLR5-

negative cells within the combined B/T cell gate 10 days post-tumor initiation, followed by an expansion in the frequencies of TLR5-negative cells during advanced stages of disease (**Figure 2.2B and C**). Many of the TLR5-negative cells were presumed to be tumor cells, which do not express the TLR5 reporter or additional fluorescent reporters. All other TLR5-negative populations did not exhibit substantial or dynamic changes throughout tumor progression. On the other hand, within the TLR5-expressing population, there was a gradual and significant increase in the frequencies of both myeloid and DC subsets in response to advancing disease burden (**Figure 2.2B and C**). Within the spleen, there was an expansion of undefined TLR5-expressing leukocytes in addition to a significant decrease in B and T cells, whereas frequencies of TLR5-expressing myeloid and DCs remained stable throughout tumor progression (**Figure 2.4A**). Very few cell types, other than cells within the combined B/T cell gate, expressed TLR5 within the tumor-draining mediastinal lymph node (**Figure 2.4B**). Together, these data indicate that TLR5-expressing myeloid and DC populations exhibit significant and dynamic expansion in response to ovarian tumors which are localized predominantly within the ovarian TME.

Myeloid cells are exquisitely responsive to the multitude of inflammatory signals within the ovarian TME[196-198], and as tumors progress, both lineage-committed[181, 199] and immature myeloid precursors[200, 201] are polarized to become pro-tumorigenic and suppressive. We sought to understand how TLR5 expression associated with phenotypic changes in both myeloid and DC populations, focusing on cytokine and cell surface molecules involved in myeloid and DC activation and/or inhibition of anti-tumor T cell function. As tumors progressed, frequencies of TLR5-expressing DCs producing IL-10 and expressing PD-L1 significantly increased (**Figure 2.2D**). Conversely, the frequencies of TLR5-negative DC populations expressing IL-10 or PD-L1 were low and remained unchanged throughout tumor progression (**Figure 2.2E**). Not only were there more TLR5-expressing PD-L1+ DCs within the TME, the amount of surface PD-L1 on these cells increased significantly as ovarian tumors progressed (**Figure 2.2F**). No differences in the

levels of PD-L1 on TLR5-negative DC populations were observed (**Figure 2.2G**). Although there was a significant increase in the frequencies of TLR5-expressing PD-L1+ myeloid cells during advanced ovarian tumor progression (**Figures 2.4C and 2.4D**), similar PD-L1 levels were detected in TLR5-negative myeloid populations (**Figures 2.4E and 2.4F**). Although TLR5-expressing myeloid cells are expanding within the ovarian TME, phenotypically we did not identify how these populations differed from TLR5-negative myeloid cells. On the other hand, tumor-associated DCs express TLR5 and exhibit a corresponding phenotypic shift in response to tumor progression, predominantly through the expression of PD-L1.

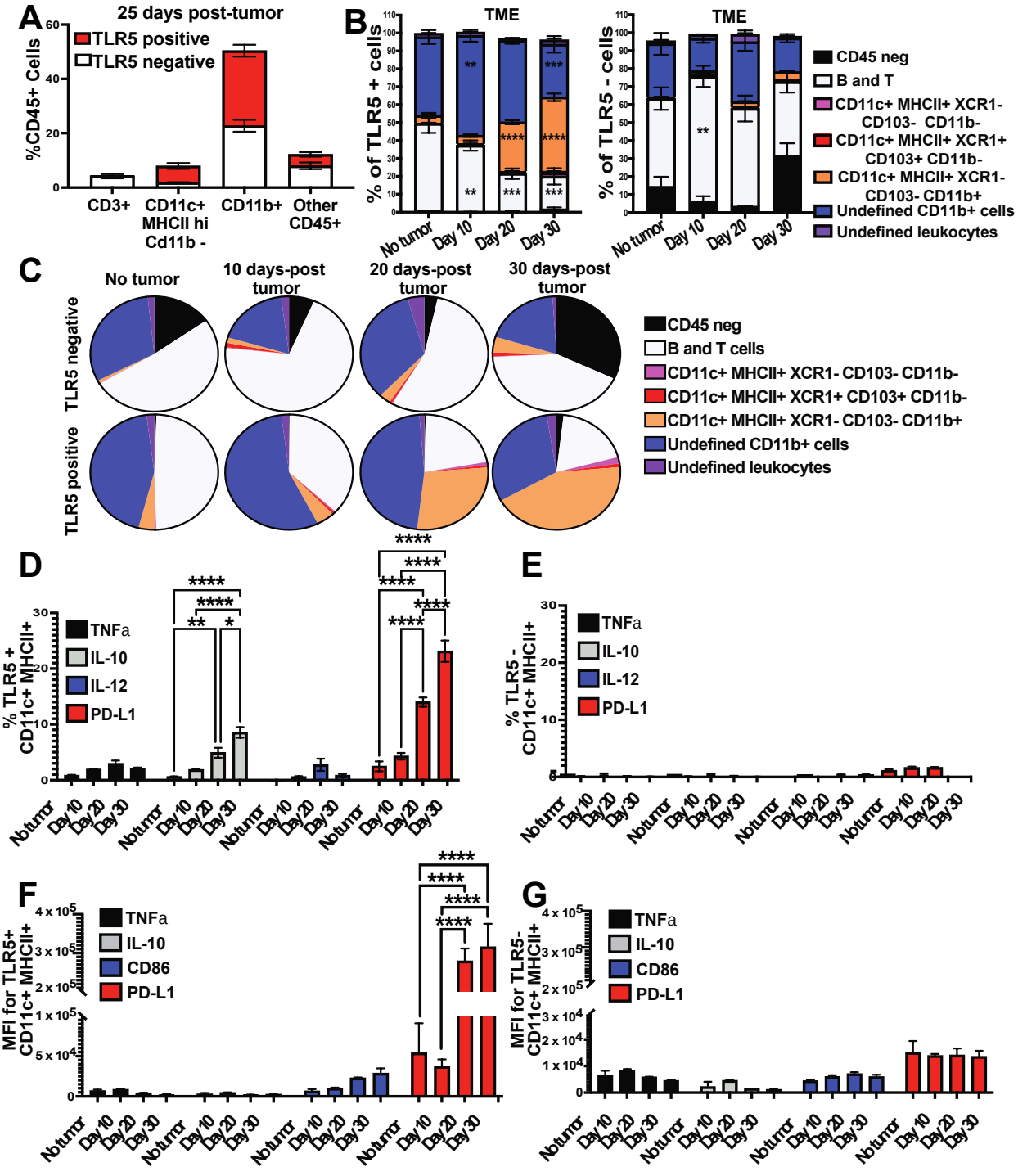

A
25 days post-tumor
TLR5 positive
TLR5 negative
%CD45+ Cells
CD3+
CD11c+ MHCII hi Cd11b -
CD11b+
Other CD45+
B
TME
% of TLR5 + cells
% of TLR5 - cells
No tumor
Day 10
Day 20
Day 30
CD45 neg
B and T
CD11c+ MHCII+ XCR1- CD103- CD11b-
CD11c+ MHCII+ XCR1+ CD103+ CD11b-
CD11c+ MHCII+ XCR1- CD103- CD11b+
Undefined CD11b+ cells
Undefined leukocytes
C
No tumor
10 days-post tumor
20 days-post tumor
30 days-post tumor
TLR5 negative
TLR5 positive
CD45 neg
B and T cells
CD11c+ MHCII+ XCR1- CD103- CD11b-
CD11c+ MHCII+ XCR1+ CD103+ CD11b-
CD11c+ MHCII+ XCR1- CD103- CD11b+
Undefined CD11b+ cells
Undefined leukocytes
D
% TLR5 + CD11c+ MHCII+
TNFa
IL-10
IL-12
PD-L1
E
% TLR5 - CD11c+ MHCII+
F
MFI for TLR5+ CD11c+ MHCII+
CD86
G
MFI for TLR5- CD11c+ MHCII+

Figure 2.2. TLR5 expression on myeloid and dendritic cells, but not T cells, corresponds with tumor-associated cellular changes within the ovarian tumor microenvironment.

(**A**) Peritoneal wash exudates from tdTomato TLR5 reporter mice bearing ID8-*Defb29/Vegf-A* ovarian tumors were analyzed by flow at day 25 for TLR5-expressing (tomato+) cells in the TME. Percentages based upon frequency of tdTomato+ or tdTomato- cells out of total leukocytes (CD45+). N=14 total. (**B**-**C**) Analysis of TLR5 expressing cell subsets in the peritoneal microenvironment of naïve mice (no tumor) and in the tumor microenvironment (TME) of mice with progressively increasing tumor burden. Peritoneal wash exudates and tumor nodules of Td-Tomato TLR5 reporter mice bearing ID8-*Defb29/Vegf-A* tumors were examined by flow cytometry at 10-, 20-, and 30-days post-tumor implantation, and compared to peritoneal wash fluid from naïve animals. N=5 per group. Percentages are calculated based upon total TLR5+ and – cell proportions and depicted as stacked bar graphs (**B**) or pie graphs (**C**). Phenotypic analysis of TLR5+ (**D and F**) or TLR5- (**E and G**) MHCII hi and CD11c+ DC cell subsets within the peritoneal environment was performed in Td-Tomato TLR5 reporter mice administered brefeldin A 6 hours prior to analysis for cytokine analysis. (**D and E**) proportions of each phenotypic DC subset from TLR5+ or TLR5- cell subsets. (**F and G**) mean fluorescent intensity (MFI) of each marker from cells in **D** and **E**. N=5 per group. Mann-Whitney unpaired t-test (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$) was used to calculate significance. Error bars represent mean ± SEM. Plots are representative of at least 2 repeats.

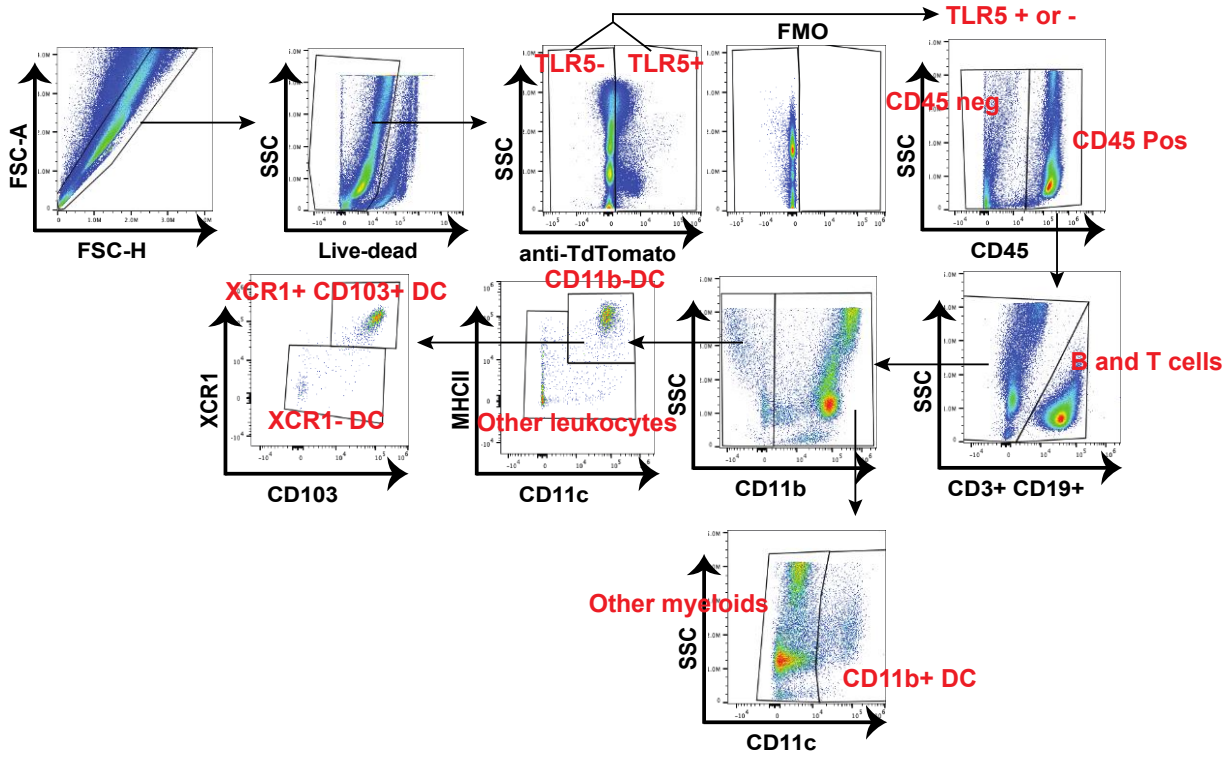

FSC-A
FSC-H
SSC
Live-dead
SSC
anti-TdTomato
TLR5-
TLR5+
FMO
TLR5 + or -
SSC
CD45
CD45 neg
CD45 Pos
SSC
CD3+ CD19+
B and T cells
SSC
CD11b
CD11b-DC
MHCII
CD11c
Other leukocytes
XCR1
CD103
XCR1+ CD103+ DC
XCR1- DC
SSC
CD11c
Other myeloids
CD11b+ DC

Figure 2.3. Hierarchical gating of TLR5-expressing cells in the ovarian Tumor Microenvironment.

Cells were harvested from the peritoneal cavities of Td-Tomato TLR5 reporter mice 30 days-post establishing ovarian tumors with ID8-*Defb29/Vegf-A* cancer cells. Gating was performed in FlowJo post antibody staining and analysis on a Cytek Auroura. Hierarchical gating was used (shown by the arrows) starting with singlets, followed by live cells, then TLR5+ or TLR5-, as indicated by Td-Tomato positive and negative, respectively. TLR5 positivity was defined using a fluorescence minus one (FMO) control of peritoneal wash/tumor nodules. TLR5+/- populations were gated separately for CD45 then a combined B/T cell gate, based upon CD3/CD19 staining. CD3-CD19- populations were further selected for expression of CD11b, CD11c, MHCII, XCR1, and CD103. Allowing for the characterization of CD11b- DCs, XCR1+CD103+ DCs, XCR1- DCs, CD11b+ DCs, other leukocytes, and other myeloid cells.

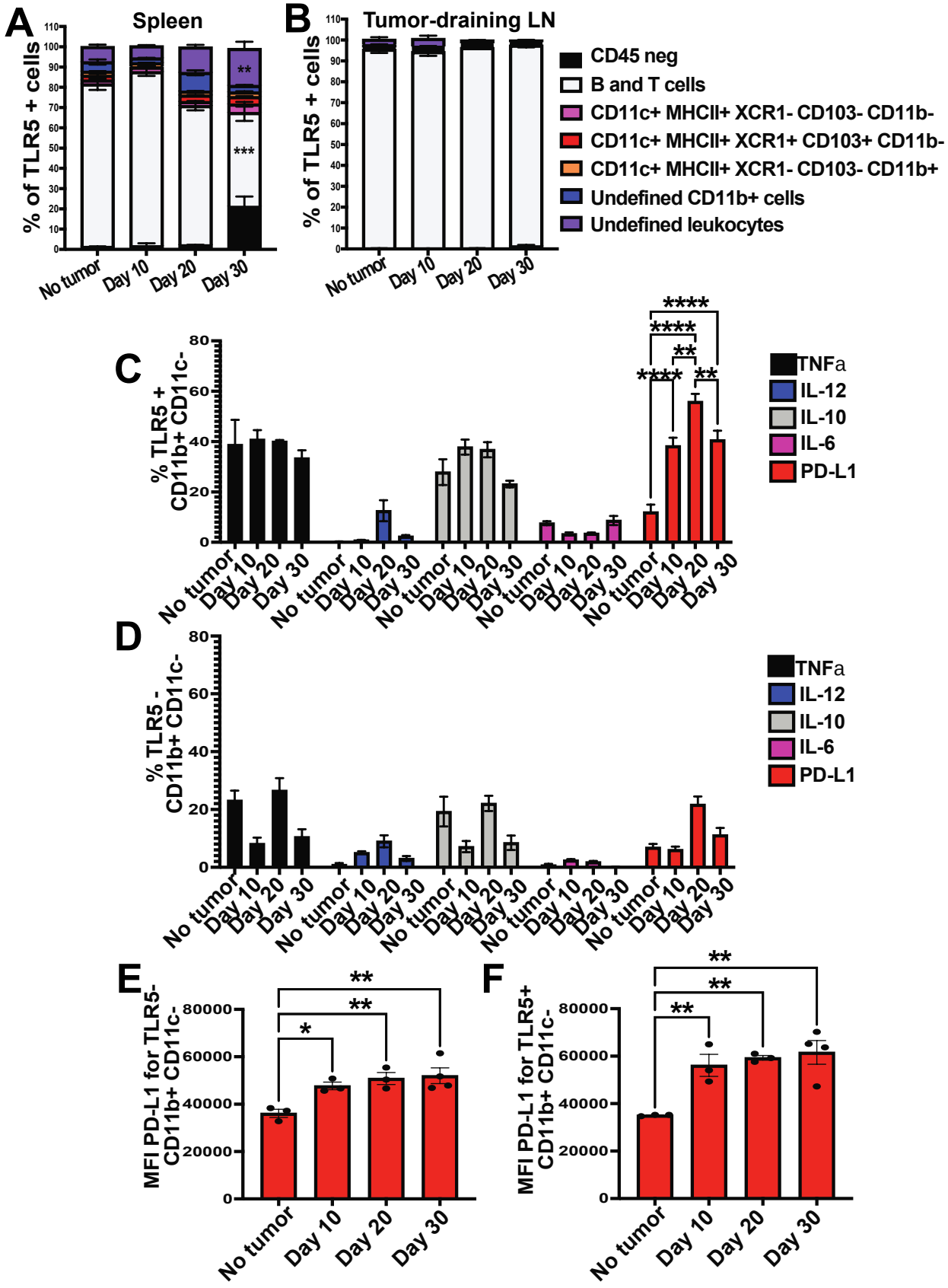

A
Spleen
% of TLR5 + cells
No tumor
Day 10
Day 20
Day 30
B
Tumor-draining LN
% of TLR5 + cells
CD45 neg
B and T cells
CD11c+ MHCII+ XCR1- CD103- CD11b-
CD11c+ MHCII+ XCR1+ CD103+ CD11b-
CD11c+ MHCII+ XCR1- CD103- CD11b+
Undefined CD11b+ cells
Undefined leukocytes
C
% TLR5 + CD11b+ CD11c-
TNFa
IL-12
IL-10
IL-6
PD-L1
D
% TLR5 - CD11b+ CD11c-
TNFa
IL-12
IL-10
IL-6
PD-L1
E
MFI PD-L1 for TLR5- CD11b+ CD11c-
F
MFI PD-L1 for TLR5+ CD11b+ CD11c-

Figure 2.4. Temporal analysis of TLR5 expression in myeloid and dendritic cell populations within tumor-draining lymph nodes, systemically within the spleen, and within the tumor microenvironment of TLR5 reporter mice.

Analysis of TLR5 expressing cell subsets from the indicated tissues of naïve Td-Tomato TLR5 reporter mice (no tumor) from mice during early (day 10) and progressively advanced stages of ovarian tumor progression (days 20 and 30). Tissues from TLR5 reporter mice bearing ID8-*Defb29/Vegf-A* tumors were examined by flow cytometry at the indicated times post tumor implantation and compared to tissues from naïve animals. **(A)** Numbers of immune cell populations in the spleen; or **(B)** tumor-draining mediastinal lymph node. Percentages are calculated based upon total TLR5+ and – cell proportions and depicted as stacked bar graphs. **(C-D)** Analysis of CD11b+ CD11c- myeloid cells from peritoneal wash and tumor nodules of Td-Tomato TLR5 reporter mice administered brefeldin A 6 hours prior to analysis for cytokines. Values indicate proportions of each functional subset indicated in the legend from either TLR5+ **(C)** or TLR5- **(D)** myeloid populations. **(E-F)** MFI of PD-L1 expression on TLR5- **(E)** or TLR5+ **(F)** CD11b+ CD11c- myeloid cells. n=5 per group. Significance was calculated using an unpaired non-parametric t-test with Mann Whitney correction (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). Error bars represent mean ± SEM

TLR5 signaling is associated with reduced accumulation of cDC1s in the ovarian tumor microenvironment.

We observed that TLR5-expressing DCs expressing high levels of PD-L1 on their surface accumulate within the ovarian TME as tumor burden increases. Given that DCs are known to orchestrate anti-tumor immunity and response to PD-L1 blockade, we hypothesized that TLR5 signaling may be detrimental to the therapeutic efficacy of anti-PD-L1 through modulation of DCs. To better understand how DC phenotype and function was affected in the absence of TLR5 signaling, we compared DC subsets between wild-type and TLR5 KO mice bearing ID8-*Defb29/Vegf-A* tumors. DCs within the TME were assessed prior to (day 7) and during (day 15) administration of anti-PD-L1. cDC1s express chemokine receptors XCR1[202] and CD103[203] and are critical for the activation of anti-tumor CD8 T cells, associating with greater survival outcomes during checkpoint therapy[37, 204]. Very few cDC1s were observed within the peritoneal cavity of either strain of mice without tumors (**Figure 2.5A**), suggesting that cDC1s are either actively recruited to or expanded in the ovarian TME. At day 7, there was a slight, but significant, increase in total and IL-12-producing cDC1s for both wild-type and TLR5 KO mice when compared to non-tumor-bearing animals (**Figure 2.5A and 2.5B**). By day 15, cDC1 numbers in TLR5 KO mice were significantly greater than wild-type mice at the same temporal point, with the total number of IL-12 producing cDC1s within the TLR5 KO ovarian TME correspondingly increased (**Figure 2.5A and B**). In addition to the increased quantity of cDC1s in the TLR5 KO mice on day 15, we also observed greater expression of functional indicators IL-12 and CD80, measured by mean fluorescent intensity (MFI) (**Figure 2.5C and 2.5D**). The cytokine IL-12 is normally produced in response to T cell-DC interactions and is critical for growth and function of T cells[205, 206]. Additionally, the ligand CD80 is a co-stimulatory factor indicative of DC maturation[207, 208]. These data suggest that TLR5 KO cDC1s are more functional and mature than wild-type cDC1s during the period in which anti-PD-L1 is administered. Although there was a significant expansion in XCR1/CD103 double negative DC subsets in the TME of TLR5 KO mice on day 15, there were

no discernible differences in maturation or function, when compared to wild-type DCs with similar phenotypic attributes (**Figure 2.6A-C**). We also observed a significant reduction in CD11b+ DCs and an increase in mucosal and migratory DCs in the TME of TLR5 KO mice on day 15 (**Figure 2.6D-F**). Therefore, at the time when PD-L1 blockade is administered, TLR5 KO mice have significantly increased numbers of functional/mature cDC1s, corresponding to an environment that is better poised to expand T cells in response to PD-L1 blockade. On the other hand, mice with intact TLR5 signaling experience reduced differentiation and/or maturation of CD103+ DCs during PD-L1 blockade. We did not observe numeric or phenotypic differences in any DC subset within the tumor-draining mediastinal lymph node (TDLN) (**Figure 2.6G-L**), suggesting that at these temporal points, the effects of TLR5 signaling on DC phenotype and function occur predominantly within the ovarian TME.

We next blocked TLR5 signaling using a neutralizing antibody against mouse TLR5 (αTLR5)[209] to define how transient blockade of TLR5 signaling impacted DC phenotype and function during advanced ovarian cancer (**Figure 2.5E**). Using t-SNE (t-distributed Stochastic Neighbor Embedding) on CD11c+ MHCII+ pre-gated cells within the TME, we observed an increased frequency of CD103+ and XCR1+ DCs after acute TLR5 blockade when compared to the IgG2a isotype, as depicted by the magenta population highlighted by the red circle and red arrows (**Figure 2.5F-H**). These data indicate that acute TLR5 inhibition is sufficient to enhance the accumulation and/or differentiation of cDC1s within the ovarian TME of wild-type mice. Validating the t-SNE results using hierarchical gating, we confirmed a significant increase in total cDC1s in response to TLR5 inhibition (**Figure 2.5I**). No significant changes to XCR1 and CD103 negative, migratory, and mucosal DCs were observed after acute TLR5 inhibition (**Figure 2.6J-L**), suggesting that differentiation of cDC1s, or a precursor population, are negatively affected by TLR5 signaling. At this advanced temporal point, we also observed that TLR5 inhibition resulted in a significant reduction of PD-L1 expression on cDC1s, relative to PD-L1 expressed on cDC1s

within the TME of mice administered IgG2a isotype (**Figure 2.5J**). Collectively, these data suggest that TLR5 signaling culminates in the reduced maturation and/or functionality of cDC1s, further implicating TLR5 signaling and its effects on DCs in the failure of PD-L1 blockade.

