Therapeutic Vascular and Immune Normalization in the Melanoma Microenvironment Using STING Agonists

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$CD8^+$ T-cells are indispensable for immune-mediated rejection of solid cancers. Hence, the conditional enhancement of intratumoral T-cell content and/or function defines a preferred outcome for successful immunotherapies. Activated anti-tumor $CD8^+$ T-cells rely on functional blood vessels for their efficient trafficking to, and extravasation into, the tumor parenchyma. Indeed, pathologic progression of solid tumors is closely associated with the development of structurally and functionally abnormal tumor blood vessels which impede T-cell infiltration into cancer lesions. In this regard, therapeutic dosing of anti-angiogenic interventional strategies fortifies or reprograms tumor blood vessels (or vascular normalization) to significantly improve intratumoral $CD8^+$ T-cell infiltration. Intriguingly, agonists of Stimulator of Interferon Genes (STING), which evolved from a class of anti-angiogenic agents, have recently demonstrated significant clinical promise for their ability to enhance $CD8^+$ T-cell recruitment into tumors but whether therapeutic changes to the tumor vasculature underlies successful immune-mediated tumor control remain only partially resolved. Indeed, in this thesis, I demonstrate that intratumoral administration of STING agonist ADU S-100 induces vascular normalization (i.e., improved vascular perfusion, enhanced pericyte coverage and increased endothelial activation) and enhances tumor infiltration by immune cells, specifically, $CD8^+$ T-cells and $CD11c^+$ dendritic cells (DC).

STING activation also increases local production of pro-inflammatory cytokines/chemokines that sponsor the development of high endothelial venules (HEV) and HEV-associated tertiary lymphoid structures (TLS) within the therapeutic melanoma tumor microenvironment (TME). HEV/TLS formation with STING agonism was further linked to evidence of local T-cell cross-priming by tumor-resident antigen presenting cells (APC) within the tumor microenvironment (TME), with the therapeutic tumor infiltrating lymphocyte (TIL) repertoire exhibiting enrichment in T cell clonotypes found in the periphery as well as those detected only within the TME. These vasculature-centric underpinnings for the efficacy of STING agonist-based interventions provide enthusiasm for improved translational value of future combinational cancer immunotherapies that seek to integrate these agents.

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List of Abbreviations

1. ACT – Adoptive T-Cell Transfer
2. ADCC – Antibody Dependent Cellular Cytotoxicity
3. AE – Adverse Events
4. ALM – Acral Lentiginous Melanoma
5. AMP – Adenosine Monophosphate
6. ANOVA – Analysis of Variance
7. APC – Antigen Presenting Cell
8. ARG2 – Arginase 2
9. BCR – B-Cell Receptor
10. BID - BH3 interacting-domain death agonist
11. BLC – B-Lymphocyte Chemoattractant
12. BMDC – Bone Marrow derived Dendritic Cell
13. CAR – Chimeric Antigen Receptor
14. CCL19 – Chemokine Ligand 19
15. CCL21 – Chemokine Ligand 21
16. CD – Cluster of Differentiation
17. CDN – Cyclic Dinucleotide
18. cDNA – Complementary DNA
19. CDR3 – Complementarity Determining Region 3
20. CLEVER - Common Lymphatic Endothelial and Vascular Endothelial Receptor-1
21. CM – Cutaneous Melanoma
22. CMA – 10-carboxymethyl-9-acridanone
23. COX-2 – Cycloxygenase-2
24. CR – Complete Response
25. CT – Cancer Testis
26. ctDNA – Circulating Tumor DNA
27. CTLA4 - Cytotoxic T-Lymphocyte-Associated protein 4
28. CTT – C-Terminal Tail
29. CXCL10 – C-X-C motif Ligand 10
30. CXCL11 – C-X-C motif Ligand 11
31. CXCL13 – C-X-C motif Ligand 13
32. DC – Dendritic Cell
33. DEG – Differential Gene Expression
34. DLK1 – Delta Like 1 homolog
35. DLK2 – Delta Like 2 homolog
36. DM – Desmoplastic Melanoma
37. DMXAA – 5,6-dimethylxanthenone-4-acetic acid
38. DNA – Deoxyribonucleic acid
39. EBV – Epstein Barr Virus
40. EC – Endothelial Cell

41. EphA2 – Ephrin type A receptor 2
42. ETBR – Endothelin Beta Receptor
43. FDA – Food and Drug Administration
44. FDC – Follicular Dendritic Cell
45. GBM - Glioblastoma
46. GC – Germinal Center
47. gDNA – genomic DNA
48. GMP – Guanosine Monophosphate
49. GO – Gene Ontology
50. GSEA – Gene Set Enrichment Analysis
51. GZMB – Granzyme B
52. HBB – Hemoglobin subunit Beta
53. HEV – High Endothelial Venule
54. HNSCC – Head and Neck Squamous Cell Carcinoma
55. HPV – Human Papilloma Virus
56. HVEM – Herpes Virus Entry Mediator
57. i.p. - intraperitoneal
58. i.t. - intratumoral
59. IAV – Influenza A Virus
60. ICAM-1 – Intercellular Adhesion Molecule 1
61. ICB – Immune Checkpoint Blockade
62. IDO – Indoleamine-2,3-Dioxygenase
63. IFM – Immuno-Fluorescence Microscopy
64. IFN - Interferon
65. IFNAR – Interferon Alpha Receptor
66. IFP – Interstitial Fluid Pressure
67. IL - Interleukin
68. IPA – Ingenuity Pathway Analysis
69. IRF3 – Interferon Regulatory Factor 3
70. JNK – c-JUN N-terminal Kinase
71. LBD – Ligand Binding Domain
72. LEC – Lymphatic Endothelial Cell
73. LFA-1 - Lymphocyte function-associated antigen 1
74. LIGHT - homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with HSV **g**lycoprotein D for binding to **h**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes
75. LLC – Lewis Lung Carcinoma
76. LMM – Lentigo Maligna Melanoma
77. LN – Lymph Node
78. LTα – Lymphotoxin Alpha
79. LTβ – Lymphotoxin Beta
80. MAGE-A1 - Melanoma-associated antigen 1
81. MAPK – Mitogen Activated Protein Kinase

82. MC1R – Melanocortin 1 Receptor
83. MCT – Metronomic Chemotherapy
84. MDR – Multi Drug Resistance
85. MDSC – Myeloid Derived Suppressor Cell
86. MHC – Major Histocompatibility Complex
87. MM – Malignant Melanoma
88. MSH – Melanocyte-Stimulating Hormone
89. MTD – Maximum Tolerable Dose
90. NK – Natural Killer cell
91. NM – Nodular Melanoma
92. NMSC – Non melanoma skin cancer
93. NSCLC – Non-Small Cell Lung Cancer
94. NY-ESO – New York Esophageal Squamous Cell Carcinoma 1
95. ORR – Objective Response Rate
96. PBS – Phosphate Buffered Saline
97. PD-1 – Programmed Death-1 (Receptor)
98. PDL1 – Programmed Death Ligand 1 (Ligand)
99. PNAd – Peripheral Node Addressin
100. PR – Partial Response
101. PRR – Pattern Recognition Receptor
102. PTEN - Phosphatase and tensin homolog
103. PTGES – Prostaglandin E Synthase
104. PVDF – Polyvinylidene Fluoride
105. RECIST – Response Evaluation Criteria in Solid Tumors
106. RGS5 - Regulator of G-protein signaling 5
107. RIP – Rat Insulin Promoter
108. RNA – Ribonucleic Acid
109. RT – Room Temperature
110. s.c. - Subcutaneous
111. SDS PAGE – Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
112. SLO – Secondary Lymphoid Organ
113. SSM – Superficial Spreading Melanoma
114. STING – Stimulator of Interferon Genes
115. TBVA – Tumor Blood Vessel-associated Antigen
116. TCGA – The Cancer Genome Atlas
117. T_{CM} – T central memory cell
118. TCR – T Cell Receptor
119. T_{EM}- T effector memory cell
120. TEM1 – Tumor Endothelial Marker 1
121. T_{FH} – T-Follicular Helper cell
122. TIL – Tumor Infiltrating Lymphocytes
123. TLS – Tertiary Lymphoid Structure
124. TME – Tumor microenvironment

125. TMEM173 – Transmembrane protein 173
126. TMZ - Temozolomide
127. TNF – Tumor Necrosis Factor
128. TNFR – Tumor Necrosis Factor Receptor
129. TNFSF14 – Tumor Necrosis Factor Super Family 14
130. TNFSF15 – Tumor Necrosis Factor Super Family 15
131. UV - Ultraviolet
132. VCAM-1 – Vascular Cell Adhesion Molecule 1
133. VEC – Vascular Endothelial Cell
134. VEGF – Vascular Endothelial Growth Factor
135. VLA-4 – Very Late Antigen 4
136. VN – Vascular Normalization
137. VTP – Vascular Targeting Peptide
138. WT – Wild-Type

1.0 Introduction

1.1 Epidemiology of melanoma

With an incidence of ~1,000,000 new cases every year, skin cancer is the most common solid malignancy in the western hemisphere and accounts for roughly one-third of all cancer diagnoses in the United States [1]. Skin cancer can be broadly categorized into non-melanoma skin cancer (NMSC) and malignant melanoma (MM) which impact different cell types in the skin [2].