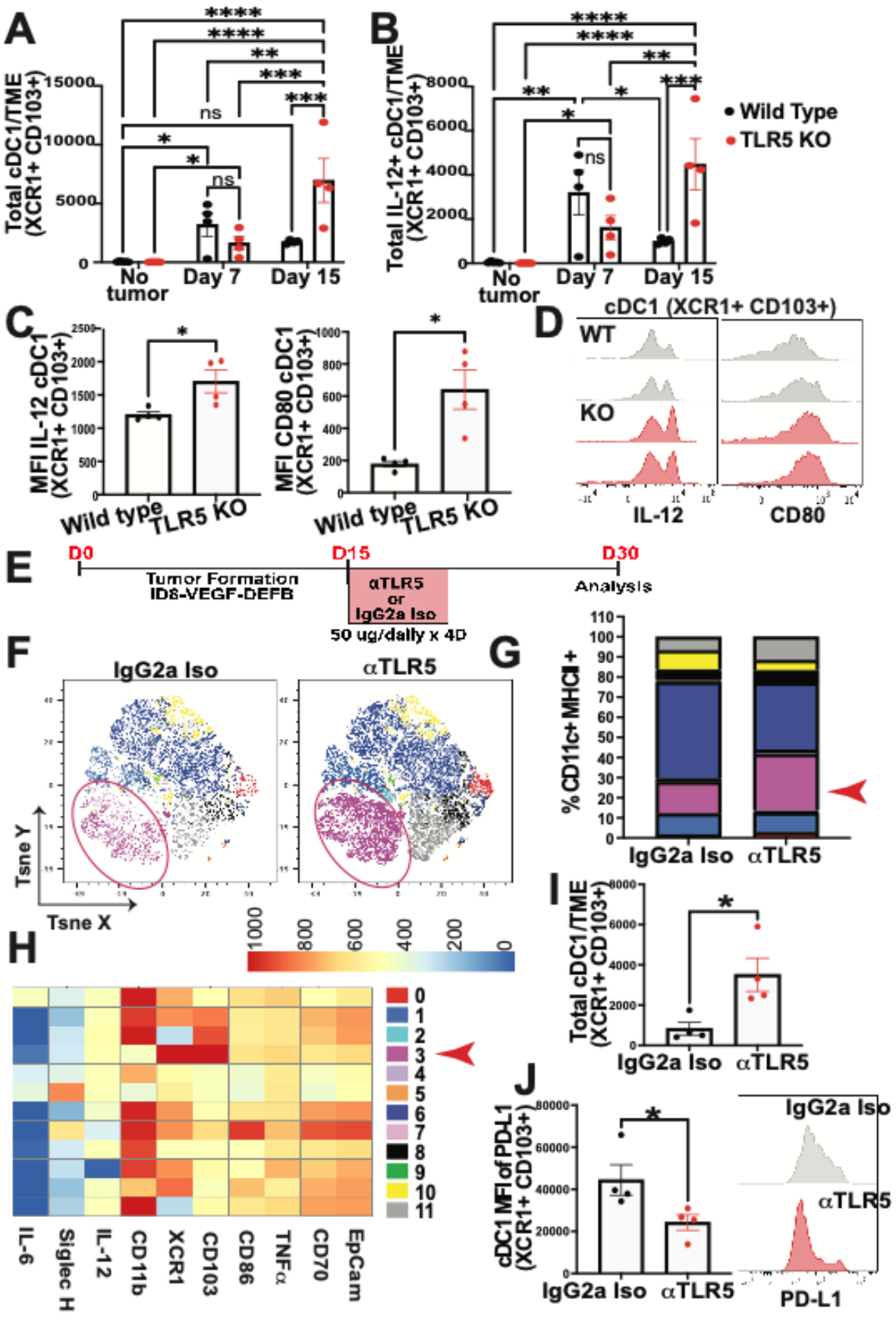

A
Total cDC1/TME (XCR1+ CD103+)
No tumor
Day 7
Day 15
B
Total IL-12+ cDC1/TME (XCR1+ CD103+)
Wild Type
TLR5 KO
C
MFI IL-12 cDC1 (XCR1+ CD103+)
MFI CD80 cDC1 (XCR1+ CD103+)
Wild type
TLR5 KO
D
cDC1 (XCR1+ CD103+)
WT
KO
IL-12
CD80
E
D0
D15
D30
Tumor Formation
ID8-VEGF-DEFB
αTLR5
or
IgG2a Iso
50 ug/daily x 4D
Analysis
F
IgG2a Iso
αTLR5
Tsne Y
Tsne X
G
% CD11c+ MHCI +
IgG2a Iso
αTLR5
H
IL-6
Siglec H
IL-12
CD11b
XCR1
CD103
CD86
TNFα
CD70
EpCam
I
Total cDC1/TME (XCR1+ CD103+)
IgG2a Iso
αTLR5
J
cDC1 MFI of PDL1 (XCR1+ CD103+)
IgG2a Iso
αTLR5
PD-L1

Figure 2.5. TLR5 signaling reduces the frequency and functionality of cDC1s in the ovarian tumor microenvironment.

(**A**) Total cDC1 and (**B**) IL-12+ cDC1 numbers in the peritoneal cavity of non-tumor-bearing or within the ovarian tumor microenvironment 7- and 15-days post-initiation of ID8-*Defb29/Vegf-A* ovarian tumors. Cells were assessed by flow cytometry. (**C**) Mean fluorescent intensity (**D**) and representative histograms of intracellular IL-12 and surface CD80 levels in cDC1s from the ovarian tumor microenvironment 15 days post-initiation of ID8-*Defb29/Vegf-A* tumors. (**E**) Schematic depicting strategy to neutralize TLR5 signaling. A neutralizing murine anti-TLR5 or IgG2a isotype control was administered for 4 consecutive days at 50μg/daily starting 15 days post-tumor initiation. Peritoneal wash samples and tumor nodules were analyzed on day 30 post-tumor initiation using spectral flow cytometry. (**F**) t-distributed stochastic neighbor embedding (t-SNE) map of pre-gated CD11c+ MHCII+ cells (putative DCs). The red circle depicts the populations highlighted by the arrow in **G** and **H**. (**G**) Population frequencies and (**H**) heat map of phenotypic marker expression by DCs from the clusters depicted in **F**. (**I**) Quantitation of cDC1 numbers and (**J**) MFI of PD-L1 levels with representative histograms after hierarchical gating analysis using FlowJo for samples from **E**. Unpaired non-parametric t-tests with Mann Whitney correction were used to calculate statistical significance (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). Error bars represent mean ± SEM. Plots are representative of at least three repeats.

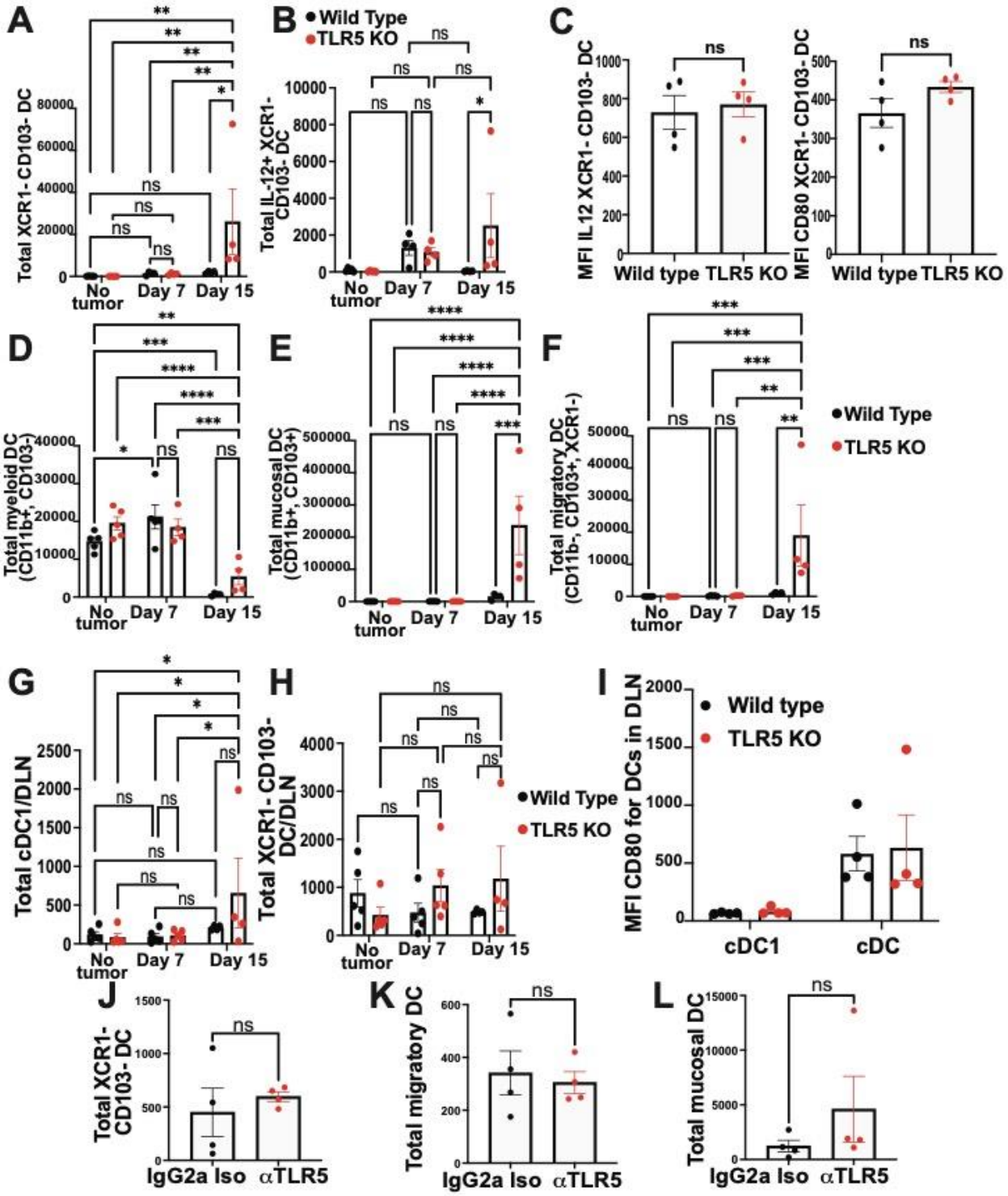

A
Total XCR1- CD103- DC
No tumor
Day 7
Day 15
B
Wild Type
TLR5 KO
Total IL-12+ XCR1- CD103- DC
C
MFI IL12 XCR1- CD103- DC
MFI CD80 XCR1- CD103- DC
Wild type
TLR5 KO
D
Total myeloid DC (CD11b+, CD103-)
E
Total mucosal DC (CD11b+, CD103+)
F
Total migratory DC (CD11b-, CD103+, XCR1-)
G
Total cDC1/DLN
H
Total XCR1- CD103- DC/DLN
I
MFI CD80 for DCs in DLN
cDC1
cDC
J
Total XCR1- CD103- DC
IgG2a Iso
αTLR5
K
Total migratory DC
L
Total mucosal DC

Figure 2.6. Dendritic cell subsets examined within the tumor microenvironment and tumor-draining lymph nodes in tumor-bearing wild type and TLR5 KO mice.

(A) Total and **(B)** IL-12+ CD103- XCR1- DCs in the ovarian TME 7- and 15 days-post administration of ID8-*Defb29/Vegf-A* tumors after assessing by flow cytometry. **(C)** MFI of IL-12 and CD80 XCR1- and CD103- DC 15-days post ID8-*Defb29/Vegf-A* tumors. **(D)** Total myeloid, **(E)** mucosal, and **(F)** migratory DC numbers overtime. **(G)** Numbers of XCR1+ CD103+ cDC1s and **(H)** XCR1- CD103- DCs in the tumor-draining mediastinal lymph node. **(I)** MFI of CD80 in CD11c+ MHCII+ DCs in the mediastinal lymph node at D15. **(J-L)** Analysis of dendritic cell subsets within the ovarian tumor microenvironment of mice bearing ID8-*Defb29/Vegf-A* tumors. At day 15, mice were treated daily with either 50μg of a neutralizing antibody for TLR5 (αTLR5) or an IgG2a isotype control for 4 consecutive days, followed by analysis at day 30. Graphs depict numbers of each DC subset from peritoneal wash and tumor nodules. **(J)** XCR1- CD103- DC. **(K)** Migratory DCs. **(L)** Mucosal DCs. Significance was calculated using an unpaired non-parametric t-test with Mann Whitney correction (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). Error bars represent mean ± SEM.

When in the same ovarian tumor microenvironment as wild-type DCs, TLR5 KO DCs retain the ability to differentiate and mature into cDC1s.

The possibility remains that differences in immune cell phenotype and response to PD-L1 blockade are mediated by indirect effects of TLR5 signaling and/or non-immune cell populations arising due to broad differences within each individual TME. To address this, we established a mixed bone marrow chimera to determine whether differences in DC phenotype are retained for TLR5 KO cells within the same ovarian tumor environment as wild-type immune cells. Lethally irradiated wild-type congenic (CD45.1) mice were reconstituted with a 1:1 mix of TLR5 KO (CD45.2) and wild-type (CD45.1) bone marrow (**Figure 2.7A**). After a 10-week rest period, ID8-*Defb29/Vegf-A* tumors were established, followed by the initiation of anti-PD-L1 or the vehicle IgG2b isotype control beginning on day 5 (**Figure 2.7A**). Anti-PD-L1 therapy was initiated early (day 5) to account for enhanced aggressiveness of tumor kinetics, which is exacerbated by irradiation. Peritoneal wash exudates/tumor nodules were harvested and assessed by flow cytometry 15 days post-tumor initiation. We observed greater frequencies of XCR1+, CD103+ TLR5 KO (CD45.2) cDC1s relative to wild-type (CD45.1) cDC1s within the same TME (**Figure 2.7B**). Differences in frequencies were accompanied by trending increases in absolute numbers of TLR5 KO versus wild-type cDC1s (**Figure 2.8A**). These data indicate that within the same TME, TLR5-deficient DCs are better able to differentiate into cDC1s relative to wild-type DCs. This is consistent with earlier observations that TLR5 deficiency or TLR5 blockade achieves a significant increase in the numbers of cDC1s within the ovarian TME (**Figure 2.5**). Additionally, these differences only manifested in ovarian tumor-bearing animals, further indicating that differences in cDC1 accumulation between wild-type and TLR5 KO cells is dependent on tumor growth (**Figure 2.7B**). Tumor-associated CD103+ DCs are found to retain T cell stimulatory capacity in advanced ovarian cancer, while CD11b-expressing DCs associate with immune suppression and poor cancer outcomes[49, 210, 211]. Proportions and total numbers of wild-type CD11b-expressing CD103+ and CD103- DCs were increased relative to TLR5 KO DCs (**Figures**

2.7C-D and Figures 2.8B-C), suggesting that TLR5 signaling has a direct impact on the differentiation and/or recruitment of cDC1s into the ovarian tumor microenvironment. Further supporting that TLR5 signaling impairs the ability of DCs to initiate and support anti-tumor T cell responses, there were significantly reduced frequencies and numbers of TLR5 KO cDC1s expressing PD-L1 compared to wild-type cDC1s, whereas there were increased frequencies of IL-12-expressing TLR5 KO cDC1s (**Figures 2.7E-F and Figures 2.8D-E**). Overall, TLR5-deficient DCs maintain a cDC1 phenotype, with low expression of PD-L1, despite being within the same TME as wild-type DCs. These phenotypic changes occurring on wild-type but not TLR5 KO cells are observed in a mixed bone marrow chimera setting, suggesting that shifts in DC phenotype or differentiation are occurring due to the direct effects of TLR5 signaling, independent of microenvironmental differences within the TME.

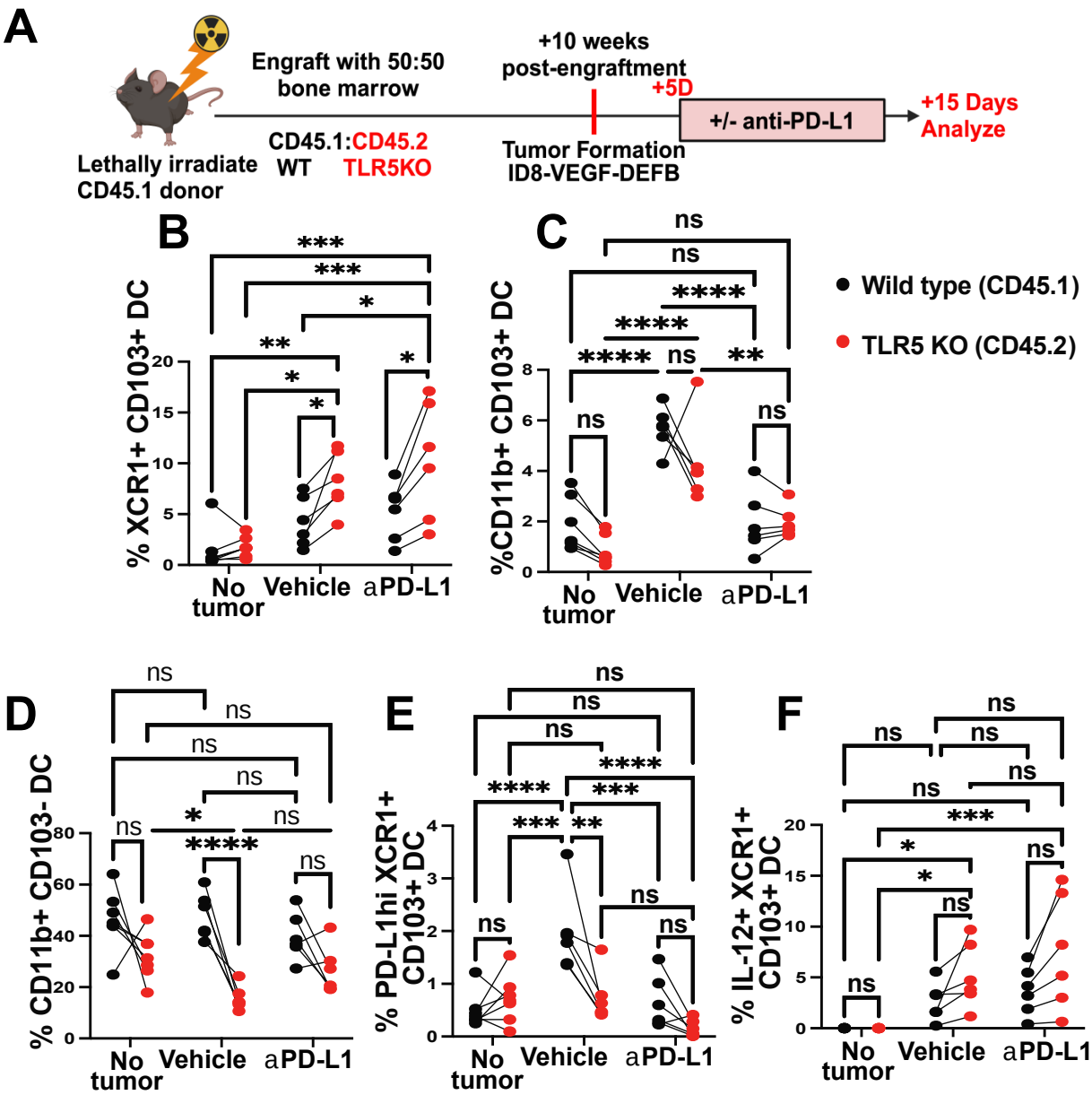

A
Lethally irradiate CD45.1 donor
Engraft with 50:50 bone marrow
CD45.1:CD45.2
WT TLR5KO
+10 weeks post-engraftment
+5D
Tumor Formation ID8-VEGF-DEFB
+/- anti-PD-L1
+15 Days Analyze
B
% XCR1+ CD103+ DC
No tumor
Vehicle
aPD-L1
C
%CD11b+ CD103+ DC
Wild type (CD45.1)
TLR5 KO (CD45.2)
D
% CD11b+ CD103- DC
E
% PD-L1hi XCR1+ CD103+ DC
F
% IL-12+ XCR1+ CD103+ DC

Figure 2.7. TLR5 signaling *in vivo* attenuates the accumulation and functionality of tumor-associated DC subsets.

(**A**) Schema for mixed bone marrow chimera and treatment regimen. Recipient wild-type (CD45.1) mice were irradiated (2 consecutive days x 600 rads/day) and reconstituted with an equal mix of donor bone marrow cells (TLR5 KO:Wild-type). ID8-*Defb29/Vegf-A* cells tumors were initiated 10-weeks post-reconstitution followed by treatment with anti-PD-L1 five days post-tumor initiation. Peritoneal wash exudates and tumor nodules were collected 15 days after tumor initiation and analyzed by flow cytometry. (**B**) Frequency of XCR1+CD103+ (cDC1), (**C**) CD11b+CD103+ (mucosal DC), (**D**) CD11b+CD103- (myeloid DC), (**E**) PD-L1hi wild-type (CD45.1) or TLR5 KO (CD45.2) cDC1 (XCR1+CD103+) with or without anti-PD-L1 therapy. (**F**) Frequency of wild-type (CD45.1) or TLR5 KO (CD45.2) IL-12+ cDC1s. Unpaired with Mann Whitney (between mice) or paired t-tests (within mice) were used to calculate significance (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). Plots are representative of three experiments.

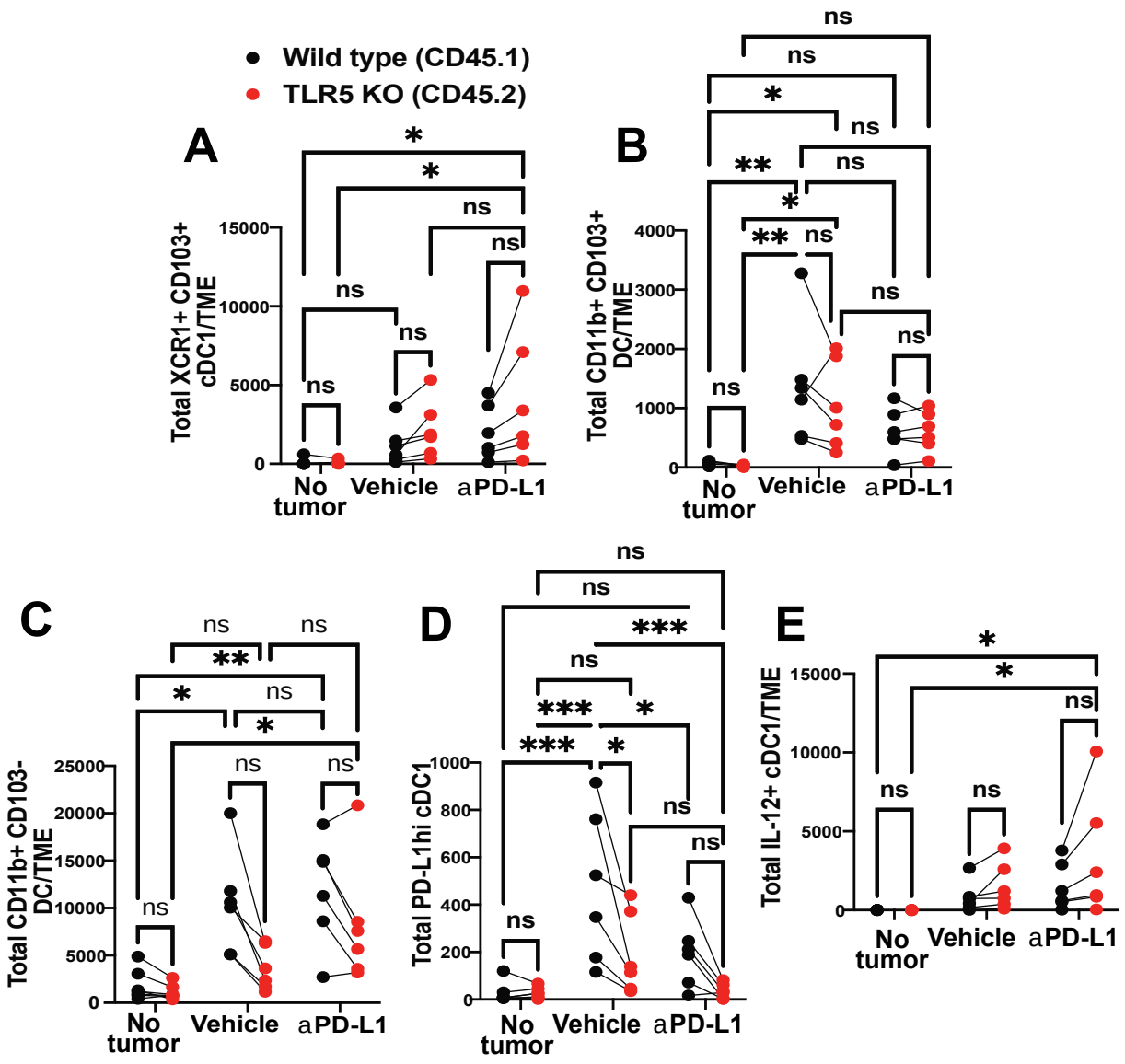

Wild type (CD45.1)
TLR5 KO (CD45.2)
A
Total XCR1+ CD103+ cDC1/TME
15000
10000
5000
0
No tumor
Vehicle
aPD-L1
B
Total CD11b+ CD103+ DC/TME
4000
3000
2000
1000
0
C
Total CD11b+ CD103- DC/TME
25000
20000
15000
10000
5000
0
D
Total PD-L1hi cDC1
1000
800
600
400
200
0
E
Total IL-12+ cDC1/TME
ns
*
**
***

Figure 2.8. Comparative analysis of WT and TLR5 KO DC subsets within the same ovarian tumor microenvironment.

Briefly, congenic CD45.1 wild type or TLR5 KO CD45.2 bone marrow was mixed 50:50 and engrafted into a lethally irradiated wild type congenic CD45.1 recipients. After allowing cells to engraft over a period of 10 weeks, ovarian tumors were initiated, and peritoneal wash exudates/tumor nodules were analyzed 15 days post-tumor initiation. **(A-C)** Absolute numbers of XCR1+CD103+ **(A),** CD11b+CD103+ **(B)**, and CD11b+CD103- **(C)** DC subsets. **(D-E)** Phenotypic analysis of cDC1 subsets, depicting absolute numbers of PD-L1hi **(D)** or IL-12-producing **(E)** cDC1s. All samples were assessed by flow cytometry. Absolute numbers were calculated using counting beads. Significance was calculated using unpaired (between mice) or paired t-tests (within mice) (* $p < .05$, ** $p < .01$, *** $p < .001$). Error bars represent mean ± SEM.

Chronic exposure of FLT3L-cultured BMDCs to bacterial flagellin results in preferential expansion of PD-L1-expressing myeloid cells.

Our data thus far indicate that TLR5 signaling mediates phenotypic changes in dendritic cells within the ovarian TME. To define the direct effects of TLR5 signaling on DC differentiation and function independently of tumor growth, we established *in vitro* cultures using bone marrow cells expanded with FLT3L, an *in vitro* method utilized to enrich bone marrow-derived DC cultures for cross-presenting cDC1s from bone marrow progenitors[212, 213]. Using the gating scheme outlined in **Figure 2.10**, we found that for both TLR5 KO and wild-type bone marrow, there was a similar expansion of both cDC1s and cDC2s when cultured in FLT3L alone, while frequencies of cDC1s were reduced only in wild-type mice after chronic exposure to flagellin (**Figure 2.9A and 2.10C-D**). The reduction in cDC1 frequencies for wild-type cultures was not due to increased cell death after chronic exposure to flagellin (**Figure 2.10B**) and was recapitulated regardless of the bacterial source of flagellin (**Figure 2.10C**). This resulted in a significant decline in proportions of cDC1s as the days of exposure to flagellin were increased (**Figure 2.10E**). Although frequencies of cDC1s were reduced in wild-type cultures after chronic exposure to flagellin, we did not observe significant differences in the total number of cDC1s within any culture conditions (**Figure 2.9B**), likely due to the expansion of other myeloid subsets within wild-type cultures. Wild-type cultures with chronic exposure to flagellin had significant increases in the numbers of monocytes and other undefined myeloid cells, whereas TLR5 KO cultures did not exhibit any changes over time in response to chronic or acute exposure to flagellin (**Figure 2.9B)**. Although we did not assess the suppressive potential of Ly6C+ monocytes, cells expressing similar phenotypic markers within the ovarian TME are known to be poor prognostic indicators[214, 215]. Functionally, there was a significant expansion of PD-L1-expressing myeloid subsets after chronic exposure to flagellin and FLT3L (**Figure 2.9C**). The role of tumors in the expansion and induction of PD-L1 on tumor-associated myeloid cells is well-defined[216, 217]. Our data indicate that in the absence of a tumor, chronic exposure to bacterial flagellin in the presence of FLT3L is sufficient to promote

differentiation of cDCs away from a phenotype that is well-equipped to activate CD8 T cells towards a myeloid phenotype that is less capable of stimulating CD8 T cells. Interferon regulatory factor-8 (IRF8) is a terminal selector for the cDC1 lineage and maintains cDC1 identity by preventing cDC1 conversion into cDC2s[218-220]. We found that both acute and chronic TLR5 signaling resulted in a significant reduction of IRF8 for wild-type DCs, whereas TLR5 KO DCs maintained similar levels of IRF8 across all conditions (**Figure 2.9D**). Therefore, it is plausible that the observed decrease in IRF8 resulted in subsequent increase in cDC2s, monocytes and other myeloid cells after chronic exposure to flagellin.

Next, we tested the ability of each mixed culture condition to activate antigen-specific CD8 T cells. To examine cross-presentation, SIINFEKL peptide was added on day 7 to the same culture system, followed by cell trace labeled OT-1 transgenic CD8 T cells on day 8. After three days of expansion, we observed that OT-1 T cells cultured with wild-type BMDC cultures exposed to chronic flagellin and FLT3L proliferated significantly less than those incubated with TLR5 KO cultures, as measured by dilution of cell trace violet (**Figure 2.9E-F**). Altogether, these data indicate that TLR5 signaling promotes expansion of PD-L1-expressing myeloid subsets over cross-presenting cDC1 populations. The mechanism by which this occurs is unclear, however, it is plausible that flagellin binds to TLR5 and the corresponding signaling cascade induces expression of genes that promote myeloid development. TLR5 signaling associates with increased IL-6 expression[126], which we find is increased in FLT3 cultures upon exposure to flagellin (**Figure 2.10F**). Chronic IL-6 signaling can induce myeloid differentiation favoring MDSCs[221]. Further studies are needed to distinguish whether TLR5 alone, or synergism between TLR5 and IL-6, are reducing the accumulation of cDC1s to favor expansion of other tumor-supporting myeloid subsets.

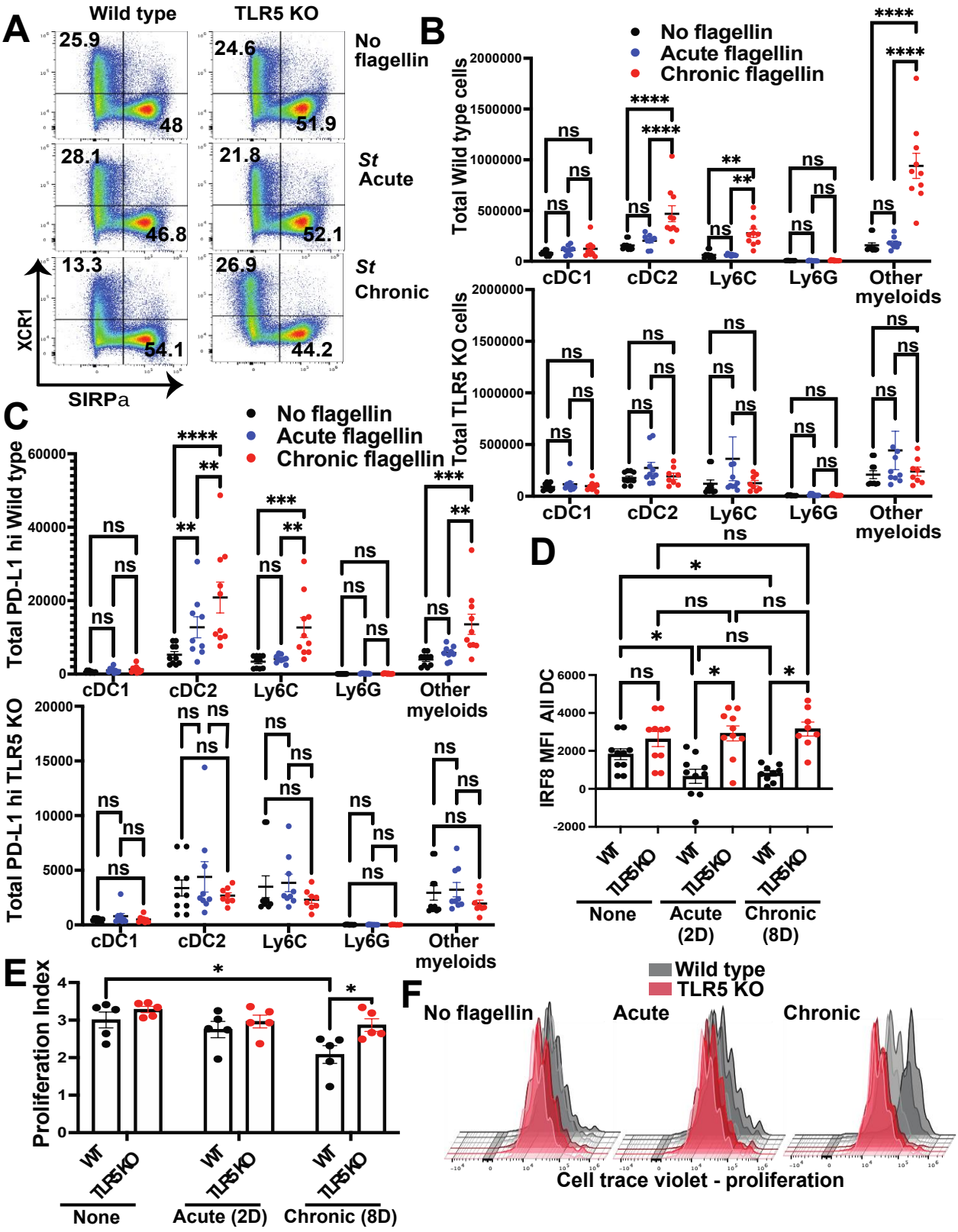

A
Wild type
TLR5 KO
No flagellin
St Acute
St Chronic
25.9
48
24.6
51.9
28.1
46.8
21.8
52.1
13.3
54.1
26.9
44.2
XCR1
SIRPa
B
No flagellin
Acute flagellin
Chronic flagellin
Total Wild type cells
Total TLR5 KO cells
cDC1
cDC2
Ly6C
Ly6G
Other myeloids
C
Total PD-L1 hi Wild type
Total PD-L1 hi TLR5 KO
D
IRF8 MFI All DC
WT
TLR5KO
None
Acute (2D)
Chronic (8D)
E
Proliferation Index
F
Wild type
TLR5 KO
No flagellin
Acute
Chronic
Cell trace violet - proliferation

Figure 2.9. Chronic TLR5 signaling by flagellin promotes differentiation and expansion of PD-L1 myeloid subsets, leading to impaired CD8 T cell activation.

Briefly, bone marrow cells from wild-type or TLR5 KO mice were cultured for 8 days with FLT3L and purified flagellin (*Salmonella typhimurium*) in an acute setting, for 2 days (2D) of culture; or a chronic setting, for 8 days (8D) of culture. (**A**) Representative dot plots of wild-type or TLR5 KO conventional dendritic cells cDC1 (XCR1+) or cDC2 (SIRPα+). Plots represent cell populations after the 8-day culture period followed by flow cytometry analysis. (**B**) Total numbers of differentiated myeloid and dendritic cell subsets in cultures of wild-type or TLR5 KO bone marrow with FLT3L +/- flagellin: cDC1s (CD11c+, MHCIIhi, XCR1+SIRPα-), cDC2s (CD11c+, MHCIIhi, XCR1-SIRPα+), Ly6C (CD11c-, CD11b+Ly6C+, Ly6G-), Ly6G (CD11c-, CD11b+, Ly6Clow, Ly6G+), and other undefined myeloid cells (CD11c-, CD11b+, Ly6C-, Ly6G-). (**C**) Total numbers of PD-L1 high expressing subsets from **B**. (**D**) Intracellular levels of IRF8 (MFI) from cultures in **B**. (**E-F**) To assess the ability of bone marrow cells cultured in FLT3L +/- flagellin to present antigen and activate CD8 T cells, bulk DC/myeloid cultures were pulsed with SIINFEKL peptide followed by incubation with cell trace violet labeled OT-1 transgenic T cells, followed by analysis of T cell proliferation on a flow cytometer. (**E**) Proliferation index and (**F**) representative histograms of OT-1 CD8 T cells co-cultured with SIINFEKL peptide pulsed bone marrow FLT3L cultures. Unpaired t-test with Mann Whitney correction was used to calculate significance. (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). Plots represent all data points combined from two experiments.

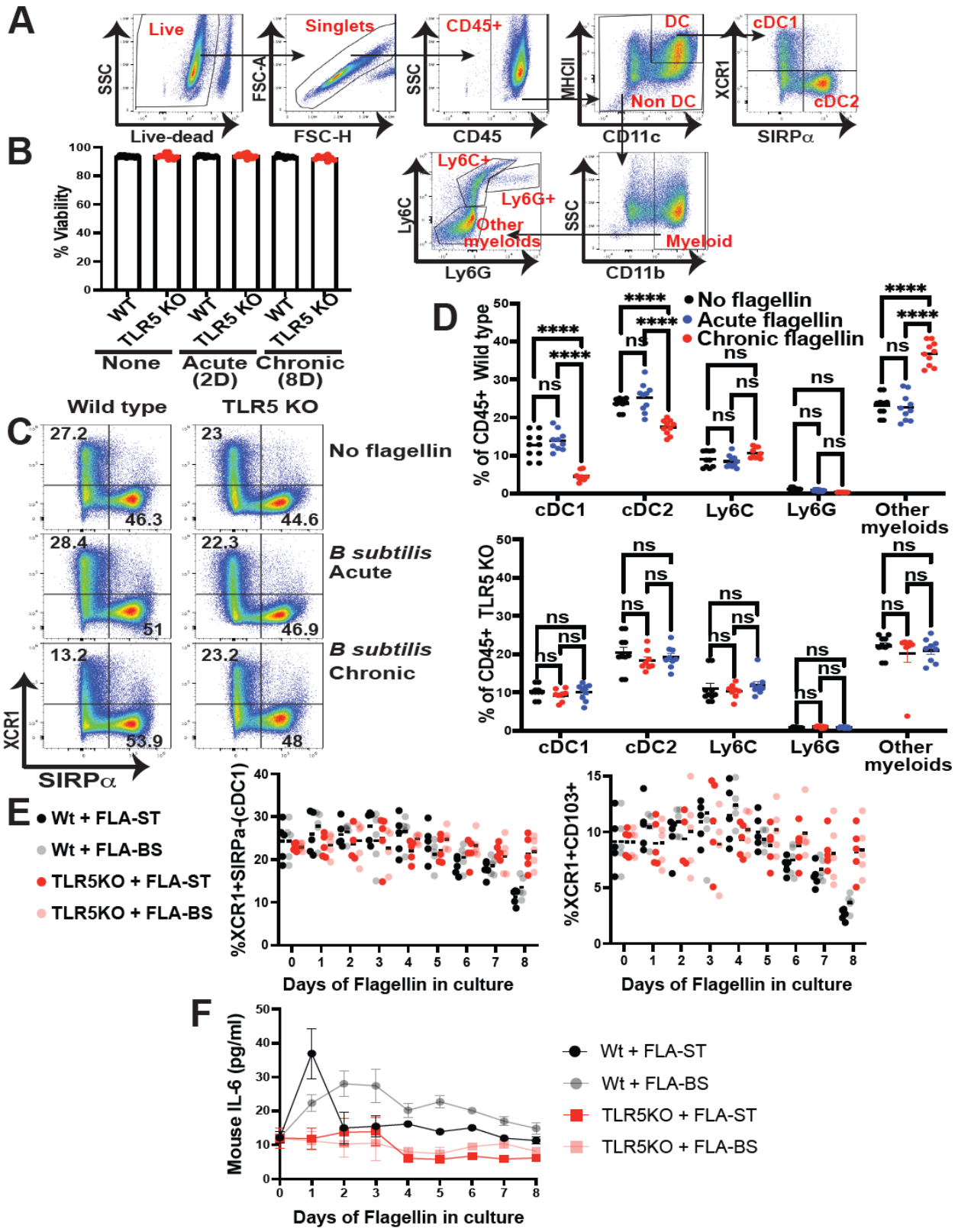

A
Live
SSC
Live-dead
Singlets
FSC-A
FSC-H
CD45+
SSC
CD45
DC
Non DC
MHCII
CD11c
cDC1
cDC2
XCR1
SIRPα
B
% Viability
WT
TLR5 KO
None
Acute (2D)
Chronic (8D)
Ly6C+
Ly6G+
Other myeloids
Ly6C
Ly6G
SSC
Myeloid
CD11b
C
Wild type
TLR5 KO
No flagellin
B subtilis Acute
B subtilis Chronic
27.2
46.3
23
44.6
28.4
51
22.3
46.9
13.2
53.9
23.2
48
XCR1
SIRPα
D
No flagellin
Acute flagellin
Chronic flagellin
% of CD45+ Wild type
% of CD45+ TLR5 KO
cDC1
cDC2
Ly6C
Ly6G
Other myeloids
ns
****
E
Wt + FLA-ST
Wt + FLA-BS
TLR5KO + FLA-ST
TLR5KO + FLA-BS
%XCR1+SIRPa-(cDC1)
%XCR1+CD103+
Days of Flagellin in culture
F
Mouse IL-6 (pg/ml)
Days of Flagellin in culture
Wt + FLA-ST
Wt + FLA-BS
TLR5KO + FLA-ST
TLR5KO + FLA-BS

Figure 2.10. Phenotypic assessment of FLT3L cultured bone marrow-derived dendritic cells with acute or chronic exposure to bacterial flagellin.

Bone marrow from wild type or TLR5 KO mice were cultured in media containing FLT3L, to establish DC cultures with an enrichment of cDC1s. Flagellin was added to cultures for 2 (acute) or 8 days (chronic), after which cells were stained and analyzed using a flow cytometer. **(A)** Gating hierarchy of cells collected after expansion of bone marrow cells in FLT3L. **(B)** Percentage of viable cells, out of total cells in the culture, as assessed by frequency of Zombie Aqua negative cells. **(C)** Representative gates of WT or TLR5 KO CD11c+MHCII+ cDC1 (XCR1+) or cDC2 (SIRPα) after 8 days of expansion with FLT3L and purified flagellin (*Bacillus subtilis*). **(D)** Frequency of differentiated DC and myeloid cells from culture in wild type or TLR5 KO mice. **(E)** Frequency of cDC1s gated based on either XCR1+ SIRPα or XCR1+ CD103+ after exposure to flagellin in culture for the indicated number of days in the X axis. Flagellin was either derived from *Salmonella typhimurium* (ST) or *Bacillus subtilis* (BS). **(F)** Levels of IL-6 from FLT3L culture supernatants taken from the culture in **E**. Significance was calculated using an unpaired non-parametric t-test with Mann Whitney correction (* $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). Error bars represent mean ± SEM.

CD8 T cells are critical for mediating survival in ovarian tumor-bearing TLR5 KO mice treated with PD-L1 blockade.

We have demonstrated that TLR5 signaling impacts the phenotype of cDC1s, a likely liaison between anti-tumor T cells and control of tumor growth in TLR5 KO mice. cDC1s are central for activating and/or priming of tumor-reactive CD8 T cells[180-184] and enhancing CD8 T cell activation and function[222], in addition to response during checkpoint blockade[223]. Considering that tumor-infiltrating CD8 and CD4 effector cells correspond with increased overall and long-term survival in ovarian cancer[224-226], and given the association between cDC1s and CD8 T cell activation, we sought to examine the role of CD8 T cells during enhanced survival in TLR5 KO ovarian tumor-bearing mice. CD8 T cells were depleted prior to tumor initiation and in combination with anti-PD-L1 therapy (**Figure 2.11A and 2.12A**). Depletion of CD8 T cells with anti-CD8a prior to and during anti-PD-L1 therapy eliminated the survival benefit observed in TLR5 KO mice, indicating that CD8 T cells are critical effectors for driving survival during PD-L1 blockade (**Figure 2.11B**). To examine how these changes in T cell phenotype correspond to the previously noted differences in DC phenotype and function, we assessed the T cell compartment within TDLN, the spleen, and the TME by flow cytometry 15 and 30 days post-ovarian tumor initiation, using the gating strategy depicted in **Figure 2.12B.** In the absence of PD-L1 therapy, TLR5 KO mice had significantly greater numbers of both total CD8 and CD4 subsets in addition to greater numbers of central memory ($CD62L^{hi}$ and $CD44^{hi}$) and effector ($CD62L^{low}$ and $CD44^{hi}$) T cells in the TME by 15 days post-tumor initiation (**Figure 2.11C and Figure 2.12C**). During advanced tumor progression (day 30), effector CD8 T cells remained significantly elevated within the TME regardless of anti-PD-L1 therapy, while the accumulation of both effector and central memory CD8 and CD4 subsets was increased in response to anti-PD-L1 therapy (**Figure 2.11C and Figure 2.12C**). Corresponding with increased numbers of functional T cells, numbers of IFNγ-producing CD8 T cells were significantly elevated in TLR5 KO mice relative to wild-type mice during both early and advanced tumor progression (**Figure 2.11D**). IFNγ-producing CD4 T cells were also detected at significantly

greater numbers in the TME of TLR5 KO mice at day 15 post-tumor and day 30 after treatment with PD-L1 blockade (**Figure 2.12D**). Phenotypic and functional differences in T cells were only observed in the TME, and not in the tumor-draining mediastinal lymph nodes (**Figure 2.12E-H**), paralleling differences observed when evaluating tumor-associated DC infiltrates in the TME of TLR5 KO mice. Together, these data indicate that in the absence of TLR5 signaling, T cells are better able to accumulate and function within the ovarian TME. Furthermore, these data suggest that the negative impact of TLR5 signaling on T cells is predominantly occurring within the ovarian TME, as opposed to tumor-draining lymph nodes.

To define how PD-L1 blockade in the absence of TLR5 signaling affects CD8 T cell function during the initiation of early effector responses and following recall against the same tumor antigens, CD8 T cells were analyzed from peritoneal wash exudates and tumor nodules 7 days post-injection of ID8-*Defb29/Vegf-A* tumors into the groups depicted in **Figure 2.11E**. Early CD8 T cell effector responses were evaluated 7 days after naïve mice were given ovarian tumors. CD8 T cell function during tumor rechallenge was evaluated 7 days post-tumor rechallenge of TLR5 KO mice that survived initial challenge with ID8-*Defb29/Vegf-A* tumors and anti-PD-L1 therapy. Compared to non-tumor-bearing TLR5 KO mice, after 7 days of tumor there was a significant expansion of IFNγ-producing and CD107a-positive CD8 T cells, markers associated with activation, degranulation, and function within the ovarian TME (**Figure 2.11F-G**). Although wild-type animals exhibited a slight increase in the accumulation of functional CD8 T cells after 7 days of tumor, numbers of activated T cells within the tumor microenvironment did not reach significance relative to that observed in non-tumor-bearing controls. TLR5 KO mice surviving a secondary challenge to ovarian tumors maintained significant numbers of CD127+ KLRG1- CD8, but not CD4, T cells within the peritoneal cavity prior to tumor rechallenge (**Figure 2.12I-J**). CD127+ and KLRG1- T cells are considered precursor cells to long-lived memory subsets, capable of rapidly expanding and responding against antigen rechallenge[227, 228]. In line with this observation, TLR5 KO mice

receiving a tumor rechallenge exhibited a robust and significant expansion of functional CD8 T cells, a response that increased in magnitude after each successive exposure to tumor antigen (**Figure 2.11F-G**). These data, in addition to the survival data depicted in **Figure 2.1**, indicate that in the absence of TLR5 signaling, PD-L1 blockade enables the acquisition of a functionally robust and durable CD8 T cell response against ovarian cancer.

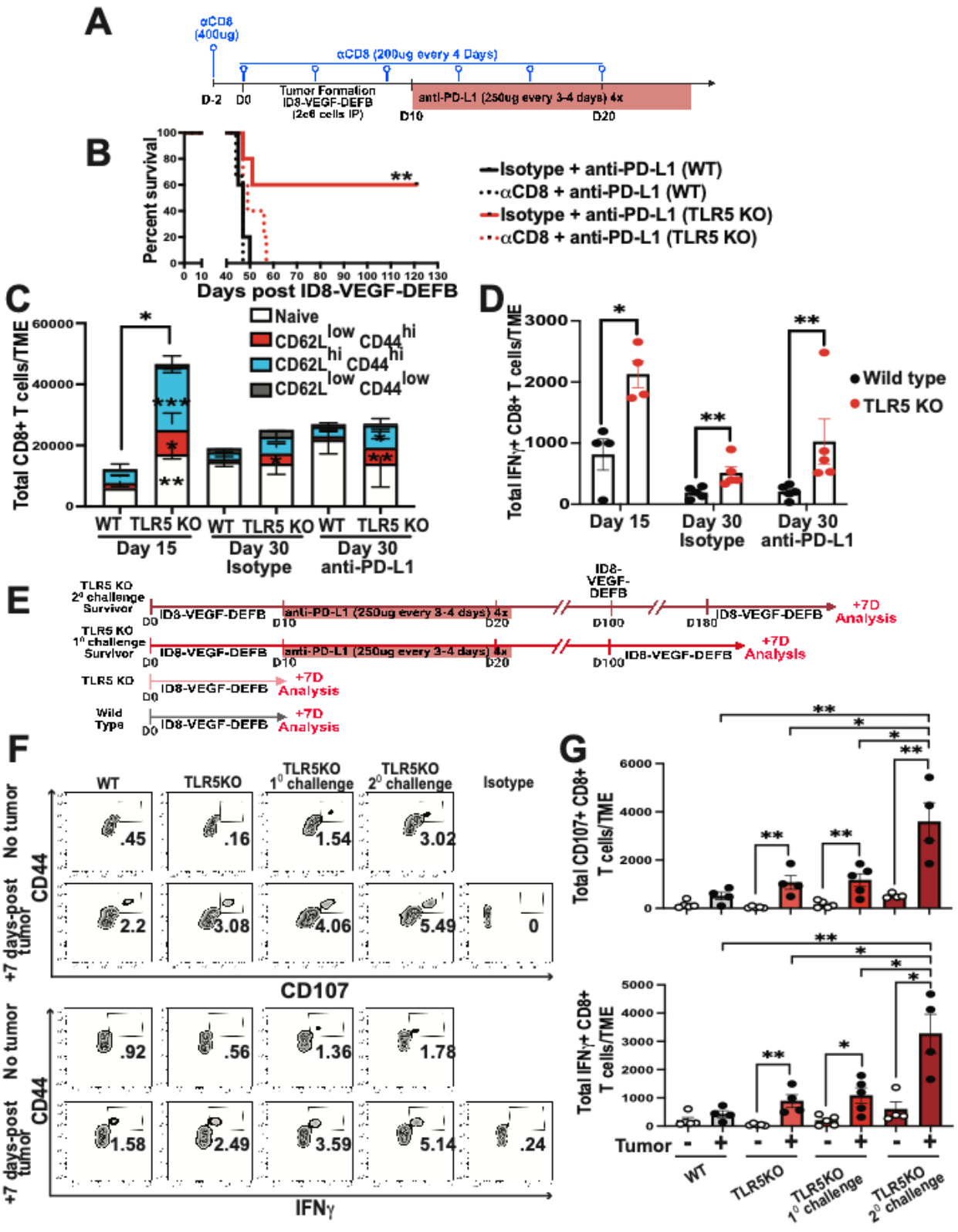

A
αCD8 (400ug)
αCD8 (200ug every 4 Days)
D-2
D0
Tumor Formation ID8-VEGF-DEFB (2e6 cells IP)
anti-PD-L1 (250ug every 3-4 days) 4x
D10
D20
B
Percent survival
Days post ID8-VEGF-DEFB
Isotype + anti-PD-L1 (WT)
αCD8 + anti-PD-L1 (WT)
Isotype + anti-PD-L1 (TLR5 KO)
αCD8 + anti-PD-L1 (TLR5 KO)
C
Total CD8+ T cells/TME
Naive
CD62L low CD44 hi
CD62L hi CD44 hi
CD62L low CD44 low
WT TLR5 KO WT TLR5 KO WT TLR5 KO
Day 15
Day 30 Isotype
Day 30 anti-PD-L1
D
Total IFNγ+ CD8+ T cells/TME
Wild type
TLR5 KO
Day 15
Day 30 Isotype
Day 30 anti-PD-L1
E
TLR5 KO 2° challenge Survivor
TLR5 KO 1° challenge Survivor
TLR5 KO
Wild Type
ID8-VEGF-DEFB
anti-PD-L1 (250ug every 3-4 days) 4x
+7D Analysis
F
WT
TLR5KO
TLR5KO 1° challenge
TLR5KO 2° challenge
Isotype
No tumor
+7 days-post tumor
CD44
CD107
IFNγ
G
Total CD107+ CD8+ T cells/TME
Total IFNγ+ CD8+ T cells/TME
Tumor
WT
TLR5KO
TLR5KO 1° challenge
TLR5KO 2° challenge

Figure 2.11. CD8 T cells are necessary for survival of TLR5 KO ovarian tumor-bearing mice treated with anti-PD-L1.