MM affects the pigment producing melanocytes residing at the interface of the epidermal-dermal junctional layer of the skin. It is the rarest amongst all forms of skin cancer with an incidence of ~160,000 new cases every year, with primary risk factors for MM including age, degree of UV exposure and the sex of a patient [3-5]. Despite a low rate of incidence compared to other forms of solid cancer, MM is a major health concern due to its aggressive nature, which gives rise to metastatic lesions in distal (and frequently surgically inaccessible) anatomic sites. MM incidence rates have sharply increased over the past few decades, with now roughly 1 in 50 Caucasian adults at significant risk of developing cutaneous malignant melanoma [6 7]. Incidence rates for MM also increase with age [8]. The following sections detail the most common risk factors for developing metastatic melanoma.

1.1.1 Risk factors

1.1.1.1 Extrinsic risk factor: ultraviolet radiation

UV radiation comprises low-wavelength, high-energy emissions from the sun which readily penetrate human skin. Short periods of exposure to solar UV radiation improves dermal biosynthesis of vitamin D necessary for physiological well-being and the production of eumelanin by melanocytes through the MSH-MC1R signaling axis, which serves to acutely shield the skin

from UV [9,10]. However, prolonged UV exposure can have deleterious genotoxic effects in exposed skin cells in support of neoplastic transformation, thus making UV a potent carcinogen at high exposures [10]. Consequently, keratinocytes and melanocytes have evolved to respond to genotoxic UV exposure by changing their cellular and physiological properties to limit further cellular/tissue damage.

Keratinocytes demonstrate a remarkably low tolerance for UV radiation and undergo rapid DNA repair and/or apoptosis upon chronic UV exposure. During this repair process, keratinocytes promote melanocytic production of eumelanin (black or brown pigment) which they readily internalize to shield against UV-induced genotoxicity [10]. In the absence of such keratinocyte-melanocyte interactions, melanocytes primarily produce pheomelanin (red pigment) which when found in large quantities may independently drive melanomagenesis by inducing ROS-dependent DNA damage in melanocytes [11,12].

On the other hand, melanocytes demonstrate a distinct ability to withstand extreme doses of UV radiation, but respond uniquely by accumulating C -> T transitional mutations, which promotes rapid cell proliferation [10,13,14]. This specialized resistance of melanocytes to UV exposure underlies the large mutational burden observed in clinical specimens of cutaneous malignant melanoma (CMM), where driver mutations in RAC1, STK19 and PPP6C in human CMM have been linked to UV-induced C->T transitional mutations [15]. Additionally, UV-A/B radiation also promotes melanocytic proliferation by accelerating cell-cycle signaling and by amplifying JNK signaling cascades [16,17].

Thus, exposure to UV radiation is considered a key melanoma risk factor due to its ability to initiate and drive oncogenic pathways in several skin-resident cell types.

1.1.1.2 Host risk factors

1.1.1.2.1 Nevi

A melanocytic nevus is a benign neoplasia consisting of melanocytes and nevus cells that together produce melanin in localized lesions [4]. While benign nevi appear early in the first two decades of life and stabilize or regress with age, cancerous melanocytic nevi appear later in life (after 40 years of age) and increase in prevalence with advanced age [18-21]. Cancerous nevi are typically 2-5 mm in diameter and fall into one of three categories depending on the location of the melanocytic nests within the skin: junctional nevi, intradermal nevi and compound nevi [22]. Regardless of their type, the presence of nevi predisposes a patient to higher melanoma risk by serving as hotbeds for melanocytic maturation and proliferation [22]. Mutations in candidate genes within the RAS, RAF and MEK/ERK family (Nras, Braf, Craf, MAPK etc.,) are strongly linked to the development of cancerous nevi and indeed play a major role in cancer progression beyond nevus formation [22-24].

In this regard, ~25%-33% of melanoma lesions observed in patients evolve from pre-existing melanocytic nevi [19], with incidence increasing to ~50% in patients with high nevus counts (>100) [21]. Therefore, genetic, histologic, and pathologic evidence suggests that, when present, nevi represent a significant risk factor for the development of melanoma.

1.1.1.2.2 Genetic mutations

1.1.1.2.2.1 CDKN2A

Cell cycle progression determines the kinetics and frequency of cell division and is therefore a strictly regulated process in normal non-malignant cells. Several germ-line encoded proteins such as cyclins, cyclin-dependent kinases and inhibitors of cyclin-dependent kinases orchestrate cell

proliferation and represent hotspots for mutagenesis in pre-malignant cells [25]. Indeed, mutations in one such cell cycle gene, Cdkn2a, have been found to be associated with ~20% of families with a history of melanoma [26].

Cdkn2a is a germ-line encoded tumor suppressor gene that is subject to alternative splicing to produce two tumor suppressor proteins; CDKN2A (p16INK4A) encoded by the alpha-variant and p14ARF encoded by the beta-variant [27] [28]. CDKN2A functions as an inhibitor of cell cycle progression by regulating CDK4/6-mediated activation of the retinoblastoma (Rb)-E2F axis by p16INK4A and by activating the tumor suppressor p53 via p14ARF [28]. Accordingly, missense and/or nonsense germline mutations in CDKN2A disrupt cell cycle progression and promote tumorigenesis. Specifically, p16INK4A mutations negatively affect binding and inhibition of CDK4/6, which leads to hyperphosphorylation of Rb and a loss of sequestration of the transcription factor E2F, which ultimately promotes G1-S transition in tumor cells [29] [30]. Additionally, p14 mutations prevent its antagonism of the p53-specific ubiquitin ligase, HDM2 which leads to the rapid degradation of the tumor suppressor p53 and the corresponding loss of G2 checkpoints [31].

1.1.1.2.2.2 BRAF

BRAF is an intermediary kinase within the MAPK (MEK-ERK) signaling cascade that initiates cellular proliferation in response to RAS activating growth factors. BRAF contains three functional domains, namely CR1-3, where CR1 (aa 120-280) detects activated RAS, CR3 activates the BRAF kinase (aa 457-717) while CR2 serves as a hinge between the two functional domains [32]. In addition to detecting RAS activation, CR1 also functions as an autoinhibitory domain for CR3 to prevent aberrant activation of the MAPK cascade [32].

Since BRAF functions as an amplifier of RAS dependent cell proliferation, mutations in any of the domains may acquire driver function in melanomagenesis. Indeed, BRAF is commonly

mutated in melanoma where ~60% of lesions harbor at least one of ~200 different mutations annotated [33]. Specifically, class I BRAF mutations allow monomeric BRAF to activate MAPK signals independently from RAS activation and therefore function as the most potent oncogenic mutations. Expectedly, ~80% of all malignant melanoma lesions harbor the class I BRAF V600E mutation [33]. BRAF may also drive melanomagenesis through fusion events where BRAF C-terminal kinase domains couple with N-terminal domains from other oncogenes such as SOX10, AGK and SEPT3 to further promote MAPK signals [32].

1.1.1.2.2.3 PTEN

PTEN is a tumor suppressor gene with functional lipid phosphatase and protein phosphatase activity. PTEN reduces intracellular PIP_3 levels through its lipid phosphatase activity and downregulates Akt/MAPK signaling to limit G1/S transition of the cell cycle [34]. When active, PTEN also modulates apoptosis by upregulating pro-apoptotic protein BID and downregulating anti-apoptotic Bcl2 to conditionally select against pre-malignant cells [34]. Consequently, loss-of-function PTEN mutations could lead to tumorigenesis, and these are indeed frequently observed in melanoma patients [34]. Given their functional convergence at the level of Akt/MAPK, concurrent gain of function BRAF mutations with loss of function PTEN mutations occur in ~20% of patients with malignant melanoma [35].

1.1.2 Types of Melanoma

1.1.2.1 Superficial Spreading Melanoma (SSM)

SSM is the most common form of melanoma accounting for ~70% of all clinically diagnosed cases and is primarily found in intermittently exposed surfaces of skin i.e., on the backs of men and on the legs of women. Sharply outlined SSM lesions acquire a range of colors from black to

bluish hues and typically appear darker than surrounding normal tissue. SSM lesions are also palpable and develop into nodules that protrude several millimeters above the surface of the skin [8 36 37].

1.1.2.2 Nodular Melanoma (NM)

NM accounts for ~5% of all melanoma cases and is also associated with intermittent exposure to sun. NM lesions appears in the trunk region and limbs and demonstrate an increased incidence in older males aged between 50-70 years. NM is characterized by a nodular growth pattern wherein nests of melanocytes grow in individual nodules before combining into a larger palpable nodule that often ulcerates. Further, dermal invasion is common with NM and often precedes metastatic growth [8 36 37].

1.1.2.3 Lentigo Maligna Melanoma (LMM)

LMM accounts for 4-15% of melanoma cases diagnosed and it primarily appears in dermal regions chronically exposed to sun and UV, such as the head and neck region. LMM is characterized by the proliferation of basal melanocytes and appears as a relatively flat lesion on the skin with only rare protruding growth [8 36 37].

1.1.2.4 Acral Lentiginous Melanoma (ALM)

ALM accounts for ~5% of melanoma cases and primarily localizes to skin of the extremities and digits such as in the palmoplantar and subungual regions. It occurs more commonly in non-Caucasian populations with a selectively higher incidence in older females in Asian, African American and Hispanic populations [8 36 37].

1.1.2.5 Desmoplastic Melanoma (DM)

DM accounts for ~2% of melanoma cases and results from chronic exposure to UV radiation and is most found in the head and neck region. DM incidence increases with age and is most commonly found in males > 60 years of age. DM lesions appear flush and are characterized by S100+ melanocytic composition and infiltration into perineural spaces within the skin [8 36 37].

Other rare forms such as balloon cell melanoma, myxoid melanoma, osteogenic melanoma and rhabdoid melanoma are only rarely diagnosed [8].

1.2 Current treatment options for patients with melanoma

Melanoma poses a significant treatment challenge given its aggressive nature and high metastatic potential. While early lesions may be successfully resected via simple surgery, treatment of late-stage metastatic disease requires the use of noninvasive systemic drugs that are designed to access disseminated, and frequently, surgically inaccessible lesions [38]. In this regard, therapeutic regimens employing neoadjuvant approaches have recently shown great promise. The following section highlights several preferred systemic treatment options for patients with melanoma.

1.2.1 Chemotherapy

1.2.1.1 Dacarbazine

Dacarbazine/DTIC/DTIC-Dome is a DNA alkylating agent that transfers methyl groups to guanosine bases of cellular DNA [39]. By inducing extensive DNA methylation, DTIC alters the spatial configuration of the DNA helix and makes genomic DNA less conducive to DNA replication, ultimately leading to cellular apoptosis [40-42]. Therefore, DTIC serves as a cytostatic and cytotoxic drug used in the control of cancer. While all nucleated cells are susceptible to DTIC,

cancer cells predictably demonstrate a heightened sensitivity due to their increased proliferative capacity. However, despite its great promise, one major limitation of DTIC-based therapy lies in its lack of tumor targeting potential which often gives rise to severe (off target) adverse events (AE) in patients receiving treatment [43]. While one class of AEs with DTIC leads to excessive gastrointestinal irritation, another class directly affects the immune system and leads to the onset of refractory cytopenia, lymphocytic anemia and neutropenia [43]. However, since being approved for clinical use by the FDA in 1975, and despite only demonstrating nominal objective response rates (15.3% with 11.2% partial response (PR) and 4.2% complete response (CR); RECIST) [38 44] , DTIC is a key chemotherapeutic agent for patients with melanoma. More recently, DTIC has been evaluated in combination regimens such as in the 'Dartmouth regimen' (Cisplatin, Dacarbazine, Tamoxifen and Carmustine), with these complex regimens demonstrating promising yet only modest improvements in tumor response rate (proportion of CR or PR; RECIST) over DTIC monotherapy (18.5% in the Dartmouth regimen arm vs 10.2% in the Dacarbazine arm) [45 46]. Furthermore, Temozolomide (MTIC, TMZ), an oral pro-drug version of the active metabolite of DTIC has also been tested in single-agent or combination regimens, however, these approaches provide no significant benefit over that provided by treatment with DTIC alone (median survival time of 7.7 months in the TMZ monotherapy arm vs 6.4 months in the DTIC monotherapy arm) [47 48]. Hence, while DTIC remains the chemotherapeutic drug of choice in the melanoma setting, modest clinical responses and the likelihood of severe AEs on-therapy highlights the need to develop newer and better tolerated chemical agents.

Overall, chemotherapies remain attractive since they are comparatively inexpensive, effectively scalable, and easily stored and distributed. However, tumors notoriously develop resistance mechanisms to limit the access and persistence of such chemotherapeutic agents in the TME.

Notably, melanoma develops multidrug resistance (MDR), which through a variety of mechanisms [49], enables rapid efflux and clearance of agents such as DTIC from tumor cells [50 51]. MDR, together with the selective pressure imposed by chemotherapies such as DTIC, therefore also favors the selection of stressor resistant tumor cells requiring the development and implementation of effective salvage therapies.

1.2.2 Immunotherapy

1.2.2.1 Cytokine therapy

Interferons are a class of cytokines that orchestrate protective immune responses under conditions of physiological stress. Type I interferons (includes IFNα and IFNβ), are produced when innate pattern recognition receptors (PRR) are activated in stressed cells, where they mediate pro-inflammatory autocrine [52], paracrine and endocrine [53] cell/tissue defense mechanisms [54]. In cancer, interstitial DNA or ctDNA from dead or dying tumor cells may also activate innate PRRs such as DNA-dependent activator of IRFs (DAI) [55], STING [56 57] and toll-like receptors (TLR) [58] to induce strong local production of IFN-I in association with enhanced intratumoral infiltration of diverse anti-tumor immune cells [55] and the processing of tumor antigens by $CD8a^{+}$ cross-presenting DCs [59].

Studies have shown that within the TME, DCs represent an indispensable source of IFN-I required for rejection of murine tumors, with therapy benefits linked to the direct activation of immune cells [55] and the upregulation of MHC-I antigen-presentation machinery in tumor cells for improved T-cell mediated immunosurveillance of cancer [60]. These pleiotropic benefits have justified the use of IFN-I as a neoadjuvant in the treatment of melanoma, and since its approval for clinical use in 1995, i.v. treatment with bolus high-dose interferon alpha 2b for advanced stage melanoma has demonstrated promising response rates in multiple clinical trials (ORR ~22%) [61].

More recently, rhIFN-α administered in a pegylated form (Peg-IFN-α-2b) has demonstrated enhanced tolerability and retention in patients [62]. However, IFNα2b and Peg-IFN treatment often leads to immune-related adverse events (irAEs) including fever, chills, nausea, autoimmune conditions and ulceration of primary cutaneous lesions which negatively impact quality of life in patients on-treatment [38]. Nevertheless, the robust inflammation achieved with IFN-I-based therapy still makes it an attractive adjuvant therapy component despite significant irAEs observed on-treatment.