(**A**) Treatment schema with IP administration of anti-CD8a (αCD8a) and anti-PD-L1. (**B**) Survival proportions of TLR5 KO and wild-type mice bearing ID8-*Defb29/Vegf-A* tumors. Log-rank test for survival compared to wild-type (** $p < .01$) N=5 per group. (**C**) CD8 T cell memory subsets and (**D**) IFNγ+ CD8 T cells from the ovarian TME were quantified after flow cytometry analysis at 15- or 30-days post-tumor initiation. Unpaired t-test with Mann Whitney correction (* $p < .05$, ** $p < .01$, *** $p < .001$). N=5 per group. (**E**) Treatment schema to investigate T cell function during acute (7 days post-tumor, +7D) or during tumor rechallenge of TLR5 KO mice surviving after ID8-*Defb29/Vegf-A* tumors and anti-PD-L1 therapy. Secondary challenge indicates surviving TLR5 KO mice that received a re-injection of ID8-*Defb29/Vegf-A* tumors 100 days after primary tumor challenge. Primary challenge indicates surviving TLR5 KO mice after primary tumor challenge and PD-L1 blockade. All groups of mice as indicated were assessed 7 days following ID8-*Defb29/Vegf-A* tumor administration. (**F**) Representative gating of CD8 T cells expressing CD107a and IFNγ. (**G**) Quantification of T cells within the ovarian TME 7 days post-tumor challenge. Unpaired t-test with Mann Whitney Correction (* $p < .05$, ** $p < .01$). Error bars represent mean ± SEM. Plots are a representative of two experiments.

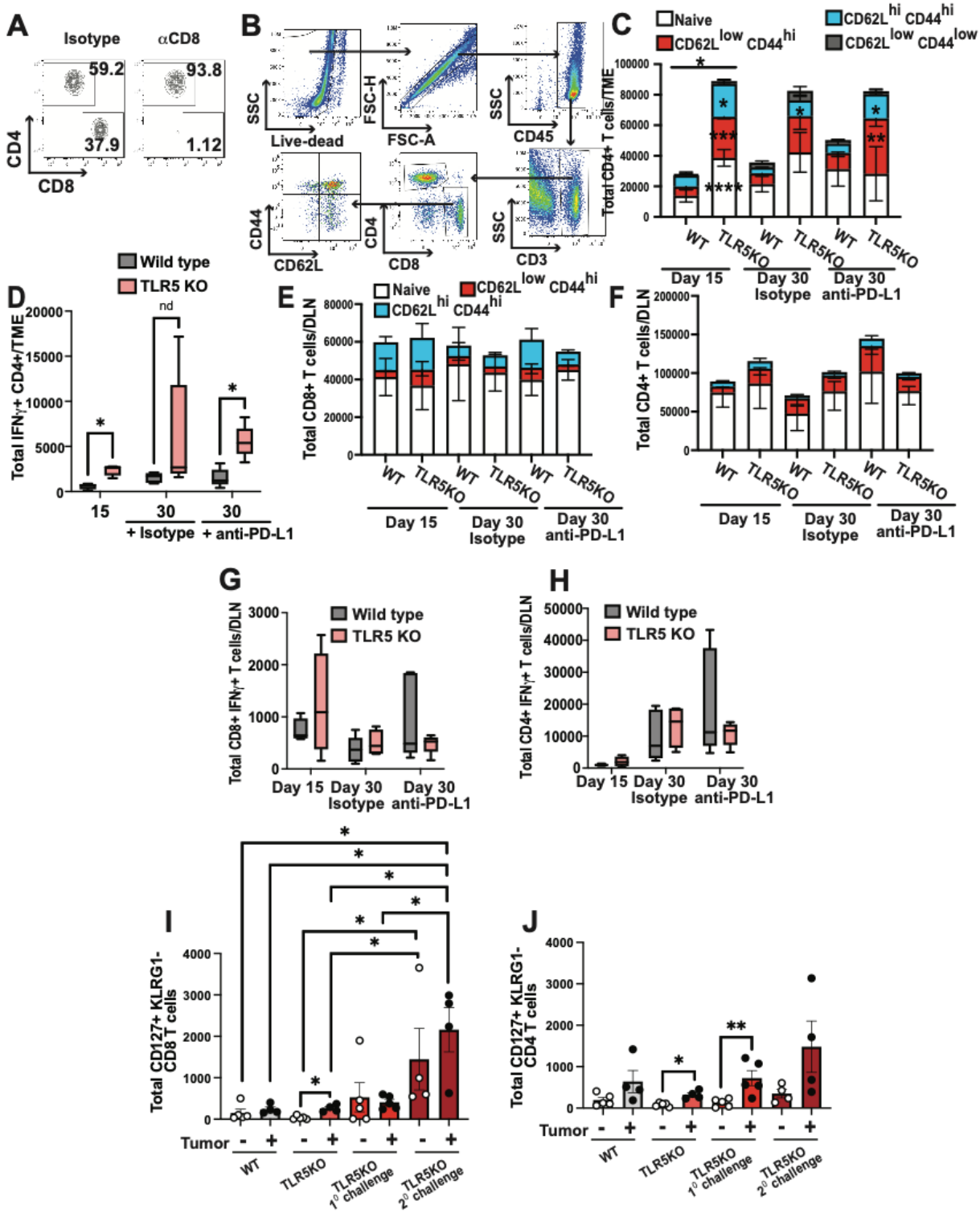

A
Isotype
αCD8
59.2
93.8
37.9
1.12
CD4
CD8
B
SSC
Live-dead
FSC-H
FSC-A
SSC
CD45
CD44
CD62L
CD4
CD8
SSC
CD3
C
Naive
CD62L low CD44 hi
CD62L hi CD44 hi
CD62L low CD44 low
Total CD4+ T cells/TME
WT
TLR5KO
Day 15
Day 30 Isotype
Day 30 anti-PD-L1
D
Wild type
TLR5 KO
nd
Total IFNγ+ CD4+/TME
15
30 + Isotype
30 + anti-PD-L1
E
Naive
CD62L hi CD44 hi
CD62L low CD44 hi
Total CD8+ T cells/DLN
F
Total CD4+ T cells/DLN
G
Wild type
TLR5 KO
Total CD8+ IFNγ+ T cells/DLN
Day 15
Day 30 Isotype
Day 30 anti-PD-L1
H
Wild type
TLR5 KO
Total CD4+ IFNγ+ T cells/DLN
I
Total CD127+ KLRG1- CD8 T cells
Tumor
WT
TLR5KO
TLR5KO 1° challenge
TLR5KO 2° challenge
J
Total CD127+ KLRG1- CD4 T cells

Figure 2.12. Assessment of temporal changes in CD4 and CD8 T cells from the ovarian tumor microenvironment and tumor-draining lymph node in wild type and TLR5 KO ovarian tumor bearing mice.

(A) Validation of CD8 depletion strategy using anti-CD8a antibody. After 4 days of CD8 T cell depletion, blood was collected from treated mice and both CD4 and CD8 T cells were analyzed. **(B)** Gating hierarchy of T cells. **(C)** CD4 T cell naïve and memory subsets and **(D)** IFNγ+ CD4 T cells within the ovarian tumor microenvironment (peritoneal wash and tumor nodules). (**E**) Numbers of CD8 and **(F)** CD4 naïve and memory subsets in the tumor-draining mediastinal LNs. **(G)** Total CD8 and **(H)** CD4 IFNγ+ T cells in the mediastinal LN. All samples were quantified using counting beads and flow cytometry analysis on days 15 or 30-post ID8-*Defb29/Vegf-A*. Anti-PD-L1 injections were initiated beginning Day 10, repeated every 3-4 days, for a total of 4 injections. **(I-J)** Numbers of KLRG1- CD127+ stem-like memory CD8 **(I)** and CD4 **(J)** T cells following the treatment schema depicted in Figure 6E. All groups of mice as indicated were assessed 7 days following ID8-*Defb29/Vegf-A* tumor administration. Significance was calculated a nonparametric unpaired t-test with Mann Whitney correction (* $p < .05$, ** $p < .01$, *** $p < .001$). n=5 per group.

TLR5-expressing CD11c+ dendritic cells are responsible for reducing survival during PD-L1 blockade for ovarian cancer.

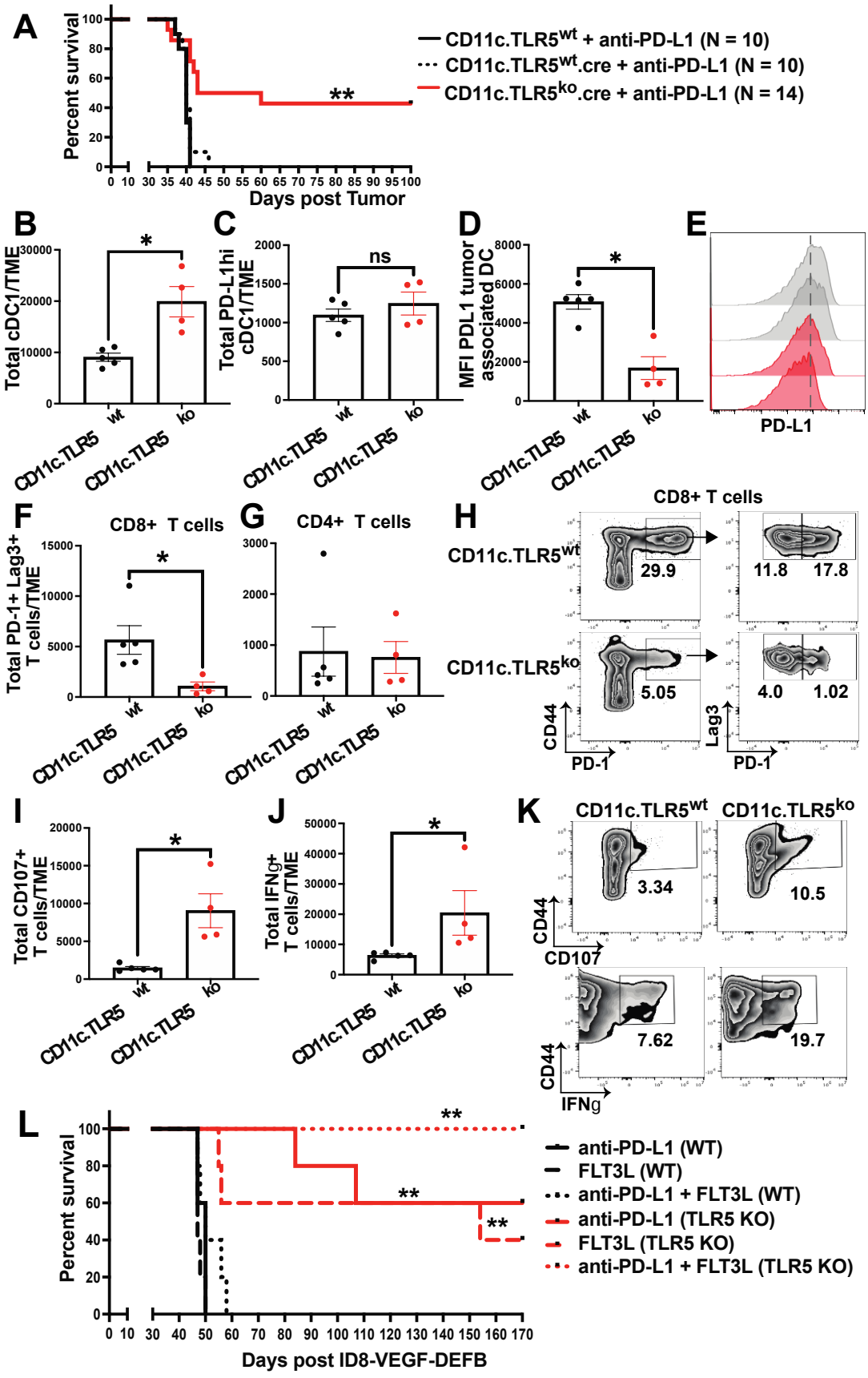
Overall, the necessity of CD8 T cells for mediating survival in our model and the parallel kinetics of cDC1 and CD8 T cell function suggests the existence of a DC-T cells axis that contributes to increased survival during PD-L1 blockade, and is impaired by TLR5 signaling. To test the hypothesis that TLR5 signaling on DCs prevents efficacy of PD-L1 blockade against ovarian cancer, we established a mouse model in which DCs lack TLR5 expression. To genetically target a TLR5 deficiency to dendritic cells, floxed-TLR5 mice[229] (TLR5 fl/fl) were crossed with *Itgax-cre* (CD11c Cre^{pos}) mice, establishing CD11c.TLR5^{ko} mice. Validating the phenotype of TLR5-deleted DCs, CD11c+ cells purified from the spleens of CD11c.TLR5^{ko} mice and co-cultured with flagellin did not enhance IL-6 production (**Figure 2.14A-B**). This was a similar response as observed after CD11c+ cells isolated from TLR5 KO mice were exposed to flagellin, indicating that CD11c+ cells from CD11c.TLR5^{ko} mice lack the ability to signal through TLR5 in response to flagellin. Ovarian tumors were initiated (ID8-*Defb29/Vegf-A*) in TLR5fl/fl x CD11c cre^{neg}, CD11c Cre^{pos}, and TLR5fl/fl x CD11c Cre^{pos} mice, followed by treatment with anti-PD-L1 therapy. In the absence of TLR5 signaling on DCs, TLR5 fl/fl x CD11c Cre^{pos} mice exhibited a significant survival benefit in response to anti-PD-L1 therapy compared to controls with intact TLR5 signaling on DCs (**Figure 2.13A**).

Next, we assessed the immune landscape of the ovarian TME in TLR5 fl/fl x CD11c Cre^{pos} mice with the goal of determining how the ovarian TME evolves in the absence of TLR5 signaling on CD11c+ cells. Mice bearing ovarian tumors for 25 days, in the absence of PD-L1 blockade, were assessed. Compared to CD11c.TLR5^{wt} animals, CD11c.TLR5^{ko} mice had significantly greater numbers of cDC1s within the ovarian TME (**Figure 2.13B**). We observed a significant increase in the accumulation of mucosal DCs into the TME of CD11c.TLR5^{ko} mice, but observed few differences in other DC subsets (**Figure 2.14C-F**). Comparison of PD-L1 expression revealed no

difference in the numbers of cDC1s expressing PD-L1 within the TME, although CD11c.TLR5^{ko} mice had significantly lower levels of PD-L1 expressed on the cell surface (**Figure 2.13C-E**). Congruent with increased tumor-associated cDC1 numbers, there was a corresponding increase in functional CD8 T cells within the TME. Specifically, CD11c.TLR5^{wt} mice with intact TLR5 signaling on CD11c cells had significantly greater numbers of CD8, but not CD4, T cells expressing both PD-1 and Lag3, markers associated with reduced T cell function and anti-tumor responses[230, 231] (**Figure 2.13F-H**). Numbers of CD8 T cell effector and memory populations were unchanged, whereas CD11c.TLR5^{ko} mice exhibited a significant increase in effector CD4 T cells and total CD4 T cells within the TME (**Figure 2.14G-H**). CD11c.TLR5^{ko} mice also had significantly increased numbers of functional CD8 T cells exhibiting increased degranulation (CD107a+) and IFNγ production in the ovarian TME (**Figure 2.13I-K**). Together, these results demonstrate the negative impact of TLR5 signaling on DCs within the TME, and that this signaling axis is detrimental to anti-tumor T cell function and response to PD-L1 blockade.

Overall, deletion of TLR5 signaling on CD11c+ cells is sufficient to achieve a significant survival increase when ovarian tumor-bearing mice are treated with PD-L1 blockade. This response corresponds with enhanced accumulation of cDC1s and similarly enhanced T cell function. We next tested whether expansion of cDC1s by *in vivo* administration of FLT3L and concurrent blockade of PD-L1 was able to achieve a significant survival benefit in the presence or absence of TLR5 signaling. Blockade of PD-L1 in combination with FLT3L benefited TLR5 KO mice, resulting in 100% of animals exhibiting no evidence of disease (**Figure 2.13L**). TLR5 KO mice treated with FLT3L alone exhibited a survival response that was comparable to anti-PD-L1 alone (**Figure 2.13L**). On the other hand, wild-type mice exhibited no benefit from the single treatments or combination therapy (**Figure 2.13L**), demonstrating that expanding cDC1s during PD-L1 blockade is a potent and effective immunotherapy against ovarian cancer in the absence of TLR5 signaling. Altogether, these results demonstrate that TLR5 signaling on DCs impairs the efficacy

of PD-L1 blockade, due to the expansion of myeloid-associated cell populations poorly equipped to activate CD8 T cells. These results support the premise that inhibition of TLR5 signaling during in situ expansion of cDC1s within the ovarian TME during anti-PD-L1 therapy has the potential to overcome this barrier, a strategy likely to elicit robust CD8 T cell immunity within the ovarian TME enabling extended survival during this devastating disease.


A
Percent survival
Days post Tumor
CD11c.TLR5wt + anti-PD-L1 (N = 10)
CD11c.TLR5wt.cre + anti-PD-L1 (N = 10)
CD11c.TLR5ko.cre + anti-PD-L1 (N = 14)
**
B
Total cDC1/TME
C
Total PD-L1hi cDC1/TME
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D
MFI PDL1 tumor associated DC
E
PD-L1
CD11c.TLR5 wt
CD11c.TLR5 ko
F
CD8+ T cells
Total PD-1+ Lag3+ T cells/TME
G
CD4+ T cells
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CD8+ T cells
CD11c.TLR5wt
CD11c.TLR5ko
29.9
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1.02
CD44
PD-1
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I
Total CD107+ T cells/TME
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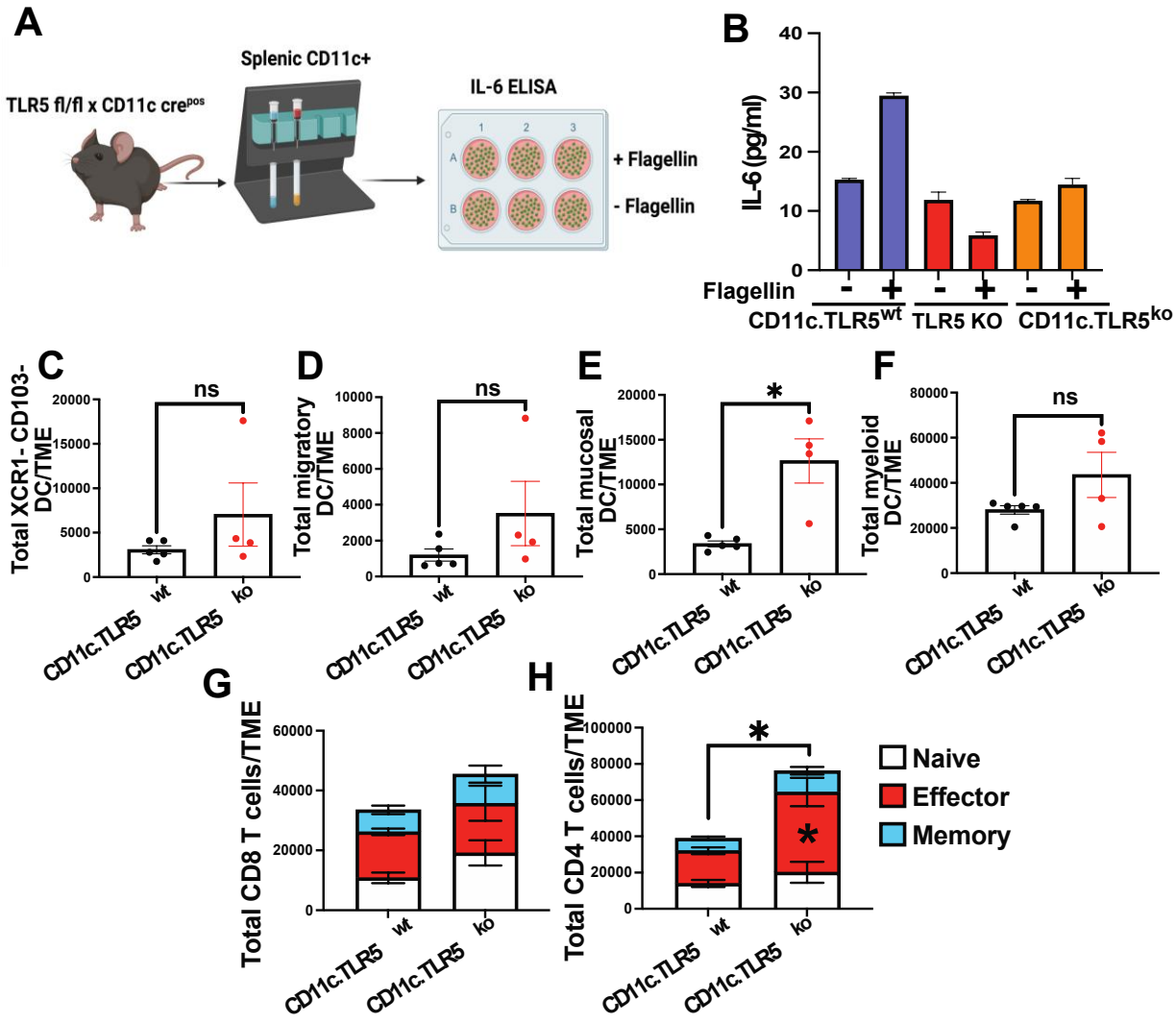
Total IFNg+ T cells/TME
K
CD11c.TLR5wt
CD11c.TLR5ko
3.34
10.5
CD107
7.62
19.7
IFNg
L
Percent survival
Days post ID8-VEGF-DEFB
anti-PD-L1 (WT)
FLT3L (WT)
anti-PD-L1 + FLT3L (WT)
anti-PD-L1 (TLR5 KO)
FLT3L (TLR5 KO)
anti-PD-L1 + FLT3L (TLR5 KO)

Figure 2.13. Dendritic cell-specific deletion of TLR5 results in significantly increased survival during anti-PD-L1 therapy, corresponding with enhanced cDC1 frequencies and increased CD8 T cell function.

(**A**) Survival proportions of TLR5fl/fl x CD11c cre negative (CD11c.TLR5^{wt}.cre^{neg}), CD11c Cre positive (CD11c.TLR5^{wt}.cre), and TLR5fl/fl x CD11c Cre positive (CD11c.TLR5^{ko}.cre) mice bearing ID8-*Defb29/Vegf-A* ovarian tumors and treated with anti-PD-L1 beginning 10 days post-tumor initiation. Log-rank test for survival compared to wild type (** $p < .01$). (**B-J**) 25 days after initiating ID8-*Defb29/Vegf-A* tumors in CD11c.TLR5^{wt} (cre negative) and CD11c.TLR5^{ko} mice (cre positive), in the absence of anti-PD-L1, peritoneal wash exudates and tumor nodules were evaluated for the immune subsets indicated. (**B**) Total numbers of cDC1s, (**C**) PD-L1hi cDC1s, (**D**) cDC MFI of PD-L1 and (**E**) representative histograms of PD-L1 expression on CD11c+ MHCII subsets. (**F**) Total numbers of PD-1+ Lag3+ CD8 T cells, (**G**) and CD4 T cells. (**H**) Representative gating of PD-1 high CD8 T cells co-expressing Lag3. Percentages represent population of PD-1 expressing. (**I**) Total CD3+CD107a+ and (**J**) CD3+IFNγ+, (**K**) with corresponding representative gating of CD3+ T cells. Unpaired t-test with Mann Whitney correction was used to calculate significance (* $p < .05$). Error bars represent mean ± SEM. (**L**) Survival proportions of TLR5 KO and wild-type mice bearing ID8-*Defb29/Vegf-A* tumors and treated with FLT3L (10μg) for 6 injections starting at day 5 post-initiation and/or anti-PD-L1 which was initiated on day 10 for a total of 4 injections. Log-rank test for survival compared to wild type (** $p < .01$) N=5 per group. Plots are representative of at least three experiments.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IL-6 (pg/ml)
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TLR5 KO
CD11c.TLR5^ko
C
Total XCR1- CD103- DC/TME
ns
D
Total migratory DC/TME
ns
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Total mucosal DC/TME
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Total myeloid DC/TME
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Total CD8 T cells/TME
H
Total CD4 T cells/TME
*
Naive
Effector
Memory
wt
ko
CD11c.TLR5

Figure 2.14. Immune phenotyping of TLR5fl/fl x CD11c Cre mice bearing ovarian tumors.

(A) Strategy to validate loss of TLR5 on CD11c+ cells. Briefly, CD11c+ cells were isolated from the spleens of the indicated strains and cultured with or without 10ng/ml of ultra-purified Flagellin for 24 hours. Following stimulation, supernatant from the cultures was collected and used to measure IL-6 levels using an ELISA. **(B)** IL-6 levels of CD11c+ cells after stimulation with flagellin for 24H. **(C-H)** 25 days after initiating ID8-*Defb29/Vegf-A* tumors in CD11c.TLR5^{wt} (cre negative) and CD11c.TLR5^{ko} mice (cre positive), in the absence of anti-PD-L1, peritoneal wash exudates and tumor nodules were evaluated for the immune subsets indicated. **(C)** Total XCR1-CD103-, **(D)** migratory, **(E)** mucosal and **(F)** myeloid DCs 25 days post initiation of ID8-*Defb29/Vegf-A* ovarian tumors in CD11c.TLR5^{wt} and CD11c.TLR5^{ko}. **(G)** Total CD8 and **(H)** CD4 T cell memory subsets. All samples were measured from the ovarian tumor microenvironment, from peritoneal wash and tumor nodules. Significance was calculated as a nonparametric unpaired t-test with Mann Whitney correction (* $p < .05$). Error bars represent mean ± SEM.