1.2.2.2 Cellular therapy

1.2.2.2.1 DC Vaccines

DCs are found in high numbers throughout different (barrier) tissue sites in the body, where they are believed to primarily serve as activators of antigen-specific T-cells in lymph nodes [63 64]. Amongst a variety of DC subtypes characterized to date, conventional $CD11c^+$ DCs (murine) are considered indispensable for the activation of T-cells [65]. Under homeostatic conditions, peripheral $CD11c^+$ DCs exist in an immature state wherein they specialize in sensing extra-organismal presence through phagocytosis or trogocytosis [66]. However, upon successful detection of PAMPs or DAMPs through PRRs, immature DCs undergo rapid maturation to specialize in antigen presentation [67 68]. Correspondingly, biomarkers of DC maturation include increased surface expression of phagocytosed antigens in MHC complexes, enhanced expression of co-stimulatory molecules, such as CD80, CD86 and CD40 and improved secretion of cytokines for the functional skewing of cognate T-cell differentiation [69]. These functional and molecular programs together make (mature) DCs excellent cellular adjuvants in promoting targeted T-cell responses against tumor-associated antigens [68]. Indeed, DC precursors can be isolated from peripheral blood, differentiated and matured into type 1 DCs (producing IL-12, IL-15, IFN-I and IL-23) and loaded

with autologous tumor lysates or tumor antigen-derived peptides competent to bind to MHC-I molecules in vitro for clinical use as personalized cancer vaccines [70] [71]. A variety of human melanoma studies have used such DC vaccines loaded (peptide pulsed or adenovirally transduced) with autologous tumor or known shared melanoma antigens (MART1, MAGE-A1, MAGE-A3, MAGE-A6, gp100, tyrosinase etc.,) to successfully prime melanoma antigen specific $CD8^+$ and $CD4^+$ T-cells [70-73]. Despite holding significant conceptual promise, a meta-analysis of thirty eight independent DC vaccine trials (1996-2007) treating advanced melanoma patients revealed only modest clinical benefit (9% CR or PR; RECIST) with treatment [74] [75] [76].

1.2.2.2.2 Adoptive cell therapy (ACT)

Activated cytotoxic T-cells may also be used directly as cellular agents to control cancer growth. In a number of clinical trials, robust intratumoral T-cell infiltration correlates with objective clinical response, suggesting that the adoptive transfer of autologous, ex-vivo primed anti-tumor CTLs may represent an effective therapeutic strategy to supplement endogenously generated anti-tumor CTLs in patients [38] [77] [78]. Early efforts using ACT-based interventional approaches yielded encouraging success against EBV^+ nasopharyngeal carcinoma, where the administration of autologous, ex-vivo primed CTLs reactive against EBV antigens induced durable (>2 years) disease remission in patients with early-stage posttranslational lymphoproliferative disease [79] [80]. Subsequent advances in computational biology have allowed for the identification of shared and unique tumor-associated antigens (TAA), which has furthered the development of ACT approaches for solid cancer [81] [82]. Melanoma especially represents a model cancer type for the study and implementation of ACT regimens, given its high mutational burden leading to the accrual of immunogenic TAAs (differentiation antigens and overexpressed antigens) or aberrant expression of typically oncofetal cancer-testis (CT) antigens [78] [83]. Indeed, adoptive transfer of CTL reactive

against CT antigens such as NY-ESO-1[84] or melanocyte-differentiation antigens including MART1/MelanA [85] and gp100 [86] have all shown promising clinical response rates (~35%, RECIST), leading to better overall survival and relapse free survival (53.5 months in $MART1^+$ ACT cohort vs 3.5 months in $MART1^-$ ACT cohort) in treated melanoma patients [85]. The high mutational burden in melanoma also leads to the development of spontaneous point mutations which may manifest as tumor unique, lesion-specific neoantigens [87]. Unique neoantigens may function as superior therapeutic targets due to their highly-restricted expression in cancer cells which reduces concerns for off-tumor toxicities associated with adoptive T-cell therapies. Additionally, the development of such unique antigens throughout the course of disease progression suggests that neoantigens may be critical to tumor survival and malignancy and therefore may serve as an 'Achilles' heel' for targeted rejection of the tumor by the adaptive immune system [88-90]. Despite these beneficial features, the complexities associated with longitudinal profiling [91], validating and directing anti-tumor immunity against such intralesionally diverse [92 93] neoantigens expressed variably in tumor clonotypes composing heterogeneous tumor lesions, likely diminish their therapeutic utility and impact within the context of current ACT regimens.

Regardless of the type of antigen targeted, several novel strategies have also served to enhance the clinical efficacy of ACT-based treatment approaches. Notably, different patient-derived T-cell subpopulations are understood to confer different anti-tumor efficacies in ACT trials. Restifo and colleagues have elegantly demonstrated that CTLs derived and expanded from autologous central memory T-cells (T_{CM}) persisted longer in patients' peripheral circulation, migrated to SLOs more efficiently and reconstituted effector memory (T_{EM}) and T_{CM} more robustly when compared to CTLs derived and expanded from autologous T_{EM} [94]. Alternatively, other modifications to ACT

protocols such as i) immunoregulatory cell depletion using cyclophosphamide, docetaxel and/or total body irradiation to ablate Treg and MDSC populations [95] [96] and ii) administration of (IL-2R) common gamma chain cytokines rhIL-7 and rhIL-15 or bolus high-dose rhIL-2 have been shown to enhance the efficacy of combination ACT regimens [97].

1.2.2.2.3 Engineered T-cell therapies

Autologous patient derived T-cells may also be genetically modified using viral vectors to express

receptors allowing for targeted recognition of antigens (over)expressed by tumor cells, leading to improved therapeutic benefit.

1.2.2.2.3.1 Chimeric Antigen Receptor T-cells (CAR-T cells):

A key limitation associated with endogenous anti-tumor T-cell responses is that CD4+ or CD8+ T-cell recognition of tumor cells requires the appropriate presentation of tumor antigens by MHC molecules expressed on the surface of cancer cells. This requirement limits T-cell recognition of cancer to short, linear peptide epitopes that are ~8-15 amino acids in length which may be differentially processed from precursor tumor antigens across individual tumor clonotypes [98]. CAR-T cells or engineered T-cells expressing a chimeric antigen receptor (CAR) seek to address this limitation by directly recognizing the intact extracellular domains of proteins accessible on the cancer cell surface. Traditional CARs possess i) an antibody-based extracellular recognition domain that allows CAR-T-cells to detect native 3D surface proteins on cancer cells and ii) an intracellular signaling domain to promote rapid and robust T-cell activation following antigen detection. CAR-T cells may therefore be designed to reject cancer by recognizing both unmodified and post-translationally modified tumor-associated surface proteins (such as differentially glycosylated tumor proteins) [99]. CAR based T-cell technologies offer a flexible platform to expand

targetability (by altering the antibody extracellular domain) and fine-tune activity (by altering intracellular activation or costimulatory domains) of T-cells against antigenically heterogenous diseases like cancer [100]. However, since CARs possess antibody-based receptors, CAR-T cells are not effective in detecting inaccessible or intracellular tumor-associated proteins and as such, are limited to the detection of tumor-associated surface proteins [101]. Unfortunately, many cancer-associated surface proteins are also expressed in varying degrees on normal cells leading to reports of on-target off-tumor toxicities in CAR-T-cell-based therapies [102 103]. Despite such concerns for irAEs, carefully dosed CAR-T cell therapies targeting melanoma-associated proteins GD2, c-Met, VEGFR2 and CD70 are currently being tested in phase I/II trials [104].

1.2.2.2.3.2 T-Cell Receptor Transgenic T-cells (TCR-T cells):

A second class of engineered T-cells are T-cells engineered to express T-cell receptors (TCRs) reactive to MHC-associated tumor peptides. TCR-T cell technologies, unlike CAR-T-cells, continue to rely on MHC-restricted recognition of tumor antigens but seek to enhance tumor reactivity of autologous T-cell products through the selective transgenic expression of superior (high affinity) tumor reactive TCRs. To develop TCR-T cells, polyclonal autologous T-cells are first co-cultured with specific pre-determined tumor-associated antigens to identify T-cell clones with greatest reactivity against the tested tumor antigen [101]. TCRs of the most reactive T-cell clones are then sequenced to generate paired-α/β TCR libraries, cloned into viral vectors, and transduced into freshly isolated autologous T-cells to generate large numbers of highly reactive anti-tumor TCR-T-cells with known anti-tumor specificities [101]. TCR-T cells can therefore enhance tumor rejection through improved recognition (i.e., high affinity and/or avidity) of MHC-presented tumor antigen-derived peptide epitopes [105 106]. However, the requirement to first know the identity of immunogenic tumor-associated antigens in order to generate TCR libraries, as well as, clinical

instances of severe, and sometimes fatal, off-target toxicities using TCR-T regimens [106-108], continue to represent major hurdles for the clinical advancement of these T-cell modalities as a potential standard of care treatment for solid cancers. Despite these challenges, several anti-melanoma TCR-T cell products including those recognizing MART-1, gp100, NY-ESO-1 and MAGE-A3 are being evaluated in phase I/II clinical trials [101].