2.6 DISCUSSION

Using both *in vivo* and *in vitro* systems, we demonstrate that chronic TLR5 signaling induces the expansion of PD-L1-expressing myeloid cells at the expense of mature cross-presenting cDC1s, limiting activation of CD8 T cells in the ovarian TME. This signaling pathway culminates in failure of PD-L1 blockade, presumably due to the expansion of tolorogenic and suppressive myeloid populations within the TME[232]. In the absence of TLR5 signaling, the ovarian TME is infiltrated with mature cDC1s and functional CD8 T cells capable of eliciting tumor control. cDC1s are the most well-equipped cell type to cross-present antigens to CD8 T cells[210] and are vital for the regulation of response to immune checkpoint blockade, particularly anti-PD-L1/anti-PD-1[233, 234]. For TLR5 KO mice, PD-L1 blockade achieves significant and durable survival in ovarian tumor-bearing mice. Blockade of tumor- or myeloid-associated PD-L1 from interacting with the T cell inhibitory receptor PD-1 has shown promise in ovarian cancer patients as a single therapy[187-189] and in combination with other therapies[190]. Unfortunately, few patients respond, and of those that do, many develop recurrent disease. Mechanisms leading to impaired response to PD-L1 blockade include a lack of tumor antigenicity in addition to mechanisms involving tumor- and immune-mediated suppression of T cells. Our study unveils a host-intrinsic mechanism governing failure of PD-L1 blockade, in which ovarian tumors enhance bioavailability of commensal bacteria, as has been reported in other extraintestinal tumor models[235], enabling chronic TLR5 signaling and impaired accumulation of cDC1 into the ovarian TME.

These findings have clinical significance, as approximately 7-8% of the general population harbor a single nucleotide polymorphism (1174 C>T amino acid substitution) encoding a transcriptional termination site in place of arginine at codon 392 (referred to as *TLR5*^{R392X}) within the flagellin binding domain of TLR5. This polymorphism acts in a dominant-negative fashion, reducing TLR5 signaling by 50-80%[126,125]. For patients with ovarian cancer, heterozygous and homozygous carriers experience increased long-term survival[126]. Our data indicate that *TLR5*^{R392X} patients may

immediately benefit from immune checkpoint therapy targeting the immune suppressive PD-L1 pathway in combination with therapies that drive accumulation of cDC1s into the TME. Conversely, in patients homozygous for the ancestral allele, TLR5 antagonism has the potential to increase anti-tumor T cell function and survival. Paradoxically, TLR5 signaling is associated with enhanced survival outcomes in breast and liver cancers[126, 141]. Possibilities for these differences include tumor microenvironmental mechanisms, differences in the availability of flagellated bacteria within the TME, or the composition of TLR5-expressing tumor- and tissue-associated myeloid and DC subsets. Future studies will aim to assess tumor microenvironmental differences and outcomes in *TLR5*^{R392X} carriers relative to individuals who are homozygous for the ancestral allele across individual cancers. The goal will be to define the broad clinical applicability of our findings for ovarian cancer in addition to other cancer types.

Using TLR5 tdTomato reporter mice, we found that the majority of TLR5-expressing immune subsets were of myeloid origin. Importantly, as ovarian tumors progressed, TLR5-expressing DCs exhibited a phenotypic shift towards suppressive and tolerogenic states, as indicated by increased IL-10 and PD-L1 expression. These data are in line with previously published findings for ovarian cancer, demonstrating that over time, ovarian tumor-associated DCs become more suppressive due to tumor-mediated immune[39] and metabolic[170, 236] constraints within the TME. Our data suggest that chronic TLR5 signaling is not only contributes to alterations in DC function, but culminates in the conversion of precursors towards monocytes and macrophages. *In vitro* cultures of bone marrow cells with FLT3L and chronic exposure to bacterial flagellin support the premise that TLR5 signaling skews myeloid progenitors away from functional cDC1s and instead promotes myeloid subsets that are associated with immune suppression and failure of PD-L1 blockade. Given that PD-L1 blockade in combination with FLT3L-mediated expansion of cDCs in situ resulted in 100% survival of ovarian tumor-bearing TLR5 KO mice, we speculate that regardless of tumor microenvironmental differences, enhancing cDC1 accumulation into the TME would

enhance the efficacy of PD-L1 blockade in the absence of TLR5 signaling. On the other hand, *in vivo* FLT3L administration is likely to expand other myeloid subsets at the expense of cDC1s in the presence of TLR5 signaling. However, this scenario is likely to be dependent upon the unique attributes of the TME and the response of TLR5-expressing cell subsets to chronic TLR5 signaling. Although clinical studies utilizing *in vivo* administration of FLT3L have shown that most patients tolerate the therapy with minimal toxicity, studies demonstrating clinical benefit are still lacking[237]. Our work provides an additional context in which expansion of cDC1s in situ, coupled with PD-L1 blockade and in the absence of TLR5 signaling, has the potential to achieve significant benefit for the treatment of ovarian cancer.

The cellular pathways governing TLR5-mediated changes in myeloid and dendritic cell fates are unknown and form the basis of future investigation. Given that acute TLR signaling is typically associated with activation of cDCs and induction of adaptive immunity, our results suggest that at the molecular level, chronic TLR5 signaling impairs the differentiation of DCs through signaling in DC precursor populations and/or downmodulation of IRF8. When bone marrow cells were cultured with FLT3L and exposed to bacterial flagellin, we observed a significant and sustained downmodulation of IRF8, even during acute exposure to bacterial flagellin. These data suggest that TLR5 signaling, and possibly other TLR pathways, impairs the ability of cDCs to differentiate and prime CD8 T cell responses. Mechanistically, one possibility is that cytosolic sensing of flagellin through NLRC4 and activation of the inflammasome pathway leads to downmodulation of IRF8 and subsequent impairment of T cell priming. McDaniel et al. previously demonstrated that inflammasome activation in DC and myeloid cells reduced IRF8 and IRF4 expression, increasing DC sensitivity to the inflammasome sensing machinery, and ultimately reducing the ability of DCs to effectively prime T cells[238]. However, we did not observe changes in IRF4 expression or cell death, suggesting the cellular mechanism may be unique. Another possible mechanism could involve the effects of PD-L1 expression on TLR5-modulated DC subsets, given

recent evidence that PD-L1 signaling not only acts to reduce T cell activation, but instead acts in a cell-autonomous mechanism to sequester co-stimulatory ligand CD80[234]. To further contextualize these findings, it will be important to interrogate how the chronicity of TLR5 signaling and stimulation via additional TLR agonists affect cDC differentiation in the context of FLT3L cultures, IRF8, PD-L1 and CD80 expression.

Overall, we find that removing or suppressing TLR5 signaling using an inhibitory antibody for TLR5 enhances accumulation of more functional cDC1 in the TME of wild-type mice. These findings suggest that antagonism of TLR5 signaling has the potential to benefit ovarian cancer patients. Congruent with the increase in cDC1 numbers, we observed there are more functional tumor-associated CD8 T cells in TLR5 deficient mice. Given that CD8 T cells are necessary for survival of TLR5 KO mice given anti-PD-L1, there is an implication that TLR5 signaling disrupts a critical DC-T cell axis during ovarian cancer. This is further validated by a similar survival increase in mice with TLR5 deleted only on CD11c+ cells during treatment with anti-PD-L1 therapy. Importantly, we observe corresponding improvements in cDC1 numbers and T cell function within the TME of mice lacking TLR5 only on CD11c cells. These data suggest that TLR5 signaling limits DC-T cell interactions and disables efficacy of anti-PD-L1 therapy. Ultimately, our study implicates that blocking TLR5 signaling specifically on DCs, or systemically, in combination with anti-PD-L1 may serve as a promising strategy to overcome failure of immunotherapy against ovarian cancer.

2.7 AUTHOR CONTRIBUTIONS

M. McGinty: Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing-original draft. **S.H. Kolli:** Investigation. **A.M. Putelo:** Investigation and editing. **T.Y. Feng:** Investigation and editing. **M.R. Dietl:** Investigation and editing. **C. N. Hatzinger:** Investigation and editing. **S. Bajgai:** Investigation and editing. **M.K. Poblete:** Investigation and editing. **F.N. Azar:** Investigation. **M.R. Rutkowski:** Conceptualization,

resources, analysis, visualization, supervision, funding acquisition, writing–original draft, writing–review and editing.

2.8 ACKNOWLEDGMENTS

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Chapter 3: Discussion

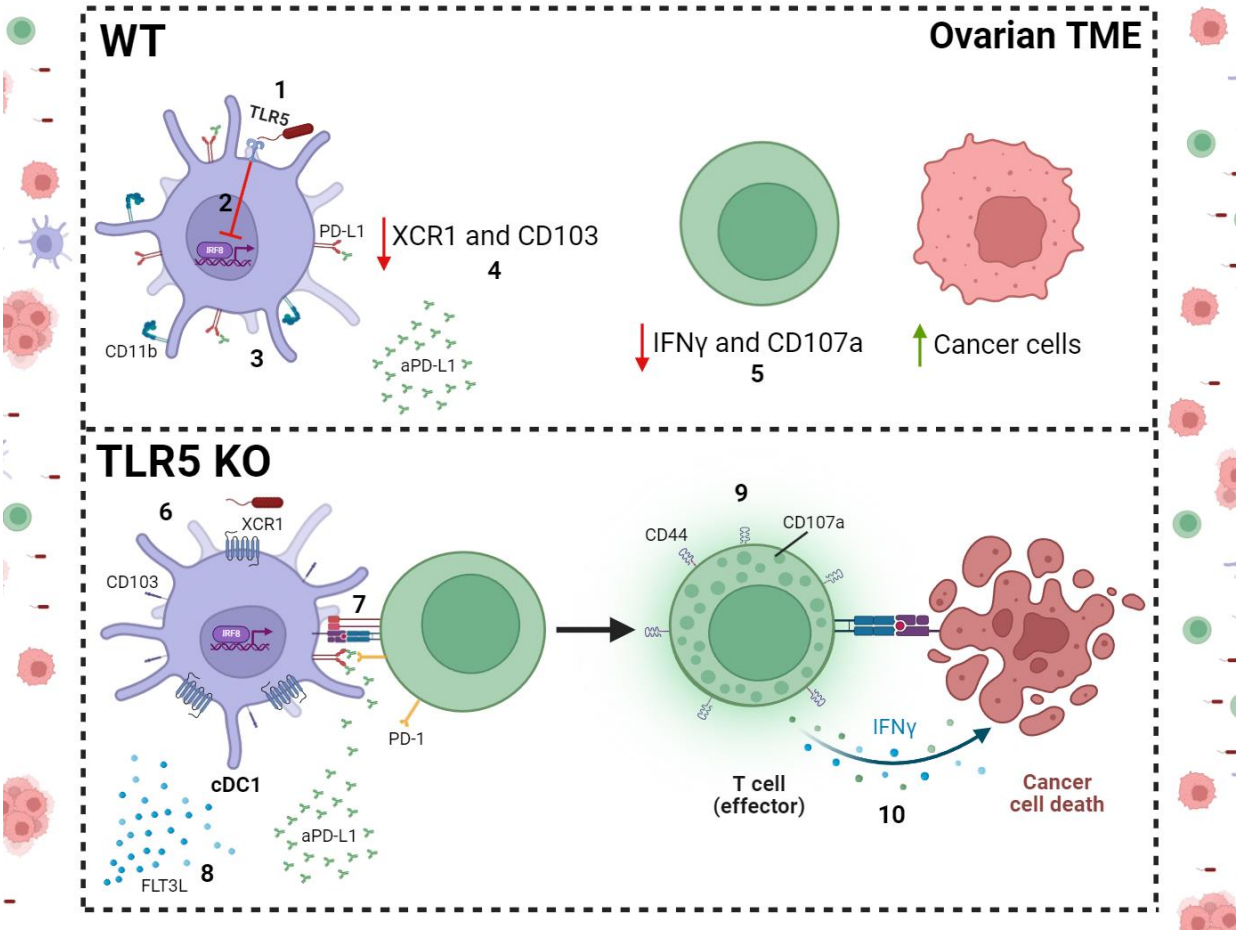


Figure 3.1. Proposed model for efficacy of anti-PD-L1 against ovarian cancer with TLR5 deficiency

1. DCs express TLR5 (figure 2.2) which binds to flagellated bacteria or free flagellum in the ovarian TME. Flagellin is present within the ovarian TME as observed via a TLR5 reporter cell line on tumor extracts (unpublished).
2. IRF8 protein and RNA are reduced in FLT3L-BMDC cultures with flagellin (Figure 2.9 and 3.2).
3. IRF8 is the master regulator of cDC1 differentiation, function, and maintenance. Thus, TLR5-induced IRF8 reduction may lead to less XCR1 and CD103 expression (cDC1 markers). TLR5 knock out or TLR5 signaling neutralization results in more cDC1s in the ovarian TME (Figure 2.5).
4. Reduced XCR1 and CD103 chemokine and migratory receptor expression results in less DCs interacting with T cells.
5. Less IFNγ+ and CD107a+ T cells were observed when CD11c+ cells can recognize flagellin in the ovarian TME (Figure 2.13). Indicative of a reduced anti-tumor response.
6. TLR5 KO DCs do not express the TLR5 receptor, therefore IRF8 expression remains constant, and XCR1 and CD103 expression is maintained.
7. More DCs engage and stimulate T cells, allowing for a greater opportunity for blockade of PD-L1 to prevent inhibitory receptor engagement.
8. FLT3L, which expands cDC1 *in vivo*, boosts TLR5 KO mice survival when combined with anti-PD-L1 (Figure 2.13).
9. More activated CD8 T cells (CD44+CD107a+) in TLR5 KO mice are observed when treated with anti-PD-L1 (Figure 2.11).
10. Activated CD8 T cell effectors kill tumor cells, leading to a survival improvement in our TLR5 KO mice treated anti-PD-L1 bearing ovarian tumors. Verified as critical for survival by CD8 T cell depletion (Figure 2.11).

3.1 How these studies address outstanding questions in the field.

Here, we demonstrated that treating ovarian tumor-bearing mice with checkpoint inhibitors significantly increased survival in the absence of TLR5 signaling. To establish an immunological mechanism to explain this observation, we first sought to define the cell types in the ovarian tumor microenvironment that express TLR5. Previous studies have assessed TLR5 expression in the intestines using reporter mice; however, this has not been done in a cancer setting. TLR5 tdTomato reporter mice were utilized to comprehensively define the phenotypic and functional attributes of TLR5-expressing immune subsets within the ovarian TME. We found that within the ovarian TME, most TLR5-expressing immune subsets were of myeloid origin. Interestingly, there was a significant increase in the frequencies of both TLR5-expressing myeloid and DC subsets in response to advancing disease burden. These results implicated myeloid cells as the driving force in diminishing response to immune therapy in mice capable of signaling through TLR5. This idea was further supported by increased frequencies of TLR5-expressing DCs producing IL-10 and expressing high levels of PD-L1 within the TME of wild-type mice.

We next wanted to address how TLR5 signaling on myeloid cells was leading to reduced efficacy of checkpoint therapy. This is an outstanding question in the field as the cause of checkpoint therapy failure is unknown for ovarian cancer. We found that in wild-type mice, many of the tumor-associated DCs expressed TLR5 and exhibited a corresponding phenotypic shift in response to tumor progression through the expression of PD-L1, resulting in reduced survival benefit during immune therapy. Conversely, we observed TLR5 KO mice had significantly greater numbers of cDC1s within their tumor microenvironment, which corresponded with significant survival benefits during immune therapy; it supported our hypothesis that TLR5 signaling likely attenuated the accumulation of cDC1 into the ovarian TME. There is literature supporting the idea that cDC1s are critical for orchestrating anti-tumor immunity in the context of melanoma and essential for the efficacy of checkpoint therapy[67, 239, 240]. Therefore, we hypothesized that TLR5 signaling is

detrimental to the therapeutic efficacy of anti-PD-L1 through modulation of DCs. To understand how TLR5 signaling was affecting DC phenotype and function, we established a WT:TLR5 KO 50:50 mixed bone marrow chimera to determine whether direct TLR5 signaling or TLR5-mediated changes in the tumor environment were affecting DC phenotype and function. We observed a greater frequency of TLR5 KO cDC1s in this setting relative to congenic wild-type cDC1s in the same tumor-bearing host. Together, this indicates that TLR5 signaling is detrimental to either cDC1 expansion, differentiation, or recruitment into the ovarian TME. These findings further underscore that DCs are vulnerable to TLR5 signaling and that this pathway impairs the efficacy of checkpoint therapy for ovarian cancer. To more directly test the hypothesis that direct TLR5 signaling on DCs prevents the efficacy of PD-L1 blockade against ovarian cancer, we established a mouse model in which CD11c+ cells, a marker expressed by DCs, lack TLR5 expression. In the absence of TLR5 signaling on DCs, TLR5 fl/fl x CD11c Cre^{pos} mice exhibited a significant survival benefit in response to anti-PD-L1 therapy compared to controls with intact TLR5 signaling on DCs. Using this model, we found that mice exhibited a significant survival benefit in response to anti-PD-L1 therapy compared to controls with intact TLR5 signaling on DCs. Furthermore, boosting cDC1 expansion using FLT3L in combination with anti-PD-L1 significantly increased survival of TLR5 KO mice, resulting in 100% of animals exhibiting no evidence of disease. On the other hand, wild-type animals received zero benefit from this therapeutic combination. Addressing the question of what cell type was the orchestrator of checkpoint failure and outlining a TLR5 signaling DC-based mechanism for checkpoint failure in OvCa.

3.2 Future Directions

The work presented thus far expands our understanding of the role of TLR5 signaling on dendritic cells in the ovarian TME. However, significant work remains to understand the relationship between flagellated bacteria, dendritic cells, the ovarian TME, and TLR5 expression. In addition, new therapeutic strategies are needed for ovarian cancer where the standard of care is still limited

to cytoreduction followed by carboplatin and paclitaxel. From what we have learned from our studies, new therapeutic avenues are now possible. These will be explored in this section.

3.2.1 Do bacteria drive an increase in TLR5+ myeloid cells in the ovarian TME?

Our findings demonstrate an increase and phenotypic shift in TLR5+ cells in the ovarian TME that corresponds with tumor outgrowth. However, it remains unclear what changes in the microbiome would promote an increase in immune cells that recognize flagellin in the TME. Using a TLR5 reporter cell line (HEK-Blue™ mTLR5 cells) to quantify bacterial flagellin, we have measured increased levels of flagellin within the peritoneal tumor microenvironment that progressively increases with disease burden. This may indicate that more flagellated bacteria or free bacterial flagellin are present within the ovarian TME during tumor progression. This is consistent with findings that the tumorigenic process induces gut leakage or is more specifically associated with ileal mucosa atrophy, constriction of the microvascular villous circulation, and drop in parasympathetic signaling in the mucosa[241]. Based on these findings, it may be plausible that bacteria accumulation in the ovarian TME drives an increase in immune cells that express TLR5. Considering that we observed TLR5 signaling is detrimental to the efficacy of checkpoint therapy, it is essential to understand if gut-leakage-mediated accumulation of flagellin modulates TLR5 expression on myeloid cells within the ovarian TME. To address this experimentally, TLR5 reporter mice will be given ovarian tumors similar to that depicted in Figure 2.1, followed by treatment by oral gavage with antibiotics to determine the impact of bacteria on the presence of TLR5-expressing myeloid cells. An antibiotic cocktail will be administered by oral gavage or IV for 25 days beginning on the same day as tumors are initiated to reduce intratumoral bacterial load. Day 25 post-tumor initiation will be chosen as we have already extensively assessed the phenotypic and functional attributes of TLR5-expressing immune subsets within the ovarian TME at this time point (**Figure 2.2**). Antibiotics will be implemented via oral gavage or intravenously to help differentiate between targeting bacteria specifically in the gut versus systemically.

Specifically, rifaximin will be administered by oral gavage, which is a non-absorbable antibiotic that does not break down in the GI tract, thus only reducing the bacterial load within the gut. Rifaximin has a broad spectrum of activity against gram-negative and gram-positive anaerobic and aerobic bacteria[242]. To match this broad-spectrum activity systemically, rifampin will be administered IV, which belongs to the same class of rifamycin antibiotics as rifaximin. Germ-free TLR5-tdTomato may be considered as an alternative to systemic depletion of bacteria by IV antibiotics. However, the absence of bacteria during development may affect the education of myeloid cells, leading to an entirely different response to ovarian cancer. Furthermore, additional groups of mice treated by IV or oral gavage of antibiotics will be treated with dextran sodium sulfate (DSS) to induce gut leakage and ensure bacterial dissemination with or without tumor burden.

To assess TLR5+ immune composition, tumors, bone marrow, mediastinal LNs, and spleens will be collected on Days 10, 20, and 30 and evaluated by flow cytometry to measure the quantity and frequency of TLR5+ myeloid cells. Additionally, the composition of bacteria will be calculated on the same samples using metagenomic sequencing and quantity of flagellin by co-culturing tissue samples with HEK-Blue™ mTLR5 cells. Specifically, metagenomic sequencing will be performed on tumors and spleens to assess how antibiotics impact bacterial populations intratumorally and systemically, respectively. This will also be informative when paired with flagellin quantification to see if there are differences between shed flagella and whole microorganisms. Previous studies have identified unique bacterial profiles in OvCa tissues, including an increased abundance of Acinetobacter[243] and altered Proteobacteria/Firmicutes ratios[244]. These microbial changes may influence tumor development by modulating the local immune environment. Assessment of commensal microorganisms within the ovarian TME over time will enable us to determine whether distinct commensals are associated with the accumulation and/or polarization of TLR5-expressing immune populations into the TME. Regarding immune composition, differences in numbers of

TLR5-expressing myeloid cells will be compared between IV, oral gavaged, or non-treated and their corresponding DSS-treated controls to determine if bacteria drive the accumulation of TLR5-expressing myeloid cells within the TME. It is anticipated that non-treated tumors from TLR5-tdTomato mice will contain increasing amounts of TLR5+ myeloid cells corresponding with tumor burden. That may reach levels similar to mice treated with DSS without antibiotics if gut leakage is comparable. Oral-gavaged mouse tumors are also expected to express fewer TLR5+ myeloid cells than non-treated mice if intratumoral flagellated bacteria are primarily derived from the gut. Additionally, IV antibiotic-treated mouse tumors will have the lowest bacterial burden overall, assuming antibiotics reach the tumors, and are expected to have the lowest number of myeloid populations that recognize TLR5. If there are no differences in TLR5+ myeloid cells, there could be phenotypic and functional changes between immune populations. For example, increasing cDC1/MDSC ratios with oral gavage relative to non-treated mice may indicate that gut leakage promotes a more antitumorigenic immune landscape. Overall, the presence of TLR5-expressing immune populations is detrimental to the efficacy of checkpoint therapy against ovarian cancer; thus, establishing a potential cause of their accumulation in the ovarian TME may open new therapeutic avenues to treat this cancer.

3.2.2 Does TLR5 signaling interfere with cDC1 recruitment to the ovarian TME?

We observed that TLR5 signaling reduces the frequency of cDC1s in the ovarian tumor microenvironment. However, it remains unclear how this change in cDC1 numbers is manifesting. One possible explanation for the low number of cDC1s in the TME is that TLR5 signaling interferes with recruitment. Specifically, chronic TLR5 signaling may hinder the expression of chemokine receptors necessary for cDC1s to find the ovarian TME or the physical processes required for cellular movement. We have preliminary evidence to support reduced migration in response to XCL1 as we have observed reductions in XCR1 expression with chronic TLR5 signaling on DCs in culture by both flow and scRNAseq (**Figures 2.9 and 3.2**). Furthermore, most of the differences

in cDC1 numbers we observed were found in the ovarian TME but not systemically in spleens or draining lymph nodes. It has been observed that DCs temporarily reduce their migratory capacity after LPS or pathogen challenge[245]. Furthermore, TLR signals and other activators of DC maturation, such as PGE2, trigger the disassembly of actin-rich podosomes, which promote cell migration[246]. These papers suggest that maturation and migration may be mutually exclusive processes. It has been observed that PGE2 produced by tumor cells acts on cDC1s to suppress responsiveness to the chemokines[66]. Therefore, it is plausible that chronic TLR5 signaling may also work in a similar fashion in the ovarian TME by reducing cDC1 sensitivity to chemoattractants and the ability to migrate.

This can be examined experimentally by *in vitro* migration assays. Bone marrow cells from wild-type or TLR5 KO mice will be cultured for 8 days with FLT3L and purified flagellin (*Salmonella typhimurium*) in an acute setting, for 2 days (2D) of culture; or a chronic setting, for 8 days (8D) of culture or without flagellin. cDC1s will be sorted from these cultures and loaded in RPMI in the upper chamber of a transwell apparatus (5-μm pore size), while RPMI with or without chemokines (CCL4, CCL5, CCL19, CCL20, CCL21, XCL1; 200 ng/ml) will be added in the lower chamber. After 1.5 h at 37°C, migrated cells will be harvested from the lower chamber and counted by flow cytometry, using absolute counting beads and stained for the corresponding chemokine receptors[247]. *In vitro* migration analysis will indicate what chemokines cDC1s lose responsiveness to after chronic TLR5 stimulus as measured by reduced numbers in the lower chamber of the transwell apparatus relative to the non-TLR5 stimulated controls. This may suggest that TLR5 signaling interferes with recruitment into the TME, which may manifest experimentally with less sensitivity to CCL4, CCL5, and CCL20 (form gradient to TME) relative to CCL19 and CCL21 (gradient to LNs) by chronically stimulated WT cDC1s. It also anticipated that less sensitivity to a chemokine will also manifest with the downregulation of its corresponding receptor when measured by flow cytometry. Additionally, to define if downregulation of chemokine receptors is

occurring *in vivo* in response to TLR5 signaling, a mixed bone marrow (MBC) approach could also be taken, similar to Figure 2.7. Multiplex ELISAs can measure chemokines, and receptors can be measured by flow cytometry from the TME and bone marrow of 50:50 WT: TLR5 KO MBC mice, with the expectation that WT (CD45.1+) cDC1s will express less chemokine receptors relative to TLR5 KO (CD45.2+) cDC1s within the same TME as measured by mean fluorescent intensity. Future studies could explore whether TLR5 signaling may impact the differentiation of cDC1s. This could be addressed by adoptively transferring WT, or TLR5 KO CFSE labeled cDC1s into WT tumor-bearing mice and measuring CFSE dilution as a metric of proliferation. Overall, these experiments proposed will bring us closer to understanding if TLR5 signaling interferes with cDC1 recruitment to the ovarian TME.