1.2.2.3 Immune checkpoint blockade antibodies

The immune system protects against tumorigenesis through a finely tuned series of checks and balances where inflammatory 'on' cues such as MHC-TCR, B7-CD28 and cytokine signals facilitate tumor protection mechanisms, while 'off' or tolerance programs protect against excessive autoimmune damage. In this regard, there exists two levels of immune tolerance; central tolerance mechanisms which function at the level of lymphocyte selection in primary lymphoid organs and peripheral tolerance mechanisms which function as a rheostat to prevent self-toxicity at peripheral sites of chronic inflammation. Immune cells such as Tregs, MDSCs and TAMs, and surface checkpoint proteins such as PD-L1/PD-1 and CTLA-4 represent a few key mediators involved in peripheral tolerance and unsurprisingly, these are often hijacked by the tumor to facilitate tumor progression. Each of these cells and axes therefore serve as key targets in interventional approaches to enhance the efficacy of therapeutic peripheral anti-tumor T-cell responses. Specifically, neutralizing antibodies against the immune checkpoint molecules PD-1 and CTLA4 have revolutionized cancer immunotherapy since receiving FDA approval in 2011 and these agents are briefly discussed below:

1.2.2.3.1 Anti-CTLA4

Cytotoxic T Lymphocyte Associate protein 4 (CTLA4) is an activation-induced surface molecule that mediates peripheral tolerance of T lymphocytes [109]. CTLA4 is upregulated acutely

within 2-3 days of TCR activation [110] and serves as a direct competitor for costimulatory CD28 ligands, B7-1 (CD80) and B7-2 (CD86), which are canonically expressed by mature APCs [111]. Based on a 4-fold increased affinity for B7-1/2 [112-114], CTLA4 directly outcompetes CD28 for binding to costimulatory ligands, thereby preventing proximal TCR signaling [115 116]. In this regard, CTLA4 neutralizing antibodies have been designed to enhance activation of tumor infiltrating T-cells in solid malignancies including melanoma. **Ipilimumab (Yervoy)**, the human monoclonal antibody raised against CTLA4, was approved for clinical use in 2011, with results from phase II trials in patients with advanced melanoma an overall response rate of 17% (23 of 139 patients) [117]. Notably, three patients developed complete or durable responses for up to 53 months post-treatment [117]. Furthermore, overall survival in treated patients was extended to ~16 months, however effective treatment often coincided with severe irAEs such as enterocolitis and hypophysitis [38 118].

1.2.2.3.2 Anti-PD-1

In contrast to CTLA4 which functions as a sink for costimulatory ligands, Programmed Death-1 (PD-1) is an inflammation-induced immunomodulatory receptor that is expressed on activated T-cells, serving to limit proximal TCR signals at peripheral sites of chronic inflammation [119]. PD-1 ligands, namely PD-L1 and PD-L2, are promptly upregulated on APCs, stromal cells and tumor cells in response to IFNγ [120] and these counter-regulate T-cell activation via their interaction with PD-1^+ T-cells where ligation of PD-1 subsequently recruits and activates the TCR phosphatases SHP1/2 to ablate CD3ζ-TCR signaling [121-123]. The importance of PD-1 in the maintenance of homeostasis is evidenced by the development of severe autoimmunity including SLE and cardiomyopathy in Pdcd1 allelic knockout animal models [124 125]. While acute expression of PD-1 serves as a marker of T-cell activation, the chronic expression of PD-1 is also associated with the

increased expression of T-cell exhaustion markers TIM3 and LAG3 [126]. PD-1 activation can also limit T-cell function by skewing T-cell metabolism in favor of fatty acid oxidation, which slows T-cell proliferation and lowers T_{EM} production of cytokines and tumoricidal proteins such as perforin [127 128]. Given these deleterious effects in PD-1 signaling in T-cells, **Nivolumab (Opdivo)**, a neutralizing human antibody against PD-1, was approved for clinical use in 2014 and has since shown great promise in numerous trials. Since PD-L1 is expressed at sites of inflammation, Nivolumab mediates therapy benefit by reinvigorating exhausted intratumoral T-cells, supporting the prerequisite that patients have pre-existing tumor infiltrating lymphocytes (TILs) for optimum clinical response to PD-1 blockade regimens [129 130]. Indeed, recent studies by Ribas et al. support this paradigm, since beneficial clinical outcome to Nivolumab correlates with the presence of intratumoral $CD8^+$ T-cells pre-therapy and with enhanced TCR clonality on-treatment, suggesting that Nivolumab induces the clonal expansion of pre-existing TIL within the TME [130]; an effect that has also been observed in other solid tumors such as NSCLC [131].

Together, blockade of the PD-1 axis induces therapeutic anti-tumor responses by rescuing exhausted T-cells via the restoration of proximal TCR signaling, leading to the reinvigoration and expansion of pre-existing TIL within the TME.

1.3 Challenges in current treatments for melanoma

1.3.1 The tumor microenvironment: an immune desert

Despite the availability of diverse treatment modalities, many cancer patients fail to respond to therapeutic intervention. It is therefore critical to evaluate whether the major roadblock to existing therapies can be attributed to the generally poor immunogenicity of tumors [132], to the insufficient activation of therapeutic anti-tumor T-cells [133 134] and/or to the inefficient mobilization/recruitment

of effector T-cells into the TME [135] to reformulate and improve the therapeutic design of next-generation protocols.

Early studies by Klein and colleagues shed light on this question by showing that activated anti-tumor T-cells are indeed found in the draining lymph nodes of MCA sarcoma-bearing mice shortly after tumor engraftment [89]. Interestingly, these tumor reactive T-cells do not limit the growth of the established primary tumor, but are competent to reject subsequent tumor challenges, suggesting that established tumors develop a 'barrier-like' microenvironment that limits T-cells access into the TME [89]. Boon and colleagues further show that the majority of melanoma patients indeed have detectable levels of anti-MAGE-A1 T-cell clones in their peripheral blood, suggesting that melanomas are indeed immunogenic and that they can be successfully recognized by the immune system under appropriate conditions [136]. Finally, Rosenberg and colleagues noted melanoma recurrence in the absence of emerging antigen-loss variants, even amongst clinical responders in a DC-based vaccine trial, implicating that tumor-extrinsic, microenvironmental factors contribute to the observed acquired therapy resistance [137]. These findings suggest that treatment-resistant TMEs support tumor progression in part by strongly impeding recruitment of anti-tumor T-cells. Indeed, Galon and colleagues have elegantly shown that in solid cancers, the exclusion of tumor reactive T-cells from the tumor parenchyma correlates negatively with clinical outcome[138]. Regardless of the type of treatment, the exclusion of T-cells from the tumor compartment represents a major mechanism underlying poor clinical response amongst patients with melanoma.

1.3.1.1 T-cell exclusion in targeted therapy

Melanoma progression is driven by activating BRAF mutations[15] in 35% of cases and loss-of-function PTEN mutations[139] in 10% of cases, where both of these mutations impede T-cell infiltration into the TME. First, melanoma patients harboring oncogenic BRAF mutations

demonstrate the poorest infiltration of endogenous [140-142] T-cells and adoptively transferred ACT products, suggesting an inverse relationship between oncogenic BRAF activation and intratumoral T-cell infiltration. Besides activating pro-tumorigenic MAPK signals, the most common $BRAF^{V600E}$ mutation also upregulates local VEGF production [143] which hinders T-cell infiltration by promoting dysfunctional angiogenesis and/or dense ECM polymerization [135 144 145]. In this regard, treatment of melanoma with inhibitors that disrupt the BRAF signaling cascade (Dabrafenib and Vemurafenib (BRAF inhibitors) or Trametinib (MEK inhibitor)) substantially enhances melanoma antigenicity [146] and/or immunogenicity [147], leading to improved infiltration of and immunosurveillance by endogenous and/or adoptively transferred T-cells [140 148-150]. Furthermore, $PTEN^{-/-}$ mutations often synergize with $BRAF^{V600E}$ mutations [35] to limit T-cell infiltration. Hwu et al. recently confirmed this relationship in pre-clinical models as well as in TCGA melanoma cohorts where PTEN copy number correlated positively with $CD8^{+}$ T-cell infiltration, and intratumoral granzyme B/IFNγ expression [151]. Mechanistically, $Pten^{-/-}$ mutations also activate PI3K to further facilitate T-cell exclusion [152]. In the case of $BRAF^{V600E}$ $Pten^{-/-}$ melanomas, TIL content can be partially rescued by administration of PI3Kb inhibitors [151].