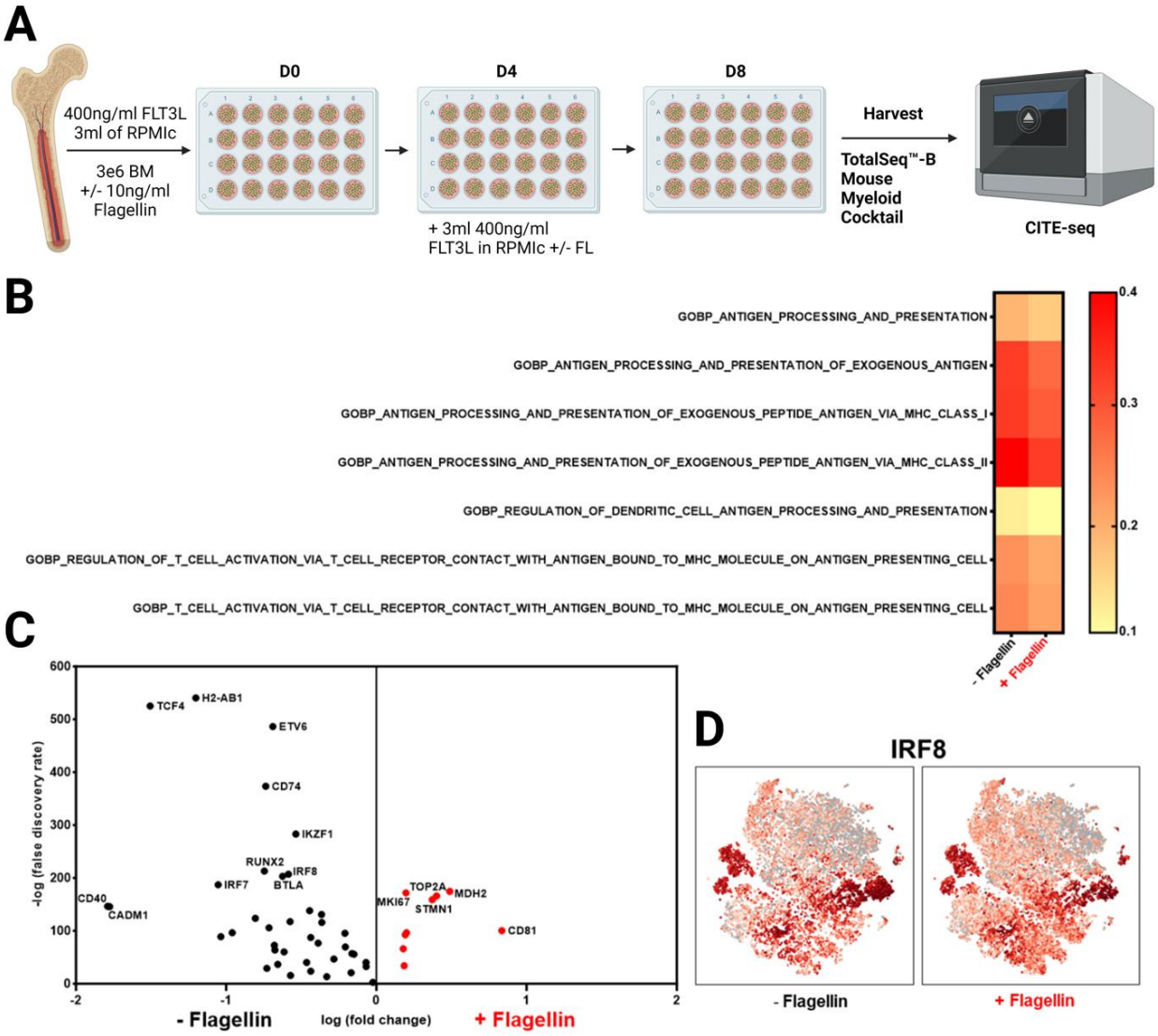

A
400ng/ml FLT3L
3ml of RPMIc
3e6 BM
+/- 10ng/ml
Flagellin
D0
D4
D8
+ 3ml 400ng/ml
FLT3L in RPMIc +/- FL
Harvest
TotalSeq™-B
Mouse
Myeloid
Cocktail
CITE-seq
B
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_EXOGENOUS_ANTIGEN
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_EXOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I
GOBP_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_EXOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_II
GOBP_REGULATION_OF_DENDRITIC_CELL_ANTIGEN_PROCESSING_AND_PRESENTATION
GOBP_REGULATION_OF_T_CELL_ACTIVATION_VIA_T_CELL_RECEPTOR_CONTACT_WITH_ANTIGEN_BOUND_TO_MHC_MOLECULE_ON_ANTIGEN_PRESENTING_CELL
GOBP_T_CELL_ACTIVATION_VIA_T_CELL_RECEPTOR_CONTACT_WITH_ANTIGEN_BOUND_TO_MHC_MOLECULE_ON_ANTIGEN_PRESENTING_CELL
0.4
0.3
0.2
0.1
- Flagellin
+ Flagellin
C
-log (false discovery rate)
600
500
400
300
200
100
0
TCF4
H2-AB1
ETV6
CD74
IKZF1
RUNX2
IRF8
IRF7
BTLA
CD40
CADM1
TOP2A
MDH2
MKI67
STMN1
CD81
-2
-1
0
1
2
- Flagellin
log (fold change)
+ Flagellin
D
IRF8
- Flagellin
+ Flagellin

Figure 3.2. Chronic TLR5 signaling by flagellin downregulates expression of genes critical for dendritic cell function and differentiation

(**A**) Experiment Schematic. Bone marrow cells were collected from femurs and tibias of a wild-type mouse and cultured with 400ng/ml of FLT3L in RPMIc on day one 10ng/ml of ultra-purified Flagellin derived from *S. typhimurium* and incubated. Additional RPMIc + FLT3L with or without flagellin was added on day four. Samples were collected and stained with TotalSeq™-B Mouse Myeloid Cocktail. (**B**) Top categories of DC function from a gene ontology (GO) analysis using GSEA database comparing total sample with or without TLR5 signaling. (**C**) Volcano plot of differential gene expression of DC genes from FLT3L expanded BM with or without TLR5 signaling. (**D**) T-sne dot plot of IRF8 expression, comparing samples expanded with or without flagellin. Sequencing and Analysis was performed in collaboration with UVA genomics and bioinformatics core.

3.2.3 Does TLR5 signaling interfere with cDC1 development?

It is plausible that the low abundance of cDC1 in ovarian tumors may be due to impaired cDC1 development, potentially caused by systemic suppression of dendritic cell production in the bone marrow or in the TME. Dendritic cells originate from hematopoietic precursors in the bone marrow, with the monocyte/dendritic progenitor (MDP) being the earliest precursor[248]. Next, MDPs develop into common DC progenitors (CDP), which can give rise to pre-cDC1s and pre-cDC2s[248]. Several transcription factors including IRF8, BATF3, NFIL3, and ID2 play a role in regulating cDC1 development[219, 249-251]. However, only IRF8 is necessary for cDC1 development as cDC1s can still be generated when knocking out BATF3, NFIL3, and ID2[219, 249-251]. Removal of IRF8 leads to a defect in both pre-cDC1 and cDC1 development that cannot be rescued by upregulation of other transcription factors[218], suggesting IRF8 is the most critical transcription factor in determining cDC1 fate. It has been demonstrated that breast and pancreatic cancers can cause systemic decreases in cDC1 cells and their progenitors by downregulating IRF8, leading to impaired anti-tumor CD8+ T-cell responses[252]. In our bone marrow FLT3L culture system, we have observed that purified flagellin reduces IRF8 protein levels using flow cytometry and at the transcript level using scRNAseq (Figures 2.9 and 3.2). Given our preliminary observations and the control IRF8 exerts over cDC1 development, we will test the hypothesis that TLR5 signaling impairs cDC1 development by downregulating IRF8 and other cDC1 regulators in DC progenitor cells.

This hypothesis can be examined by verifying changes in cDC1 regulators in DC progenitor cells in the BM and TME and assessing what molecules downstream of TLR5 signaling can directly or indirectly impact their transcription or translation. Considering there are many DC genes and progenitor subsets to investigate simultaneously, one strategy would be to utilize CITE-seq, which combines flow cytometry with single-cell sequencing, allowing for the simultaneous measure of protein and RNA expression. WT and TLR5 KO mice will be injected with the OvCa cell line ID8-VEGF-DEFB IP, and then tumors and bone marrow will be harvested on D0, D7, and D15.

Samples will be stained using TotalSeq™-B Mouse Myeloid Cocktail (contains 84 surface antigens including lineage antigens barcoded and compatible with 10X chromium X and Illumina) to distinguish cDC1s, DCs, CDPs, and MDPs by surface expression and single-cell sequencing. These cell subsets can be gated and compared to genes associated with DC development, such as IRF8, BATF3, NFIL3, and ID2, and compared between WT and TLRKO mice bone marrows and TMEs. With the expectation that DC progenitors within TLR5 KO mice will exhibit more significant expression of DC developmental genes relative to WT DC progenitors. A gap in DC development gene expression is expected to grow between WT and TLR5 KO progenitors that will correspond with tumor development. These results will support the hypothesis that chronic TLR5 signaling reduces the expression of genes associated with cDC1 development, such as IRF8. Additionally, transcription of downstream components of TLR5 signaling, such as MyD88, can be measured to clarify the relationship between TLR5 and DC developmental genes and ovarian tumor burden.

Almost all TLRs transmit signals through MyD88, except for TLR3. Upon recognizing PAMPs, TLRs recruit MyD88 to initiate signal transduction, leading to the activation of NF-κB. The relationship between TLR signaling and IRF8 has only been explored in the context of Teleost fish (Miiuy croakers). The authors found that IRF8 negatively regulated the MyD88-mediated NF-κB signaling pathway through ubiquitin-proteasome degradation of MyD88[253]. Thus, one possible explanation for our observation of reduced IRF8 with TLR5 signaling is that IRF8 is sequestered or degraded after mediating chronic MyD88 signaling. Future studies could explore if there is direct binding between IRF8 and MyD88 by co-immunoprecipitation or more rigorously by Fluorescence resonance energy transfer (FRET). If IRF8 binds to or inhibits MyD88 or vice versa, this would reveal TLRs as playing a new role in directing the development of cDC1s.

3.2.4 Does TLR5 signaling convert cDC1s to becoming cDC2s or another phenotype in the ovarian TME?

We observed that chronic exposure to bacterial flagellin in the presence of FLT3L is sufficient to promote the expansion of other myeloid subsets instead of cDC1s from bone marrow (**Figure 2.9B**). IRF8 is a terminal selector for the cDC1 lineage and maintains cDC1 identity by preventing cDC1 conversion into cDC2s[218-220]. We found that both acute and chronic TLR5 signaling significantly reduced IRF8 for wild-type DCs, whereas TLR5 KO DCs maintained similar levels of IRF8 across all conditions (**Figure 2.9D**). Therefore, it is plausible that the observed decrease in cDC1s is due to conversion to another myeloid lineage after chronic exposure to flagellin.

One hypothesis is that TLR5 signaling interferes with cDC1s ability to maintain their identity by reducing the stability of IRF8 expression. This idea can be tested using cDC1 fate mapping mice. Similar to the *Foxp3*$^{tm9(EGFP/cre/ERT2)Ayr}$/J 'fate mapping mice' developed by Alexander Rudensky and available at Jackson labs to study lineage stability and genetic mapping of regulatory T cells, *IRF8*$^{tm9(EGFP/cre/ERT2)Ayr}$/J mice could be developed to explore similar questions in cDC1s. In these mice, IRF8+ cells will constitutively express GFP, and tamoxifen treatment will induce the expression of tdTomato under the IRF8 promoter. This dual reporter system would enable the identification of cells that no longer express IRF8 but have at one point in time. For example, tdTomato expression without IRF8 expression would manifest as GFP^{-}tdTomato^{+}, thereby identifying cells that were previously IRF8^{+} but have lost IRF8 expression (ex-cDC1s). These mice could be crossed to a TLR5 KO background to examine the impact of TLR5 signaling on IRF8 stability in the ovarian TME. In lieu of breeding to a TLR5 KO background, anti-TLR5 antibody could also be injected into tumor-bearing IRF8 fate mapping mice to see if TLR5 blockade can preserve IRF8 expression by DCs.

To examine this experimentally, ovarian tumors will be initiated in both WT and TLR5 KO cDC1 fate mapping mice and treated with tamoxifen at Day 0 for six days to induce tdTomato

expression. This may require optimization to determine how long to treat with tamoxifen to induce tdTomato expression in as many cDC1s in the TME as possible. On day 15, ovarian tumors will be collected and examined by flow cytometry comparing WT and TLR5 KO TMEs for ratios of ex-cDC1s (GFP-tdTomato+) to current cDC1s (GFP+tdTomato+). Additional cDC1 markers, such as XCR1 and CD103, will be included in the flow panel to help distinguish cDC1s from other IRF8-expressing populations. WT cDC1 fate-mapping mice are anticipated to have greater ratios of ex-cDC1s to active cDC1s relative to TLR5 KO cDC1 fate-mapping mice. Indicating TLR5 signaling in the ovarian TME contributes to reduced IRF8 stability in cDC1s. Suppose there is no difference in IRF8 downregulation between WT and TLR5 KO ovarian tumors. In that case, it may suggest that another cDC1 transcription factor plays a more prominent role in maintaining cDC1 phenotype or an alternative mechanism is dominating, such as recruitment or development of cDC1s. Based on preliminary CITE-seq results, ETV6 could be an interesting candidate (**Figure 3.2**). Evidence suggests that ETV6 regulates the functional differentiation of cDC1s, optimizing their ability to cross-prime CD8+ T cells and generate tumor-specific responses[254].

It is unknown whether cDC1 fate is fixed or plastic. Studies have demonstrated that DCs, in general, become tolerogenic over time in the TME, and mechanisms have been proposed involving PGE2 as a cause for systemic cDC1 reduction[66, 255]. However, there is much to be understood regarding whether cDC1s can maintain function and phenotype within a tumor microenvironment. It has been demonstrated that deletion of IRF8 in committed cDC1 leads to an IRF4-independent functional and transcriptional reprogramming of cDC1 into cDC2-like cells[220]. Thus, given our observations that TLR5 signaling reduces IRF8 RNA and protein levels, it is plausible that there may be phenotypic conversion at play in cDC1s. If we observe that TLR5 signaling drives a conversion of cDC1s to become ex-cDC1s, more experiments will be required to assess the function of ex-cDC1s. Future studies may involve sorting the GFP-tdTomato+ DCs from tumors and evaluating their ability to present antigen in either an *in vitro* pulse-present setting

with OT-1 CD8 T cells or with an in OVA expressing ovarian cancer cell line such as ID8-Ova. Additional studies may also be necessary to understand the transcriptional nature of ex-cDC1s in the ovarian TME via single-cell RNA seq, comparing cDC1s in the absence of TLR5 signaling. Furthermore, it would also be interesting to explore how changes to the microbiome via antibiotics or seeding specific bacterial populations may impact cDC1 conversion in the ovarian TME.

3.2.5 Can FLT3L and anti-PD-L1 be successful clinically for ovarian cancer?

Roughly 7-8% of the general population harbor an SNP encoding a transcriptional termination site in place of arginine at codon 392 (referred to as *TLR5*^{R392X}). This dominant-negative polymorphism reduces TLR5 signaling by 50-80%[126,125]. We have observed that patients with ovarian cancer who are heterozygous or homozygous carriers for this polymorphism experience increased long-term survival relative to other TLR5 polymorphisms[126] (**Figure 3.3**). Specifically, we observed ovarian cancer patients that express *TLR5*^{K841K} and *TLR5*^{V61V} did not have significantly greater survival than patients expressing the ancestral allele. Interestingly, *TLR5*^{K841K} expressing patients are close to achieving statistical significance. This could be due to *TLR5*^{K841K} being a non-silent synonymous SNP where one base in a gene's exon is substituted with another, but the amino acid sequence of the resulting protein is not changed. Synonymous SNPs can affect messenger RNA splicing, stability, and structure, as well as protein folding[256]. Therefore, *TLR5*^{K841K} which is located in the cytoplasmic tail domain, could impact TLR5 signal transduction; however, this remains to be verified[125]. In comparison, the *TLR5*^{R392X} SNP prematurely truncates TLR5 in the extracellular domain, causing the loss of the transmembrane domain and the entire signaling cytoplasmic tail[125]. Consequently, this truncation reduces TLR5 signaling, which appears to be beneficial for the survival of ovarian cancer patients but not melanoma, breast, or colorectal cancer patients (**Figure 3.3**).

Our data using two orthotopic models of ovarian cancer indicate that *TLR5*^{R392X} patients may immediately benefit from immune checkpoint therapy targeting the immune suppressive PD-L1

pathway in combination with FLT3L. One goal will be to define the broad clinical applicability of our findings for ovarian cancer by assessing tumor microenvironmental differences, outcomes, and immunological read-outs in *TLR5*^{R392X} carriers relative to homozygous individuals for the ancestral allele. This analysis would then be extended to other solid tumors as well.

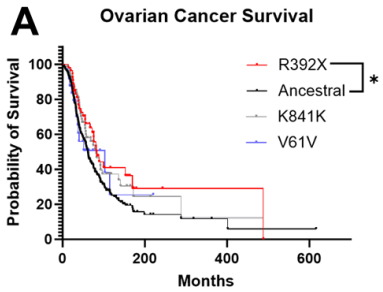
To achieve this goal, cohorts of ovarian cancer patients' peripheral blood mononuclear cells (PBMCs), tumors, and ascites will be assessed for indicators of DC and T cell activity. The first step will be to establish the genotype of patients. Heterozygous, homozygous, and ancestral alleles for *TLR5*^{R392X} will be assessed by TaqMan sample to SNP genotyping kit, which enables genotyping of patient blood or tumor samples using a QuantStudio Real-Time PCR system. We have used this kit and performed functional assays to confirm a reduction of TLR5 signaling by collecting 25,000 PBMCs, resting them for 18 hours in RPMI media, and then culturing them for 18 hours with purified flagellin and measuring IL-6/IL-8 release by ELISA (**Figure 3.4**). We find heterozygous R392X patient PBMCs release less IL-6 and IL-8 when stimulated with flagellin. This observation is consistent with the study that first identified the *TLR5*^{R392X} polymorphism[125]. Once patients have been identified as heterozygous or homozygous for the *TLR5*^{R39X} or the ancestral allele, we will analyze ascites and tumors by spectral flow cytometry and bulk RNAseq by CIBERSORT. Considering we observed TLR5 KO mice expressed more cDC1s that exhibited a more functional phenotype in the ovarian TME, our initial hypothesis will be that patients bearing an *TLR5*^{R392X} allele will have more cDC1s expressing less inhibitory receptors and greater numbers of T cells in their ascites and tumors relative to patients expressing both ancestral alleles. It would be intriguing to assess samples expressing *TLR5*^{R392X} to determine if the cytokine response to flagellin aligns with the presence of cDC1 or DC activity in the tumor or ascites samples. It is worth noting that *TLR5*^{R392X} does not result in a complete loss of TLR5 signaling in heterozygous carriers. Our expectation is that a reduced flagellin-mediated cytokine response will correspond with a more pronounced cDC1 and T cell response and function.

To compare gene expression changes amongst the multiple immune cell subsets within the TME of patients with or without the *TLR5*^{R392X} polymorphism, we will use CIBERSORT[257]. CIBERSORT is a machine learning algorithm able to deconvolute individual immune cell signatures from bulk tissue sequences, with the capability of resolving individual immune cell populations in samples with low signal/noise ratios (e.g., poorly immune infiltrated tumors)[257]. We will identify the clinical features or immunological attributes from genomic data analyzed from ovarian cancer patients, followed by modeling and prediction to determine the effects of the TLR5 polymorphism on the immune composition and outcomes.

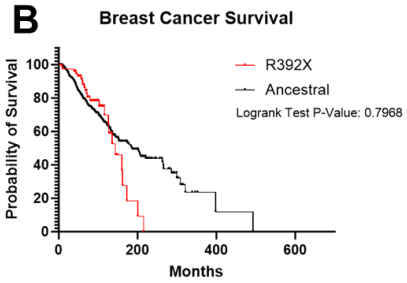
Human DC subsets are scarce within PBMCs, ranging from less than 0.2% of PBMCs for cDC2s and pDCs to less than 0.08% of PBMCs in the case of cDC1s[258]. DC subsets can be isolated with higher purity and yield than cell sorting or magnetic-microbead kits via a multistep depletion protocol using a MultiMACS Cell24 Separator Plus. The protocol consists of multiple steps including depletion of monocytes (CD14$^+$), B cells (CD19$^+$), T cells (CD3$^+$), and NK cells (CD56$^+$) from PBMCs, followed by DC isolation using magnetic microbeads specific for each DC subset: cDC2s (CD1c$^+$), cDC1s (CD141$^+$), and pDCs (CD304$^+$)[259].

DC subsets from patient PBMCs will be examined for function by a mixed lymphocyte reaction. This technique is used to evaluate the potential impact of a small therapeutic molecule or biologic on the immune system. In this case, it will be used to examine the immunomodulatory effect of anti-PD-L1 or FLT3L independently and in combination on *TLR5*^{R392X} DCs *in vitro*. With the goal of addressing if anti-PD-L1 and FLT3L would be a beneficial therapy for ovarian cancer patients that are *TLR5*^{R392X} carriers. To assess this isolated cDC1s, cDC2s, and pDCs will be independently cultured with CFSE-labeled allogeneic T cells from a healthy patient donor in with anti-PD-L1 and FLT3L individually and in combination. This experiment will be set up as follows: 50,000 isolated DCs (cDC1s, cDC2s, and pDCs) from ancestral and *TLR5*^{R392X} carriers will be co-cultured with 50,000 CFSE-labeled allogeneic CD3+ T cells for five days at three different DC: T

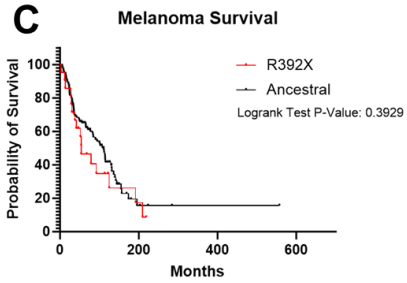
cell ratios 1:5, 1:2, and 1:1 with anti-PD-L1 and/or FLT3L. Furthermore, anti-CTLA4 must also be considered in combination with anti-PD-L1 and FLT3L due to our published findings that anti-CTLA4 drives survival in TLR5 KO ovarian tumor-bearing mice (**Figure 3.5**). Proliferation of T cells will be calculated by the reduction in fluorescence of CFSE as the fluorescence is halved with each division and measured by flow cytometry. Additionally, CD4 v CD8 expansion ratios will be examined to see if *TLR5*^{R392X} DCs skew differentiation toward specific T cell phenotypes. cDC1s derived from *TLR5*^{R392X} patients are hypothesized to drive more significant CD8 T cell proliferation relative to ancestral-derived cDC1s when co-cultured with anti-PD-L1 and FLT3L. Chronically stimulating DCs with flagellin before culturing with T cells may be considered in another experiment to simulate the presence of bacteria in the ovarian TME. Alternative strategies may include using cord blood to examine cDC1 development with chronic TLR5 signaling in an FLT3L culture, which would enable confirmation that *TLR5*^{R392X} DCs recapitulate the behavior shown with mouse TLR5 KO DCs. Overall, these experiments will help bridge the clinical gap in our findings demonstrating that TLR5 signaling is detrimental to DC-mediated anti-tumor T cell responses. These studies would help to pave the way towards using anti-PD-L1/FLT3L in combination for *TLR5*^{R392X} carriers with ovarian cancer.



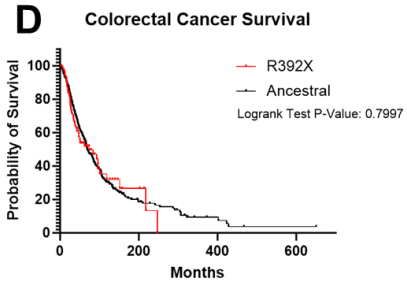
	Number of Patients	Median Survival (Months)	Log-rank (Mantel-Cox) Test to Ancestral
R392X	29	83.74	.0308
Ancestral	213	61.78	N/A
K841K	42	81.73	.0781
V61V	13	102.71	.7143



	Number of Patients	Median Survival (Months)
R392X	24	144.16
Ancestral	216	186.44



	Number of Patients	Median Survival (Months)
R392X	16	53.95
Ancestral	72	111.52



	Number of Patients	Median Survival (Months)
R392X	45	74.23
Ancestral	391	69.17

Figure 3.3. *TLR5*^{R392X} ovarian cancer patients survive significantly longer than patients expressing other TLR5 alleles

(**A**) Survival comparison of Ovarian cancer, (**B**) Breast cancer, (**C**) Melanoma, and (**D**) Colorectal cancer patients either homozygous or heterozygous for described allele. Log-rank Mantel-Cox test was used to compare survival proportions. Tables indicate number of patients or median survival within each group (* $p < .05$).