Although such inhibitors hold substantial therapeutic promise, melanoma lesions quickly develop acquired resistance in the majority of patients treated with BRAF inhibitors (BRAFi) based on *de novo* development of secondary mutations in BRAF, or in MAPK proteins, which via mechanisms discussed above, continue to sponsor a T-cell sparse TME [153-155].

1.3.1.2 Melanoma exclusion of adoptively transferred T-cells

The adoptive transfer of autologous tumor-reactive T-cells represents another promising treatment modality for the treatment of patients with melanoma. While ACT approaches significantly extend overall survival in clinical responders, a significant proportion of patients do

not respond nor benefit from such therapeutic cell transfers. Accordingly, Ganss et al. and Rosenberg et al. demonstrated that resistance to ACT treatments can indeed be attributed to the poor infiltrating yield (~1-10%) of transferred T-cells into the TME in murine models of neuroendocrine cancers and melanoma, respectively [156] [157]. In stark contrast, adoptively transferred T-cell regimens have exhibited significantly greater success in hematologic malignancies, which typically lack a physical/compartmental barrier-like microenvironment as is characteristic of solid cancers, including melanoma [158] [159]. These findings suggest that solid tumors progress, even in the presence of abundant circulating tumor-reactive T-cells through the effective barricading of T-cells from entering the TME or lack of signals that bring T-cells into the TME.

1.3.1.3 Exclusion in immune checkpoint blockade

T-cell exclusion observed within solid cancers such as melanoma also poses a significant challenge to the effectiveness of T-cell reinvigoration therapies, such as checkpoint blockade strategies. In a landmark study investigating drivers of clinical outcome in metastatic melanoma patients receiving Nivolumab, a PD-1 immune checkpoint blockade (ICB) antibody, Ribas and colleagues determined that baseline density of TIL prior to ICB therapy was highly predictive of treatment outcome wherein progressors in the trial demonstrated the poorest infiltration of T-cells prior to, and throughout the course of treatment [160]. In contrast, positive response to PD-1 ICB was associated with increased TIL clonality on-treatment, suggesting that the observed therapeutic effect with ICB was due to the reinvigoration and clonal expansion of TILs within the TME [160] [161]. Therefore, important findings from these studies suggest that reversing T-cell exclusion prior to administration of checkpoint therapy may be required for optimal efficacy of ICB [162].

Therefore, an important question arises in how tumors exclude T-cells to evade immunosurveillance, with one possible answer reflecting disease-associated defects in the tumor

endothelium. Since blood vessels provide circulating T-cells access to the TME and given that tumor angiogenesis leads to severe vascular dysfunction in solid cancers, the tumor-associated vasculature can serve as a principal suspect in preventing T-cell infiltration into the TME. The following section addresses the unique structural and functional properties of the tumor vasculature that further support/establish this idea.

1.3.2 The tumor-vascular endothelial barrier

Vascular networks serve as conduits through which peripheral antigen-experienced T-cells gain access to inflamed tissues. The rapid development of neovascular networks is a critical pathologic feature that facilitates tumor growth and is accordingly recognized as one of the hallmarks of cancer progression [163]. Since blood vessels serve as the primary highways for T-cell entry into tissues, T-cell exclusion in solid tumors may be attributed to the unique characteristics of tumor-associated blood vessels, whereby a critical understanding of vascular phenotypic differences linked to immune cell exclusion could lead to targeted approaches for improved TIL entry into the TME.

1.3.2.1 Pathologic properties of the tumor vasculature

Tumor angiogenesis is triggered by a simple imbalance in oxygen consumption versus oxygen supply. Oxygen consumption by the growing tumor mass often supersedes the rate of oxygenation by endogenous blood vessels feeding the growing lesion, resulting in local tissue hypoxia [164]. Hypoxia-associated proteins, such as Von Hippel Lindau Factor (VHL), and hypoxia-inducible transcription factors, such as HIF1α and HIF2α [165-167] in turn promote local production of vascular endothelial growth factors, including VEGFA, which supports neoangiogenesis and improved local tissue oxygenation upon binding to its cognate receptors VEGFR1/2 [168]. This key process

known as the “angiogenic switch”, is critical in the conversion of avascular neoplasia into highly-vascularized, progressive tumors [169].

Angiogenesis under homeostatic conditions is a carefully regulated process characterized by controlled cell-cycle checkpoints, orchestrated interactions between endothelial and mural populations [170 171], and the development of tight intercellular junctions [172 173], all of which are commonly dysregulated in the hypoxic TME [169]. Developmental flaws in endothelial cells that arise due to an unsupervised proliferation give rise to key differences in these vascular networks that negatively affect T-cell recruitment and function within the TME [174].

Morphologically, newly formed tumor-associated blood vessels have large diameters, exhibit random branching and develop poor cell-cell adhesion between contiguous endothelial cells and abluminal mural pericytes [175 176]. These defects lead to the formation of large, porous and leaky vessels which severely limit luminal blood flow for optimal delivery of drugs, immune cells and oxygen into the TME, and the clearance of immunosuppressive metabolic waste products from the TME [175 176]. Furthermore, leaky tumor-associated blood vessels also lead to increased local interstitial fluid pressure [177], which serves as an environmental (i.e. pressure gradient) barrier for T-cell penetrance into the diseased tissue. Through the combined effects of poor vascular flow and large pores, plasma and luminal blood pool in large vascular reservoirs leads to only limited seepage of circulating fluid contents into the TME. This phenomenon, together with an underdeveloped network of draining lymphatics within the TME, leads to further increases in the tissue interstitial fluid pressure (IFP), resulting in vessel collapse and further constraint on tissue perfusion in the face of reinforced hypoxia in the TME.

Tumor blood vessels also harbor key molecular differences that facilitate tumor immune evasion through T-cell exclusion. Of note, tumor vessels differentially upregulate expression of

apoptosis-inducing Fas Ligand (FasL), creating a harsh luminal microenvironment for perfusing Fas^+ T_{EM} [178-180]. Under these conditions, Tregs demonstrate greater intrinsic resistance to apoptosis than effector CD8 T-cells by virtue of their expression of elevated levels of the anti-apoptotic protein, cFLIP [181]. Additionally, tumor vessels also actively recruit Tregs via expression of the Treg homing ligand Common Lymphatic Endothelial and Vascular Endothelial Receptor (CLEVER) [182]. Furthermore, endothelial VEGFA-VEGFR2 and endothelin-1(ET-1)-ETβR signaling cascades downregulate the endothelial adhesion molecules Vascular Cell Adhesion Molecule 1 (VCAM1) and Intercellular Adhesion Molecule 1 (ICAM1), which also limits the recruitment of activated Very Late Antigen-4 $(VLA4)^+$, Lymphocyte function-associate antigen 1 $(LFA1)^+$ cytotoxic CD8 T-cells [157 183]. When taken together, these vascular-centric molecular alterations result in therapeutically contraindicated increased Treg:CD8 ratios in the TME of solid, vascularized cancers.

Therefore, tumor-associated vs. normal tissue blood vessels are structurally and functionally aberrant, and operationally skewed to impede effector T-cell trafficking into the TME (**Fig. 1**).

1.3.3 Therapeutic reprogramming of the solid tumor vascular network to improve T-cell infiltration

1.3.3.1 Normalizing existing tumor vasculature

Given that the abnormal tumor vasculature exacerbates disease pathology, one might expect that interventional strategies that antagonize tumor angiogenesis would give rise to a 'normalized' tumor vasculature characterized by an improved ability to recruit/sustain anti-tumor immune responses within the TME.

Since VEGFA is a major driver of tumor angiogenesis, vessel-associated pathologies (as discussed above) could theoretically be ameliorated by limiting VEGF bioavailability within the

TME [184]. Furthermore, tumor blood vessels express unique, tumor-associated antigens which could also be used to selectively deplete abnormal tumor associated vessels in support of therapeutic re-vascularization by normal endothelial cells leading to the restoration of normal homeostatic vascular behavior [185] [186]. Besides limiting T-cell infiltration through induced biophysical barriers, tumor endothelial cells are poor expressors of lymphocyte adhesion molecules VCAM1 and ICAM1, which are essential for the recruitment of LFA-1^{+}VLA-4^{+} effector T-cells. In this regard, inflammatory stimuli, such as IFN-I and TNFα, known to upregulate VEC expression of VCAM1 and ICAM1, may potentiate normal vascular function within the TME [187] [188].