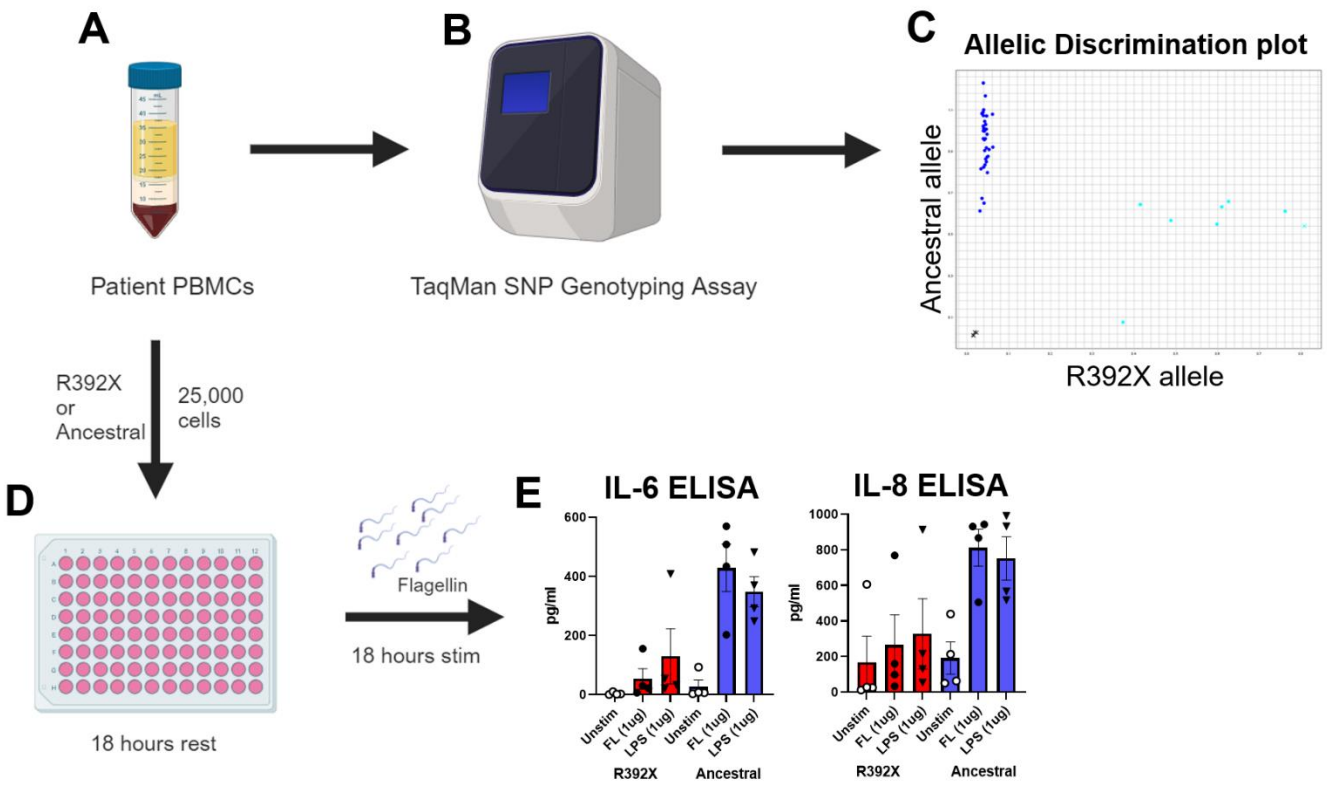

A
B
C
Allelic Discrimination plot
Ancestral allele
R392X allele
Patient PBMCs
TaqMan SNP Genotyping Assay
R392X
or
Ancestral
25,000
cells
D
18 hours rest
Flagellin
18 hours stim
E
IL-6 ELISA
IL-8 ELISA
pg/ml
Unstim
FL (1ug)
LPS (1ug)
R392X
Ancestral

Figure 3.4. *TLR5*^{R392X} genotyping and phenotyping by ELISA

(**A**) Frozen PBMCs were thawed, and genomic DNA was extracted and purified. (**B**) TaqMan SNP Genotyping assay was performed on patient samples. (**C**) Allelic discrimination plots to distinguish between samples expressing *TLR5*^{R392X} allele (light blue) v ancestral allele (navy blue). (**D**) For phenotypic validation, 25,000 PBMCs were counted and rested for 18 hours in RPMIc media, and then cultured for 18 hours with purified flagellin (1ug) or LPS (1ug) and (**E**) measured IL-6/IL-8 release by ELISA, red bars represent *TLR5*^{R392X} samples and blue bars represent Ancestral.

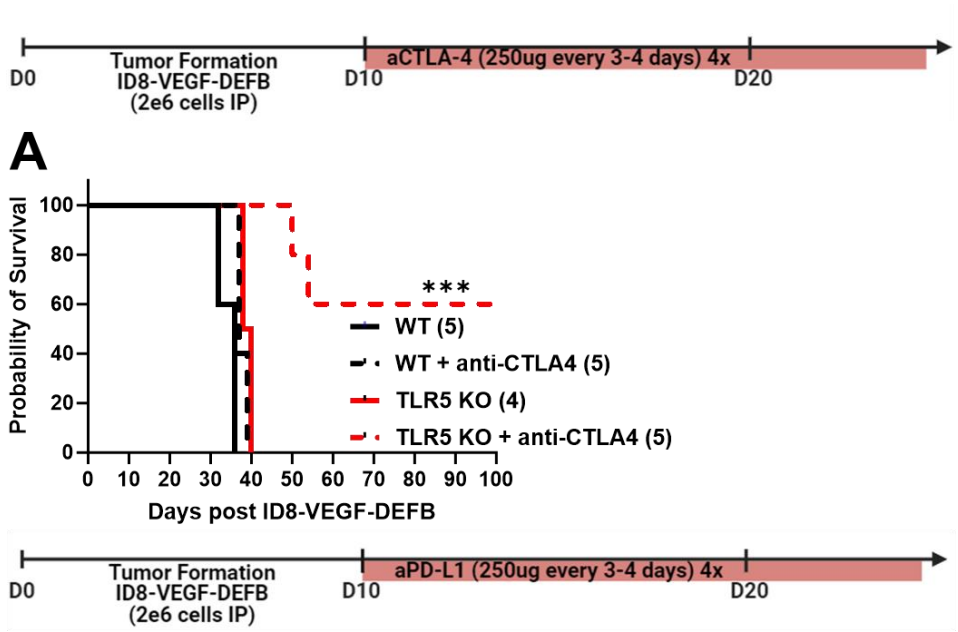

D0
Tumor Formation
ID8-VEGF-DEFB
(2e6 cells IP)
D10
aCTLA-4 (250ug every 3-4 days) 4x
D20
A
Probability of Survival
Days post ID8-VEGF-DEFB
***
WT (5)
WT + anti-CTLA4 (5)
TLR5 KO (4)
TLR5 KO + anti-CTLA4 (5)
D0
Tumor Formation
ID8-VEGF-DEFB
(2e6 cells IP)
D10
aPD-L1 (250ug every 3-4 days) 4x
D20
B
Probability of Survival
Days post ID8-VEGF-DEFB
WT (5)
WT + anti-PD-L1 (5)
TLR4 KO (5)
TLR4 KO + anti-PD-L1 (4)
D5
FLT3L (10ug daily for 6 days)
D0
Tumor Formation
PPNM
(3e6 cells IP)
D10
aPD-L1 (250ug every 3-4 days) 4x
D20
C
Probability of Survival
Days post PPNM
***
**
WT (5)
TLR5 KO (5)
WT + anti-PD-L1 (4)
TLR5 KO + anti-PD-L1 (10)
WT + FLT3L (4)
TLR5 KO + FLT3L (5)
WT+ FLT3L + anti-PD-L1 (5)
TLR5 KO + FLT3L + anti-PD-L1 (5)
*
**

Figure 3.5. TLR5 signaling impairs efficacy of CTLA-4 blockade; TLR4 signaling does not impair efficacy of anti-PD-L1 therapy; and anti-PD-L1 and FLT3L drive a synergistic survival response to ovarian cancer

(**A**) Survival of mice bearing ID8-*Defb29/Vegf-A* ovarian tumors treated with anti-CTLA-4. On day 0, tumors were initiated in wild-type (WT) or TLR5 KO mice. Anti-CTLA-4 was initiated on day 10 for a total of 4 injections. (**B**) Survival of mice bearing ID8-*Defb29/Vegf-A* ovarian tumors treated with anti-PD-L1. On day 0, tumors were initiated in wild-type (WT) or TLR4 KO mice. Anti-PD-L1 was initiated on day 10 for a total of 4 injections. (**C**) Survival proportions of TLR5 KO and wild-type mice bearing *PPNM* tumors and treated with FLT3L (10μg) for 6 injections starting at day 5 post-initiation and/or anti-PD-L1 which was initiated on day 10 for a total of 4 injections. Plots are representative of two experiments. Numbers in parentheses indicate numbers of mice within each group. Log-rank test for survival compared to wild type (* $p < .05$, ** $p < .01$, *** $p < .001$).

3.2.6 Strategies to overcome the failure of immunotherapy against ovarian cancer.

The idea that TLR5 signaling triggers a loss in anti-tumor immune function is paradoxical, considering that TLR signaling in myeloid cells is canonically associated with the induction of adaptive immunity. This has resulted in significant efforts to define combinations of TLR agonists and other immune adjuvants for anti-cancer vaccines and therapies[260, 261]. However, there is a growing body of evidence that suggests chronic TLR engagement can tip the balance toward an environment favoring tumor progression via excessive inflammation[262, 263]. The ovarian TME is highly immune infiltrated where microbial and tumor cell-derived DAMPs and PAMPs accumulate, leading to suppressed DCs, ultimately culminating in T cell exhaustion. Thus, removing a signal can potentially be a promising strategy to tip the scales from tolerance/exhaustion toward an environment that enables immune-mediated tumor control/killing. Currently, there are no clinical trials utilizing TLR5 antagonism in any capacity, and the trials attempting to agonize TLR5 have had little success (**Table 3.1**). We find that reducing or eliminating chronic TLR5 signaling within the ovarian tumor microenvironment increases the accumulation and functional maturation of cross-presenting cDC1 dendritic cells. cDC1s are the most well-equipped cell type to cross-present antigens to CD8 T cells and are vital for regulating immune checkpoint blockade therapeutic response, mainly when using anti-PD-L1/anti-PD-1. Blockade of tumor or myeloid-associated PD-L1 from interacting with the T cell inhibitory receptor PD-1 has shown promise in cancer patients as a single therapy and in combination with other treatments. Unfortunately, few ovarian cancer patients respond, and of those that do, many will go on to develop recurrent disease[106].

To develop therapeutic interventions that effectively and broadly overcome the limited efficacy of PD-L1/PD-1 blockade, it is critical to define the mechanisms underlying the failure of this therapy. We find that chronic TLR5 signaling impairs the efficacy of PD-1/PD-L1 blockade by disrupting the accumulation and maturation of cDC1 into the tumor microenvironment. Therefore, it is plausible that the efficacy of PD-L1 blockade could be enhanced by blocking TLR5 signaling. We

hypothesize that using a bispecific antibody in which an inhibitory anti-TLR5 antibody is fused via the FC domain to an inhibitory PD-L1 antibody will overcome the inhibitory effects of TLR5 signaling while enhancing the efficacy of PD-L1 blockade. We speculate that the bioavailability of an anti-PD-L1/anti-TLR5 bi-specific would be more limited to the TME, where we know that there are multiple TLR5-expressing myeloid cells that also highly express PD-L1 thus reducing off-target effects. We could fuse the anti-murine TLR5 IgG (clone Q23D11) for pre-clinical validation in murine studies to anti-mouse PD-L1 IgG2b (clone 10F.9G2). For clinical use, we could fuse the anti-human TLR5 Ig2A (clone Q2G4) with anti-human PD-L1 IgG1 (clone Atezolizumab). The anti-TLR5 antibody has been documented to neutralize the biological activity of human or murine TLR5 in response to bacterial flagellin[264]. We have evidence that neutralization of TLR5 signaling within the ovarian tumor microenvironment reduces the number of PD-L1 expressing myeloid cells within the tumor microenvironment and instead favors the accumulation of cross-presenting cDC1s (**Figure 2.5**). Despite the positive effects of inhibition of TLR5 signaling on reducing numbers of PD-L1 expressing myeloid cells and increasing cDC1s within the tumor microenvironment, there was not a significant increase in survival (**Figure 3.6**). This may be due to the low dosage of four 20ug IP injections for the survival study compared to the four 50ug IP injections for the phenotypic study. On the other hand, in the absence of TLR5 signaling, PD-L1 blockade achieves a significant survival benefit in multiple models of ovarian cancer (ID8, UPK10, PPNM) (**Figures 2.1 and 3.5**). For a potential trial, at least four dosages at 250ug anti-PD-L1-anti-TLR5 would be required for efficacy. This matches the 250ug anti-PD-L1 needed to achieve durable survival in TLR5 KO mice bearing ovarian tumors. Considering what we have learned regarding the relationship between TLR5 and DCs throughout our studies, it may be possible to increase the efficacy of anti-TLR5 treatment by binding it to anti-CD11c in a bispecific antibody combination, potentially decreasing the amount of anti-TLR5 needed to achieve therapeutic efficacy. This strategy could be further enhanced with FLT3L to increase DC numbers in the ovarian TME, thus allowing for more opportunities for anti-TLR5 to bind to DCs.

FLT3L is a potent dendritic cell growth factor and drives a signaling pathway researchers exploit to generate *in vitro* cultures that produce bona fide conventional dendritic cells[212, 213]. Given the critical importance of cDC1s for anti-tumor immunity and response to immune checkpoint blockade, and because FLT3L can expand cDC1 *in vitro*, researchers began administering FLT3L *in vivo* in cancer-bearing hosts. Initial reports indicated that *in vivo* administration of FLT3L was relatively well-tolerated and enhanced systemic numbers of cDC1s in lymph nodes and the spleen[265]. However, few trials have demonstrated apparent clinical efficacy of using FLT3L as a new immune therapy approach[237]. We observed chronic exposure of *in vitro* FLT3L cultures to bacterial flagellin promoted the differentiation of cDC1s away from a phenotype that is well-equipped to activate CD8 T cells and instead expanded myeloid cells that are less capable of stimulating CD8 T cells due to high expression levels of PD-L1 (**Figure 2.9 and 2.10**). Furthermore, *in vivo* blockade of PD-L1 in combination with FLT3L boosted the survival of TLR5 KO mice, resulting in 100% of animals exhibiting no evidence of disease against ID8-VEGF-DEFB and 80% against PPNM (**Figures 2.13 and 3.5**). TLR5 KO mice treated with FLT3L or PD-L1 alone also showed a significant survival response. However, 40% of animals still succumbed to the disease. On the other hand, in the presence of TLR5 signaling, wild-type mice exhibited no benefit from the single treatments or combination therapy. These data demonstrate that in the absence of TLR5 signaling, expanding cDC1s using FLT3L during PD-L1 blockade has translational potential as an immunotherapy.

To circumvent the adverse effects of TLR5 signaling on *in vivo* expansion of cDC1s using FLT3L, we will generate a bi-specific antibody/protein fusion consisting of a neutralizing TLR5 antibody fused to the FC domain of the Ig-FLT3L fusion protein or an FLT3 agonist. The Ig-FLT3L fusion increases the pharmacokinetics of the FLT3L protein. We will leverage this design to fuse an anti-TLR5 antibody to the FC domain. To this end, we propose the construction of two constructs. We will fuse the anti-murine TLR5 IgG (clone Q23D11) to recombinant FLT3L-IgG1 for pre-clinical

validation in murine studies. For clinical use, we will fuse the anti-human TLR5 Ig2A (clone Q2G4) with the FLT3 agonist-Fc fusion GS-3583.

We have identified a new host-intrinsic mechanism governing the accumulation and functional maturation of cDC1 within the ovarian tumor microenvironment involving TLR5 signaling. By combining FLT3 agonism with blockade of TLR5, it is expected that expansion of cDC1s will occur despite chronic TLR5 signaling within the tumor environment. Of FLT3-expressing progenitor and mature cell types, TLR5 is expressed predominantly on myeloid progenitor populations, not lymphocyte progenitors[266]. Thus, targeting FLT3L to TLR5-expressing subsets is expected to enhance the specificity of this therapeutic approach, reducing the amount and duration of FLT3L exposure patients need to endure.

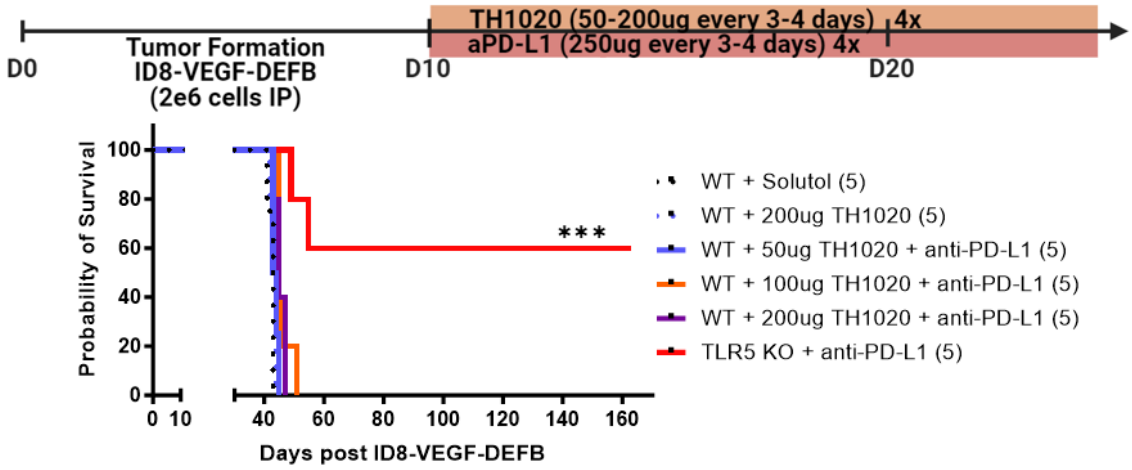
Dendritic cell-based cancer vaccines have shown promise in animal models but have largely failed to demonstrate significant clinical efficacy in human trials[267, 268]. Currently, there is only one clinically approved DC vaccine for the treatment of cancer, Sipuleucel-T (PROVENGE; Dendreon), a DC vaccine that is formulated to stimulate an immune response by targeting prostate acid phosphatase (PAP), a tissue antigen expressed by prostate cancer cells[269]. Several factors contribute to the lack of successful DC therapies, including tumor-mediated immunosuppression, downregulation of MHC molecules, lack of costimulatory molecules, and secretion of immunoinhibitory cytokines[267, 270]. To improve DC vaccine efficacy, progress is needed to optimize antigen loading techniques, enhance DC activation, preserve DC function, and find the right combination of checkpoint inhibitors[271, 272]. Based on our findings, TLR5 KO DCs appear resistant to immunosuppression and favor a cross-presenting phenotype in the ovarian TME. One strategy to improve DC-based vaccines may be eliminating TLR5 expression in autologous DCs. Broadly, DC adoptive transfers involve isolating and expanding autologous DCs *in vitro*, loading them with antigens, and returning them to patients. Thus, during the expansion phase, TLR5 expression can be eliminated by CRISPR gene editing or blocked by pre-incubation

with anti-TLR5. This strategy can be supplemented with anti-PD-L1 and FLT3L to promote *in vivo* DC expansion and prevent T-cell inhibition. This approach could overcome the difficulties of systemically blocking TLR5 on endogenous DCs by an antibody blockade and mitigate off target effects.

Table 3.1. Current clinical trials investigating TLR5 agonism or antagonism in any context

Ligand	Phase	Application	Target	aborators	NCT number	Goal
Mobilan (M-VM3)	Phase 1 and 2	Prostate cancer	TLR5 agonist/ adjuvant	Panacela Labs LLC	NCT02844699	Induce infiltration of neutrophils and NK cells and induction of a CD8 T cell response against prostate cancer
VAX102 (flagellin.HuM2e)	Phase 1	Influenza	TLR5 agonist/ adjuvant	VaxInnate Corporation; Bill & Melinda Gates Foundation	NCT00603811	Elicit cross-protective immunity against most human influenza A virus strains
VAX125	Phase 2	Influenza	TLR5 agonist/ adjuvant	VaxInnate Corporation	NCT00966238	Overcome poor immune responses in the elderly against influenza
Entolimod (CBLB502)	Phase 1	Unspecified adult solid tumor	TLR5 agonist/ adjuvant	Roswell Park Cancer Institute, NCI, Cleveland BioLabs Inc	NCT01527136	Immune stimulation to stop tumor cell growth
Entolimod (CBLB502)	Phase 2	Colorectal Cance	TLR5 agonist/ adjuvant	BioLab 612 LLC (Russian Federation)	NCT02715882	Induce immune activity in patients with Colorectal Cancer (Neo-adjuvant Treatment)
Entolimod (radiation therapy)	Phase 1	Mucositis, various types of squamous cell carcinoma of various tissues	TLR5 agonist/ adjuvant	Roswell Park Cancer Institute, NCI, Cleveland BioLabs Inc	NCT01728480	Prevent side effects caused by chemotherapy with cisplatin and radiation therapy

*ClinicalTrials.gov on 10/5/2024


TH1020 (50-200ug every 3-4 days) 4x
aPD-L1 (250ug every 3-4 days) 4x
D0
Tumor Formation
ID8-VEGF-DEFB
(2e6 cells IP)
D10
D20
Probability of Survival
100
80
60
40
20
0
0 10
40
60
80
100
120
140
160
***
Days post ID8-VEGF-DEFB
WT + Solutol (5)
WT + 200ug TH1020 (5)
WT + 50ug TH1020 + anti-PD-L1 (5)
WT + 100ug TH1020 + anti-PD-L1 (5)
WT + 200ug TH1020 + anti-PD-L1 (5)
TLR5 KO + anti-PD-L1 (5)

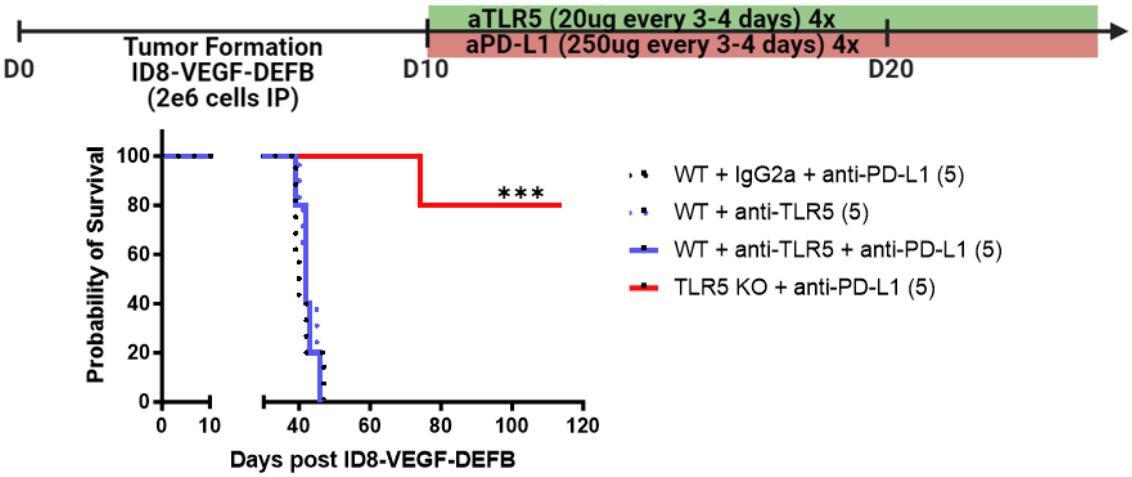

aTLR5 (20ug every 3-4 days) 4x
aPD-L1 (250ug every 3-4 days) 4x
D0
Tumor Formation
ID8-VEGF-DEFB
(2e6 cells IP)
D10
D20
Probability of Survival
100
80
60
40
20
0
0 10
40
60
80
100
120
***
Days post ID8-VEGF-DEFB
WT + IgG2a + anti-PD-L1 (5)
WT + anti-TLR5 (5)
WT + anti-TLR5 + anti-PD-L1 (5)
TLR5 KO + anti-PD-L1 (5)

Figure 3.6. Extrinsic blockade of TLR5 is unable to improve survival of WT mice in combination with anti-PD-L1

(**A**) Survival of mice bearing ID8-*Defb29/Vegf-A* ovarian tumors treated with anti-PD-L1 and TH1020 (small molecule inhibitor of TLR5). On day 0, tumors were initiated in wild-type (WT) or TLR5 KO mice. Anti-PD-L1 and TH1020 (50ug, 100ug, or 200ug) was initiated on day 10 for a total of 4 injections. (**B**) Survival of mice bearing ID8-*Defb29/Vegf-A* ovarian tumors treated with anti-PD-L1 and anti-TLR5. On day 0, tumors were initiated in wild-type (WT) or TLR5 KO mice. Anti-PD-L1 and anti-TLR5 (20ug) was initiated on day 10 for a total of 4 injections. Numbers in parentheses indicate numbers of mice within each group. Log-rank test for survival compared to wild type (* $p < .05$, ** $p < .01$, *** $p < .001$).

Concluding Remarks

Most studies investigating TLRs in the context of cancer focus on using them to stimulate or propagate an anti-cancer immune response. Stimulation of TLR on immune cells can have an anti-cancer effect by enhancing DC antigen processing and presentation[172]. However, activation in tumor cells can often lead to immunosuppression through IL-6 or promote tumor growth and angiogenesis through an NF-κB signaling cascade[154]. This makes targeting TLRs a double-edged sword. The role of TLR5 in cancer research has been largely overlooked in favor of other TLRs, such as TLR4, potentially due to being perceived as a redundant anti-bacterial mechanism. One study by the Conejo-Garcia lab set the groundwork for understanding the relationship between TLR5 signaling in ovarian cancer. Discovering that TLR5 signaling, when abrogated by a hypomorphic polymorphism, differentially influences inflammation, anti-tumor immunity, and the clinical outcome of ovarian and breast cancer patients[126]. This publication found significant differences in IL-6 transcript levels between TLR5-responsive and nonresponsive ovarian tumor specimens but not between TLR5-responsive and nonresponsive ER^+ breast tumor specimens linking tumor-derived IL-6 to a potential source of survival disparity[126]. It can be speculated that this difference in IL-6 levels between ovarian and breast cancer patients may be due to differences in the quantity or phenotypes of TLR5-expressing immune populations within their respective TMEs. Until this point, studies seldom examined TLR5 signaling in the context of cancer. On top of this, ovarian cancer remains one of the few cancers in which no FDA-approved immune therapies exist to improve the standard of care.