Therefore, vascular normalizing strategies may potentiate anti-tumor responses within the TME, and a few such strategies are discussed below:

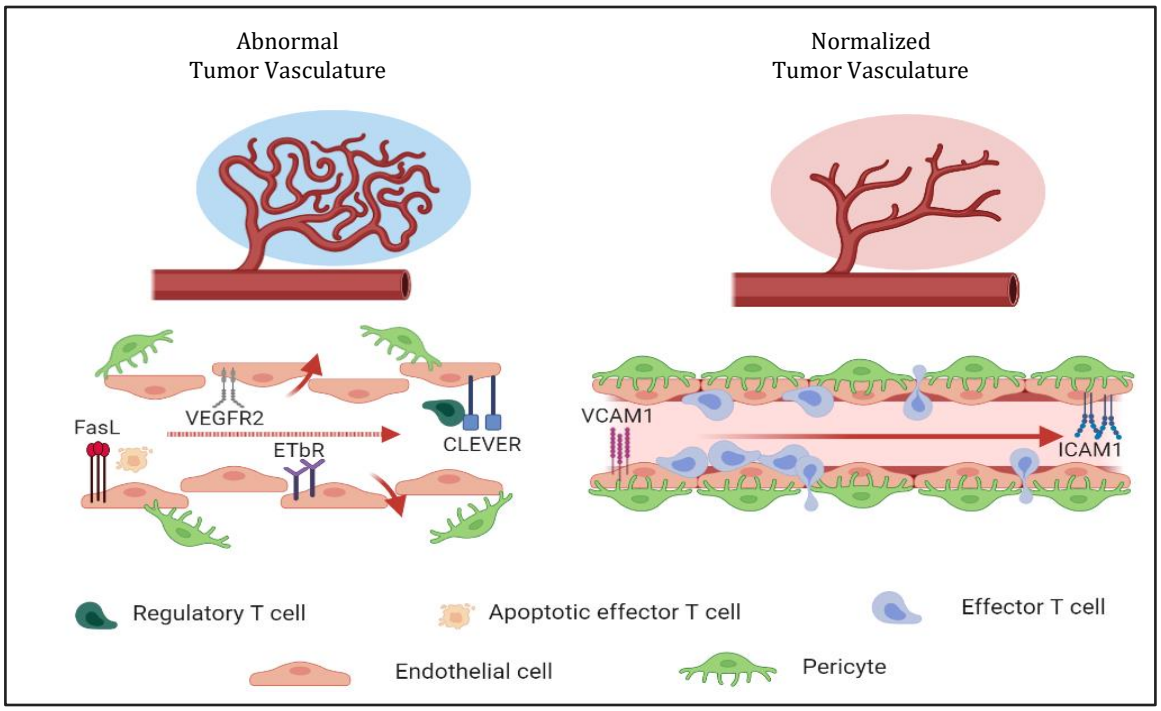

Figure 1*: Structural and functional characteristics of constitutive vs. therapeutically normalized blood vessels and their impact on immune cell recruitment into the TME*

Pictorial comparison of the untreated tumor vasculature vs. the therapeutically normalized tumor vasculature. TME with untreated vasculature are characterized by enhanced vessel permeability leading to increased tissue hypoxia and IFP. Endothelial cells (EC) in the untreated TME are also associated with poor EC-EC adhesion, poor EC-pericyte interaction and enhanced endothelial expression of Fas Ligand (FasL), Endothelin Beta Receptor (ETbR), Common Lymphatic Endothelial and Vascular Endothelial Receptor (CLEVER) and Vascular Endothelial Growth Factor 2 (VEGFR2). The vasculature in the normalized TME demonstrates improved vessel integrity, vessel perfusion and tissue normoxia. ECs in normalized TME are tightly associated with other ECs and with abluminal pericytes, and they express elevated levels of the effector T-cell adhesion molecules Vascular Cell Adhesion Molecule 1 (VCAM1) and Intracellular Adhesion Molecule 1 (ICAM1).

1.3.3.1.1 Targeting VEGF signaling for vascular normalization (VN)

VEGFA is produced in large quantities within the TME of solid tumors and is well known for its role in promoting dysregulated tumor-associated angiogenesis [189-191]. This suggests that VEGFA is a central player in sponsoring an aberrant vasculature, and that by limiting the bioavailability VEGFA in the TME, one might predictably reverse angiogenesis-associated pathology within the TME [192]. This hypothesis led to the development of VEGF neutralizing

monoclonal antibodies, including the first-generation murine antibody A4.6.1 and its humanized monoclonal successor, Bevacizumab (Avastin), that have ultimately been shown to normalize the tumor vasculature [193] [194].

In murine models of GBM, melanoma and colorectal carcinoma. Jain and colleagues demonstrated that the intratumoral delivery of A4.6.1 resulted in the normalization of the tumor vasculature as characterized by reduced microvessel density, reduced vascular permeability, reduced tumor interstitial fluid pressure, improved vessel perfusion and improved tissue normoxia, suggesting that VEGF/VEGFR blockade indeed normalizes blood vessels in the TME [193] [195] [196]. Bevacizumab conferred similar therapeutic changes to the tumor vasculature in humanized mouse models of neuroblastoma, breast cancer, melanoma, and ovarian cancer and in melanoma patients. [197] [198]. Furthermore, in melanoma patients, treatment with Bevacizumab and Ipilimumab demonstrated therapeutic synergy (vs. either monotherapy) in association with normalized blood vessels, an improved degree of intratumoral T-cell infiltration and a median overall survival of 25.1 months [199] [200].

These findings suggest that abnormal tumor vessels can be normalized by limiting VEGF activity within the TME, either directly by removing the bioactive VEGF ligand, or indirectly via enhanced local production of natural VEGFR2 antagonists such as VEGI (also known as TNFSF15) [201]. Ultimately, such therapeutic changes in the tumor vasculature may facilitate the recruitment and retention of impactful levels of therapeutic TIL.

1.3.3.1.2 Vaccines targeting tumor blood vessel antigens to promote VN

Cancer cells accrue a significant number of mutations during the oncogenic process and as such, they express starkly different gene expression profiles from their normal healthy counterparts. Although less appreciated, stromal cells (including vascular cells) are also subject to aberrant

growth conditions within the TME (i.e. hypoxia, acidosis, high IFP) that can alter their epigenetic programming. Indeed, gene set enrichment analyses of tumor-derived endothelial cells and pericytes have identified tumor-associated overexpression (i.e. 10-500 fold) of blood vessel antigens (TBVA) such as TEM1 [202 203], DLK1 [204 205], DLK2 [205], HBB [206], EphA2[207], RGS5 [208] and NRP1 [209] that allow for differential recognition of tumor vs. normal tissue-associated VEC/pericytes by antigen-specific $CD8^+$ T-cells. These TBVA have been successfully integrated into prophylactic/therapeutic DC- and DNA-based vaccines to instigate differential T-cell-targeting of tumor blood vessels. This strategy is designed to fortify tumor vasculature by promoting immune-mediated "trimming" and local development of a proinflammatory TME. Indeed, therapy using DC/TBVA-based vaccines in pre-clinical models of melanoma, colon carcinoma and lung cancer supports the therapeutic relevance of vascular normalization since the delay in tumor growth is associated with reduced tumor vascular density, improved tumor vessel functionality and enhanced infiltration of tumor reactive- and/or TBVA reactive- T-cells within the TME on-treatment. Through performance of a pilot phase II clinical trial, our group has also demonstrated the efficacy of this DC/TBVA peptide-based vaccine in normalizing the vasculature and the TME in HLA-$A2^+$ patients with metastatic melanoma, wherein we also observed a robust expansion of TBVA-specific T-cells in peripheral blood selectively in patients with objective clinical responses (Chelvanambi et al., manuscript in preparation). Interestingly, objective clinical response on this trial was also associated with epitope spreading in the T cell response of clinical responders, since over the course of treatment these patients developed an expanded repertoire of T-cells reactive against a number of vaccine-unrelated but melanoma-associated antigens. These findings suggest that vascular normalization, by improving initial T-cell mediated tumor apoptosis, may also support the release of new tumor associated antigens (TAA) that can be subsequently

internalized and cross-presented by endogenous APCs (in draining lymph nodes) to ultimately diversify the overall immune recognition of cancer. In unrelated studies, DNA-based vaccines targeting other TBVAs such as VEGFR2/Flk1[210] [211] and TEM1/CD248 [212], have also shown significant pre-clinical promise in normalizing the tumor vasculature (increased vascular perfusion, decreased vascular leakage, decreased microvessel density) and promoting the intratumoral infiltration of tumor specific $CD8^+$ T-cells in animal models of melanoma, and carcinomas of the breast, colon and lung.