This study is the first to investigate TLR5 signaling in the context of immunotherapy. The findings presented in the thesis further our understanding of the role of TLR5 signaling on the efficacy of checkpoint therapy in the ovarian tumor microenvironment. Although the effect of TLR5 signaling on an anti-tumor immune response is multi-factorial, our data suggests bacterial flagellin leads to failure of immune therapy through modulation of dendritic cell differentiation or phenotype and

function. These studies establish an underappreciated link between the microbiome and cancer. Mechanistically, we demonstrate that chronic TLR5 signaling impairs the XCR1+ CD103+ cDC1 subsets within the TME and biases precursor cells towards myeloid-associated subsets expressing high levels of PD-L1. Introducing a new understanding of how flagellin can alter the course of an immune response. We discovered eliminating TLR5 signaling on DCs enhances the efficacy of anti-PD-L1 against ovarian cancer, which can be further improved by expanding cDC1s *in vivo* with FLT3L. Thus, introducing a promising new strategy to treat ovarian cancer. Overall, this work subverts the expectation that TLR signaling promotes an appropriate anti-cancer immune response. We observed that TLR4 signaling did not impact the efficacy of anti-PD-L1 therapy in TLR4 KO mice bearing ovarian cancer, unlike TLR5 (**Figure 3.5**). Demonstrating TLR5 signaling may be uniquely detrimental to the efficacy of checkpoint therapy. Future studies will need to address if other TLR signaling pathways impact immunotherapy for ovarian cancer. Ultimately our findings demonstrate that TLR5 antagonism needs to be seriously considered as a therapy for ovarian cancer.

Chapter 4: Materials and methods

Mice

TLR5 wild-type mice were generated using transgenic *Kras*^{tm4Tyj} and *Trp53*^{tm1Brn} mice[273, 274] obtained from the National Cancer Institute (NCI) Mouse Models of Human Cancers Consortium and brought to a full C57BL/6 background[39]. These mice were then bred to TLR5-deficient (TLR5 KO) mice (B6.129S1- *Tlr5*^{tm1Flv}/J), as previously described[191], to generate TLR5 KO mice. TLR5fl/fl (B6(Cg)-*Tlr5*$^{tm1.1}$Gewr/J)[229] mice were crossed with CD11cCre (B6.Cg-Tg$^{(Itgax\text{-}cre)1\text{-}1}$Reiz/J)[275] mice, both of which were obtained from The Jackson Laboratory to delete TLR5 from CD11c cells, to generate CD11c.TLR5^{ko} mice. Cre^{+} and Cre^{-} CD11c.TLR5^{WT} littermates were maintained as controls. TLR5 tdTomato reporter mice (TLR5KI-tdTom - B6(FVB)-*Tlr5*$^{tm1.1}$Gbrt/J)[276] and TLR4KO mice (B6(Cg)-*Tlr4*$^{tm1.2Karp}$/J) are also from The Jackson Laboratory. All strains were maintained in specific-pathogen-free barrier facilities at the University of Virginia. OT-1 mice (C57BL/6-Tg(*TcraTcrb*)^{1100}Mjb/J)[277] were obtained from The Jackson Laboratory, while CD45.1 mice (B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyCrl)[278] were obtained from Charles River.

To validate CD11c Cre x TLR5fl/fl cross in CD11c.TLR5^{ko} DCs, CD11c+ cells were isolated from spleens of CD11c.TLR5^{wt}, total TLR5 KO, and CD11c.TLR5^{ko} mice using magnetic bead separation MojoSort™ Mouse CD11c Cell Isolation Kit (cat# 480078). After bead isolation, 100,000 CD11c+ cells were cultured with or without 10ng/ml of ultra-purified Flagellin (InvivoGen, cat# tlrl-epstfla-5) in RPMIc media (described below) for 24 hours and an IL-6 ELISA (Biolegend, cat# 431316) was subsequently performed on supernatants from the cultured cells.

All experiments were conducted utilizing adult (~20-week-old) female mice. All experiments in this study were approved by the University of Virginia Institutional Animal Care and Use Committee.

Cell lines and implantation

ID8 cells were provided by K. Roby (Department of Anatomy and Cell Biology, University of Kansas) and retrovirally transduced to express *Defb29* and *Vegf-A*^{192}. UPK10[39] was derived after 10 serial passages from solid tumors developed using a p53-deficient autochthonous tumor model. The PPNM (*p53*$^{-/-R172H}$*Pten*$^{-/-}$*Nf1*$^{-/-}$*Myc*^{OE} genotype)[33] cell line was provided by the Weinberg group who developed them at the Whitehead Institute as described by Iyer et al.

Cell lines were authenticated by monitoring of morphology and monthly testing for mycoplasma. To limit the opportunity for genetic drift, cells were maintained at less than five passage numbers and maintained as frozen stocks at −180°C and expanded only for inoculation into mice. Tumor cell lines were cultured in RPMI complete media (RPMIc): RPMI (11875093, Gibco), 10% FBS (Sigma), 2 mmol/L of l-glutamine (25030081, Gibco), 1 mmol/L of sodium pyruvate (11360070, Gibco), 50 μmol/L of β-mercaptoethanol (M6250, Sigma), and 100 U/mL of Penicillin/Streptomycin (15140122, Gibco). ID8-*Defb29/Vegf-A* and UPK10 tumors were initiated by intraperitoneal injection (IP) of 2e6 cells in sterile PBS at 100μl total volume. PPNM tumors were expanded in Fallopian tube media (FT-media): DMEM supplemented with 1% insulin–transferrin–selenium (Thermo Fisher Scientific; ITS-G, 41400045), EGF (2 ng/mL), 4% heat-inactivated fetal bovine serum (Thermo Fisher Scientific; IFS, F4135), and 1% penicillin and streptomycin (5 ml) tumors were initiated IP at 3e6 cells mixed in a 1:1 ratio with Matrigel (Corning Matrigel matrix, 47743-710):FT-media (1:1).

***In vivo* TLR5 inhibition, CD8 Depletion, Flt3L-Ig (FLT3L), anti-CTLA-4, and anti-PD-L1 dosage**

Mice were administered neutralizing PD-L1 via intraperitoneal (IP) injection of anti-PD-L1 (Clone 10F.9G2, BioXCell, cat# BE0361 (endotoxin-free, sterile, free of any known murine pathogens)) or anti-CTLA-4 (Clone 9H10, BioXCell, cat# BE0131) in PBS at a concentration of 250μg per injection at 100μl total volume using kinetics described within each figure. *In vivo* blockade of TLR5, 20 or 50μg/mouse of TLR5 neutralizing antibody, clone Q23D11[279] or isotype rat IgG2a

was injected IP for four consecutive days starting 15 days post-tumor initiation. TH1020 was dissolved in Solutol (MilliporeSigma, cat# 70142-34-6) at 500µg/ml prior to IP injection at amounts and kinetics described in the figure. CD8 depletions were performed with anti-CD8 BioXcell Clone 2.43 cat# BE0061 or isotype rat IgG2b IP beginning 48 hours before tumor initiation at 400µg/mouse and then twice weekly at 200µg/mouse for three weeks. FLT3L (hum/hum) cat# BE0342 was injected IP at 10ug in 100ul of total volume with sterile PBS for six consecutive days starting at day five post-tumor initiation for a total of 6 injections.

Survival

Euthanasia was performed according to the guidelines set forth by the AVMA and UVA's IACUC. For the ovarian tumors, mice were euthanized when they exhibited moderate abdominal distension due to the accumulation of ascitic fluid, hypothermia, ruffled fur, difficulty breathing, anemia, dehydration, and lethargy. When these symptoms were observed, mice were euthanized with CO_2 followed by cervical dislocation. Mice showing hunched posture, severe infection, labored breathing, and failure to eat were euthanized without delay.

Flow Cytometry

Isolated tissues were placed on ice in a sterile 6-well plate with 3 mL of RMPI (11875093, Gibco) with 5% FBS (Sigma). Ascites was harvested via PBS wash of the peritoneal cavity by syringe aspiration followed by residual fluid collection by pipetting. To make single-cell suspensions before staining with antibodies, the digested tissues were passed through 70µm cell strainers (352350, Corning) using mechanical force with the rubber end of a 5mL syringe. For *in vitro* coculture experiments, all tissues were processed in sterile conditions.

For intracellular cytokine staining, disassociated tumor specimens were stained with the LIVE/DEAD fixable Aqua Dead Cell Stain Kit (Life Technologies). Cells were then fixed with 1% methanol formaldehyde solution (Thermo Scientific) followed by permeabilization in 0.5% Saponin solution (Sigma) and intracellular staining. Proliferation, surface, and intracellular

staining were analyzed using FlowJo software. SPHERO™ AccuCount Particles (cat #ACFP-50-5) were utilized to enumerate cell counts. Flow cytometry experiments were performed on a Cytek Aurora Borealis (5 lasers) or Life Technologies Attune NxT.

To enable analysis of in situ cytokine production, mice were injected IP with 200ul of Brefeldin A (Sigma, cat# B7651-25MG) at 0.5 mg/mL in PBS 6 hours prior to harvesting tissues as described in a STAR protocol to evaluate intracellular cytokine production *ex vivo* using flow cytometry [280].

Flow Cytometry data was analyzed using FlowJo version 10.10.0. Principal component analysis was performed using FlowJo-integrated Tsne analysis software. FlowSOM version 3.0.18 was downloaded from FlowJo exchange (https://www.flowjo.com/exchange/#/) and utilized to visualize and cluster high-parameter flow cytometry data using FlowJo.

Marker	Fluorochrome	Clone	Reactivity	Catalogue #	Vendor
C/EBP alpha	Alexa Fluor 594	G-10	Mouse	sc-166258	Santa Cruz
C/EBP beta	PE	H-7	Mouse	sc-7962	Santa Cruz
CD103	BUV496	M290	Mouse	741083	BD Biosciences
CD103	BV605	2 E7	Mouse	121433	Biolegend
CD103	BUV661	M290	Mouse	741504	BD Biosciences
CD107a (LAMP-1)	BV711	1D4B	Mouse	121631	Biolegend
CD11b	BUV737	M1/70	Mouse	741722	BD Biosciences
CD11b	PE-Cy7	M1/70	Mouse, Human	101216	Biolegend
CD11b	FITC	M1/70	Mouse	101206	Biolegend
CD11b	BUV395	M1/70	Mouse	563553	BD Biosciences
CD11c	APC/Fire 750	N418	Mouse	117352	Biolegend
CD11c	BUV805	HL3	Mouse	749090	BD Biosciences
CD11c	PacBlu	N418	Mouse	117322	Biolegend
CD127	APC-Cy7	A7R34	Mouse	135040	Biolegend
CD135 (FLT3)	BV421	A2F10	Mouse	135315	Biolegend
CD152 (CTLA-4)	PE Dazzle	UC10-4B9	Mouse	106318	Biolegend
CD16/32	BV605	2.4G2	Mouse	563006	BD Biosciences
CD172a (SIRPa)	BUV805	P84	Mouse	741997	BD Biosciences
CD19	BV750	6D5	Mouse	115561	Biolegend
CD206 (MMR)	Alexa Fluor 700	C068C2	Mouse	141734	Biolegend
CD206 (MMR)	PE Dazzle	C068C2	Mouse	141731	Biolegend
CD206 (MMR)	PE-Cy7	C068C2	Mouse	141720	Biolegend
CD206 (MMR)	PerCP-Cy5.5	C068C2	Mouse	141716	Biolegend
CD27	BUV496	LG.3A10	Mouse	741094	BD Biosciences
CD27	PE-Cy7	LG.3A10	Mouse	124314	Biolegend
CD274 (B7-H1, PD-L1)	PE Dazzle	10F.9G2	Mouse	124323	Biolegend
CD274 (B7-H1, PD-L1)	PE-Cy7	10F.9G2	Mouse	124314	Biolegend
CD279 (PD-1)	APC	RMP1-30	Mouse	109112	Biolegend
CD3e	FITC	145-2C11	Mouse	100306	Biolegend

CD3	BV750	17A2	Mouse	100249	Biolegend
CD3	BV785	17A2	Mouse	100232	Biolegend
CD34	APC	HM34	Mouse	128612	Biolegend
CD366 (Tim-3)	APC Fire 750	RMT3-23	Mouse	119738	Biolegend
CD366 (Tim-3)	PerCP-Cy5.5	RMT3-23	Mouse	119718	Biolegend
CD4	Alexa Fluor 700	GK1.5	Mouse	100430	Biolegend
CD40	BUV737	3.23	Mouse	741749	BD Biosciences
CD44	BUV563	IM7	Mouse	741471	BD Biosciences
CD44	BB515	IM7	Mouse	564587	BD Biosciences
CD45	Alexa Fluor 700	30-F11	Mouse	103128	Biolegend
CD45	BV605	30-F11	Mouse	103151	Biolegend
CD45	PE	30-F11	Mouse	103106	Biolegend
CD45	FITC	30-F11	Mouse	103108	Biolegend
CD45	BV711	30-F11	Mouse	103147	Biolegend
CD45	BUV661	I3/2.3	Mouse	752413	BD Biosciences
CD45	BB515	30-F11	Mouse	564590	BD Biosciences
CD45.1	BV750	A20	Mouse	747314	BD Biosciences
CD45.1	PE-Cy5	A20	Mouse	15-0453-82	Invitrogen
CD45.2	BV570	104	Mouse	109833	Biolegend
CD45.2	BV421	104	Mouse	109832	Biolegend
CD45R (B220)	Alexa Fluor 532	RA3-6B2	Mouse	58-0452-82	Invitrogen
CD45RA (B220)	BUV805	14.8	Mouse	741940	BD Biosciences
CD49b (pan NK cells)	FITC	DX5	Mouse	108905	Biolegend
CD62L	BUV395	MEL-14	Mouse	740218	BD Biosciences
CD64	Alexa Fluor 647	X54-5/7.1	Mouse	558532	BD Biosciences
CD69	BUV563	H1.2F3	Mouse	741234	BD Biosciences
CD70	BUV661	FR70	Mouse	741564	BD Biosciences
CD80	PE Dazzle	16-10A1	Mouse	104738	Biolegend
CD86	BV650	GL-1	Mouse	105036	Biolegend
CD86	BV711	GL1	Mouse	740688	BD Biosciences
CD8a	APC/Fire 750	53-6.7	Mouse	100766	Biolegend
CD8a	BUV563	53-6.7	Mouse	748535	BD Biosciences
CD8b	BV480	H35-17.2	Mouse	746835	BD Biosciences
CX3CR1	APC	SA011F11	Mouse	149008	Biolegend
DG-TCR	PE-CF594	GL3	Mouse	563532	BD Biosciences
F4/80	BUV737	T45-2342	Mouse	749283	BD Biosciences
F4/80	PE Dazzle	BM8	Mouse	123145	Biolegend
F4/80	PerCP-Cy5.5	BM8	Mouse	123128	Biolegend
FOXP3	PacBlu	MF-14	Mouse	126410	Biolegend
Granzyme B	PacBlu	GB11	Mouse	515408	Biolegend
IFN-y	BV650	XMG1.2	Mouse	505832	Biolegend
IFN-y	PE	XMG1.2	Mouse	554412	BD Biosciences
IL-10	PerCP-Cy5.5	JES5-16E3	Mouse	505028	Biolegend
IL-10	BV421	JES5-16E3	Mouse	505022	Biolegend
IL-10	PE-Cy7	JES5-16E3	Mouse	505026	Biolegend
IL-12 (p40/p70)	V450	C15.6	Mouse	561456	BD Biosciences
IL-12/IL-23 P40	PE-Cy7	C15.6	Mouse	505209	Biolegend
IL-4	PE	11B11	Mouse	504104	Biolegend
IL-6	APC	MP5-20F3	Mouse	504508	Biolegend
IRF4	AF647	IRF4.3E4	Mouse, Human	646407	Biolegend
IRF8	eFluour 450	V3GYWCH	Mouse	48-9852-82	Invitrogen
KLRG1	BUV737	2F1	Mouse	741812	BD Biosciences
Ly-6A/E (Sca-1)	BUV563	D7	Mouse	741222	BD Biosciences

Ly-6C	PE	HK1.4	Mouse	128007	Biolegend
Ly-6C	APC/Fire 750	HK1.4	Mouse	128046	Biolegend
Ly-6G	APC	1A8	Mouse	127614	Biolegend
Ly-6G	FITC	1A8	Mouse	127606	Biolegend
Ly-6G	BUV661	1A8	Mouse	741587	BD Biosciences
Ly-6G/Ly-6C (Gr-1)	PerCP-Cy5.5	RB6-8C5	Mouse	108428	Biolegend
Ly6C	PerCP-Cy5.5	HK1.4	Mouse	128011	Biolegend
MERTK (Mer)	PE	2B10C42	Mouse	747898	BD Biosciences
MHC-II (I-A/I-E)	BV510	M5/114.15.2	Mouse	107636	Biolegend
MHCII (I-A/I-E)	Alexa Fluor 700	M5/114.15.2	Mouse	107622	Biolegend
MHCII (I-A/I-E)	BV605	M5/114.15.2	Mouse	107639	Biolegend
NK1.1	BV421	PK136	Mouse	108741	Biolegend
NKG2D	BV421	CX5	Mouse	562800	BD Biosciences
PU.1	Alexa Fluor 647	7C2C34	Mouse	681304	Biolegend
RORyt	PE	AFKJS-9	Mouse	12-6988-82	Invitrogen
Siglec-H	BV711	440c	Mouse	747671	BD Biosciences
STAT3	PE-Cy7	4G4B45	Mouse	678010	Biolegend
TGF-b1	PerCP-Cy5.5	TW7-16B4	Mouse	141410	Biolegend
T-bet	PE-Cy7	4B10	Mouse	25-5825-82	Invitrogen
TNF-a	PE	MP6-XT22	Mouse	506306	Biolegend
TNFa	BV605	MP6-XT22	Mouse	506329	Biolegend
XCR1	APC-Cy7	ZET	Mouse	148224	Biolegend
XCR1	BV785	ZET	Mouse, Rat	148225	Biolegend
FC Block (CD16/32)		93	Mouse	101302	Biolegend
CD326 (Ep-CAM)	PerCP-Cy5.5	G8.8	Mouse	118220	Biolegend
TdTomato	CF568		Human, Mouse, Rat	20477	Biotium

Mixed Bone Marrow Chimera

Bone marrow cells were prepared from femurs and tibias of TLR5 KO (CD45.2) and wild-type (CD45.1) donor mice. Recipient wild-type (CD45.1) mice were irradiated (2 consecutive days x 600 rads/day) and retro-orbitally injected with an equal 1:1 mix of donor bone marrow cells (TLR5 KO:wild-type). Tumors were initiated 10-weeks post-bone marrow reconstitution via IP injection of 2e6 ID8-*Defb29/Vegf-A* cells and treated with anti-PD-L1 five days post-tumor initiation. Tissue samples were collected 15 days after tumor initiation and analyzed by flow cytometry.

Recombinant Culture of BMDCs in FLT3L

Bone marrow cells were collected from femurs and tibias of TLR5 KO and wild-type mice and cultured with 400ng/ml of FLT3L (BioXCell, cat# BE0342) in 1ml of RPMIc in a 24-well plate (Thermo Scientific™ BioLite™ Microwell Plates, cat #01-549-765) at 1e6 cells per well on day 1 (chronic) or day 7 (acute) 10ng/ml of ultra-purified Flagellin derived from *S. typhimurium* or *B .*

subtilis (InvivoGen, cat# tlrl-epstfla-5 or tlrl-pbsfla) and incubated at 37°C in a humidified incubator containing 5% CO_2. Additional RPMIc + FLT3L with or without flagellin was added at 1ml on day four. Cells were collected, stained, and analyzed by flow cytometry on day 8 of culture or by IL-6 ELISA (Biolegend, cat# 431316). To examine cross-presentation, SIINFEKL peptide (OVA 257-264, Invivogen, cat# vac-sin) was added on day 7 to cultures, followed by adding 50,000 CellTrace™ Blue (ThermoFisher, cat# C34568) labeled OT-1 CD8 T cells on day 8. CD8 T cells were isolated by magnetic bead separation using MojoSort™ Mouse CD8 T Cell Isolation Kit (cat# 480035) from spleen and lymph nodes of OT-1 mice. After three days of culture, proliferation index was measured by dilution of CellTrace™ Blue in CD8 T cells by flow cytometry using FlowJo proliferation analysis tools.

CITE-seq

Bone marrow cells were collected from femurs and tibias a wild-type mouse and cultured with 400ng/ml of FLT3L (BioXCell, cat# BE0342) in 3ml of RPMIc in a 6-well plate (Thermo Scientific™ BioLite™ Microwell Plates, cat #12-556-004) at 3e6 cells per well on day one 10ng/ml of ultra-purified Flagellin derived from *S. typhimurium* (InvivoGen, cat# tlrl-epstfla-5) and incubated at 37°C in a humidified incubator containing 5% CO_2. Additional RPMIc + FLT3L with or without flagellin was added at 3ml on day four. Samples were collected and stained with TotalSeq™-B Mouse Myeloid Cocktail, V1.0 (Biolegend, cat# 199904) separately. RNA library preparation and sequencing was performed by the UVA Genome Analysis and Technology Core using a 10X Chromium X. The UVA bioinformatics core used Cell Ranger for demultiplexing. All cells with transcriptomes were retrieved and performed low viability (mitochondrial gene content > 10%), low staining quality, doublet, and negative HTO removal was performed. Bioturing BrowserX was utilized to identify differentially expressed genes and gene ontology analysis. Collection of gene sets for ontology analysis: https://www.gsea-msigdb.org, M5 ontology gene set from GSEA database was used.

SNP genotyping and Phenotyping of PBMCs

Frozen PBMCs were thawed, and genomic DNA was extracted and purified using PureLink Genomic DNA Purification Kit (cat# K1820-01). Genomic DNA was quantified using a NanoDrop spectrophotometry. TaqMan SNP Genotyping assay (Assay ID TLR5 C__25608804_10 A/G) was implemented using a wet DNA method. QuantStudio 6 Flex Real-Time PCR system was utilized to read plates and QuantStudio Real-Time PCR software v1.3 was used to generate allelic discrimination plots. For phenotypic validation, 25,000 PBMCs were counted and rested for 18 hours in RPMIc media, and then cultured for 18 hours with purified flagellin *S. typhimurium* (InvivoGen, cat# tlrl-epstfla-5) and measured IL-6/IL-8 release by ELISA (ELISA MAX™ Deluxe Set Human IL-6, cat# 430504 and ELISA MAX™ Deluxe Set Human IL-8, cat# 431504).

Patient Survival Assessment

Survival probability extracted from data from CBioPortal in collaboration with Aster Insights (formerly M2GEN) using ORIEN avatar.

Statistical analysis

Differences between the means of experimental groups were analyzed using the Mann-Whitney test. Mouse survival data were analyzed with the log-rank test. Data was analyzed using GraphPad Prism software (version 10.1.2; GraphPad). A P value less than 0.05 was considered significant. Graphics made using Biorender.

Reagent or Resource	Vendor	Identifier
InVivoMAb Anti-mouse PD-L1 (B7-H1)	BioXCell	Cat# BE0361
InVivoMAb Anti-mouse CTLA-4	BioXCell	Cat# BE0131
InVivoMAb Anti-mouse CD8a	BioXCell	Cat# BE0061
InVivoMAb rat IgG2b isotype control	BioXCell	Cat# BE0090
Mouse Anti-mTLR5 Neutralizing mAb	InvivoGen	mabg-mtlr5
Rat IgG2a isotype control antibody	InvivoGen	mabg2a-ctlrt
InVivoMAb recombinant Flt-3L-Ig	BioXCell	Cat# BE0098
Standard flagellin from S. typhimurium	InvivoGen	Cat# tlrl-stfla
Ultrapure flagellin from S. typhimurium	InvivoGen	Cat# tlrl-epstfla-5

Ultrapure flagellin from B . subtilis	InvivoGen	Cat# tlrl-pbsfla
OVA 257-264 (SIINFEKL)	InvivoGen	Cat# vac-sin
Primers		
KRAS Forward	CCA TGG CTT GAG TAA GTC TGC	IDT
KRAS Reverse	CGC AGA CTG TAG AGC AGC G	IDT
TLR5 fl Forward	CAG GTC TGG AAT GGG TGA AC	IDT
TLR5 fl Reverse	GTG GAA CAG AAA TGC CCA GT	IDT
CD11c Cre Forward	ACT TGG CAG CTG TCT CCA AG	IDT
CD11c Cre Reverse	GCG AAC ATC TTC AGG TTC TG	IDT
Experimental models: Mice		
C57BL/6	Wild-type	Jackson Laboratory
B6.129S1-Tlr5tm1Flv/J	TLR5 KO	Jackson Laboratory
B6(Cg)-Tlr4tm1.2Karp/J	TLR4 KO	Jackson Laboratory
B6(Cg)-Tlr5tm1.1Gewr/J	TLR5 flox/flox	Jackson Laboratory
B6.Cg-Tg(Itgax-cre)1-1Reiz/J	CD11c-Cre	Jackson Laboratory
B6(FVB)-Tlr5tm1.1Gbrt/J	TLR5Td tomato mice	Jackson Laboratory
B6.SJL-Ptprca Pepcb/BoyJ	CD45.1	Jackson Laboratory
C57BL/6-Tg(TcraTcrb)1100Mjb/J	OT-1	Jackson Laboratory
Cell Lines		
ID8-*Defb29/Vegf-A*	Ovarian Epithelial	
UPK10	Ovarian Epithelial	
PPNM	Ovarian Epithelial	
Software		
Prism 10	GraphPad	
FlowJo 10	FlowJo	
Excel	Microsoft	
SpectroFlo	Cytek	

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