This suggests that vaccine-induction of T-cells that selectively react against tumor-associated endothelial cells/pericytes may promote vascular normalization, leading to corollary cross-priming of vaccine unrelated, but therapeutically meaningful anti-tumor T-cells that are effectively recruited into the proinflammatory TME post vaccination.

1.3.3.1.3 Metronomic chemotherapy for VN

The core therapeutic principle of several classes of chemotherapeutic drugs is that they target cells with high proliferative potential, leading to selective death of tumor vs. normal cells *in vivo*. Despite this rationale, interspersed, near-MTD doses of chemotherapy have largely failed in the clinic, with seminal work by Judah Folkman and colleagues demonstrating that relapse is common with traditional dosing/scheduling regimens because surviving cancer cells retain their proliferative ability and continue to expand rapidly in the long-intervals between drug doses [213]. Instead, they proposed that repetitive sub-MTD doses of the same chemotherapeutic agent, i.e., metronomic chemotherapy (MCT), would confer better therapeutic benefit by more effectively enforcing sustained selective (apoptotic) pressure on rapidly dividing cancer (and stromal cell populations) cells within the TME [213]. Interestingly, while many cancer cells quickly develop resistance to chemotherapeutic drugs [214], rapidly proliferative tumor-associated endothelial cells,

but not normal endothelial cells, remain sensitive [215] [216] to most forms of chemotherapy. This suggests that MCT approaches, through largely anti-angiogenic mechanisms, could lead to structural and functional normalization of the tumor vasculature. Indeed, several groups have now shown that metronomic dosing of cytostatic chemotherapies (i.e. paclitaxel [217] [218], capecitabine [219] and cyclophosphamide [213]), cytokine therapies (IFNα [220]), and radiation therapy [221] can all lead to normalization of the tumor vasculature in association with delayed tumor growth in pre-clinical models of cancer.

These examples highlight a range of interventional approaches that may be invoked to promote conditional vascular normalization, leading to a pro-inflammatory TME and robust infiltration by tumor-reactive T-cells for improved treatment outcome.

1.3.3.2 Therapeutic induction of a specialized vasculature in the TME for enhanced clinical benefit.

1.3.3.2.1 High endothelial venules and tertiary lymphoid structures

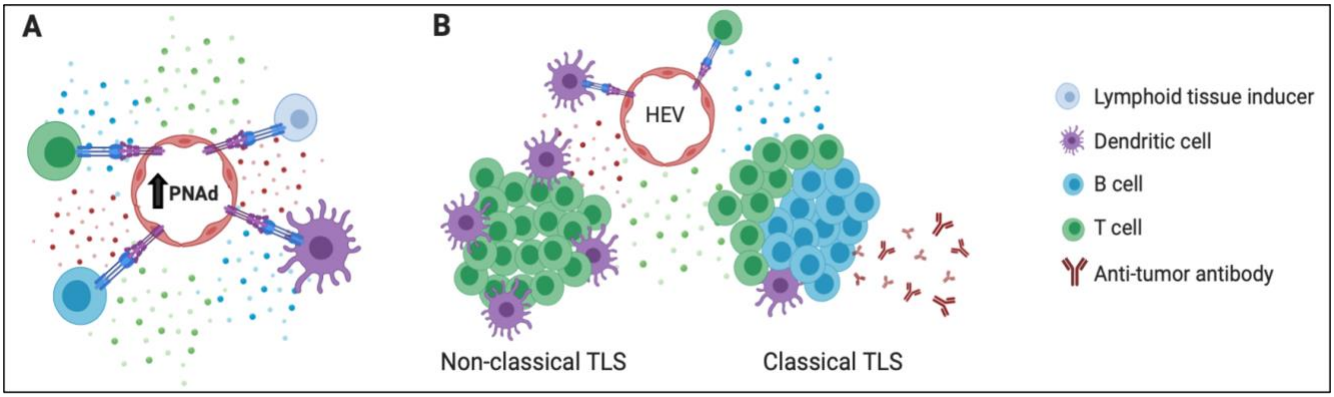

Figure 2: *High endothelial venules (HEV) and local immune cell recruitment/function and TLS formation in peripheral tissues*

A*. The induction of HEVs from flat endothelial cells (EC) is mediated by activation of EC-surface LTβR by surface* $LT\alpha_3$ *or* $LT\alpha_1\beta_2$ *expressed on tumor infiltrating immune cells. During embryogenesis, LTi cells also contribute in the lymph node anlage.* ***B****. HEVs subsequently sponsor the formation of non-classical/immature (T-cell rich, B-cell devoid) or classical/mature (GC B-cell +) TLS to mediate the local (cross)priming of lymphocytes by tissue resident APCs.*

Besides normalizing the pre-existing vasculature, one may also improve immune cell infiltration into the TME by inducing the development of high endothelial venules (HEV). HEVs are a class of specialized endothelial cells that are canonically found within secondary lymphoid organs (SLOs) such as the spleen and lymph node [222]. HEVs follow a unique developmental program where continuous LTβR stimulation confers special morphological features and physiological roles when compared to normal, flat vascular endothelial cells (**Figure 2**) [223]. HEVs are distinguishable by the presence of tall, cuboidal endothelial cells and expression of specialized adhesion molecules MAdCAM1 and PNAd that selectively recruit $CD62L^+$ naïve T/B-cells or T_{CM} cells [224-226]. However, other immune cell types including cDCs, pDCs and NK cells have also been shown to utilize HEVs to infiltrate lymphoid tissues [227] [228]. Interestingly, HEVs also develop spontaneously in highly immunogenic solid cancers and confer a virtually universal positive prognostic index when observed [229] [230]. In this regard, intratumoral HEV density also correlates positively with the number of intratumoral T and/or B-cells suggesting that these specialized vessels further improve immune access to the tumor [231-233]. In peripheral tissues with chronic inflammation such as cancer, HEVs may also be found proximal to clonally expanded T and/or B lymphocytes and APCs to form tertiary lymphoid structures (TLS); a non-encapsulated functional equivalent of SLOs. TLS demonstrate significant contextual and compositional diversity and have been commonly profiled either via germinal center B-cell zones[232] [234] [235], T-cell zones, HEVs [236-238] and/or a LN-like gene signatures [239] where in each instance, these TLS associated biomarkers have predicted a positive clinical response in human cancer [230] [240].

Therefore, HEV neovascularization, in isolation or in association with mature TLS, imparts therapeutic value by first promoting robust immune recruitment and subsequently facilitating the local expansion of unique anti-tumor T and/or B-cell repertoires within the TME.

The following sections highlight a few key concepts in HEV/TLS neogenesis.

1.3.3.2.2 SLOs vs TLSs

Characteristic	SLO	TLS
Structure	Encapsulated organs	Non encapsulated aggregate of immune cells
Formation	Preprogrammed during ontogenesis	Formed as a result of chronic inflammation
Anatomy	Specialized and found at predetermined anatomical locations	Develop in peripheral tissues and demonstrates high degree of plasticity
Lifespan	Lasts through lifetime of organism	Highly transient and resolve over time with discontinuation of inflammation
Vasculature	Intricate crosstalk between vascular networks of HEVs, afferent and efferent lymphatic vessels	Varied involvement of HEVs and uncharacterized involvement of lymphatic vessels

Table 1: *Notable differences and similarities between SLO (lymph node, spleen) and TLS*

*** summarized from Pimenta et al.** [241]

1.3.3.2.3 Types of TLS

Type	Classical TLS	Non-Classical TLS
Cellular composition	Contains: GC B-cells, T-cells ($CD4^+$, $CD8^+$), T_{FH}, DCs, FDCs, HEVs	Contains some parts of classical/mature TLS; often lacking GC foci [242]
Spatial arrangement	Distinct B ($BCL6^+$) and T-cell zones surrounded by HEVs, interdigitating presence of FDCs and T_{FH}s	Zones of $CD4^+$/$CD8^+$ T-cells and $CD11c^+$ APCs proximal to HEVs

Table 2: *Features of different types of TLS*

1.3.3.2.4 Cellular mediators of HEV and/or TLS neogenesis in cancer

While the roles and molecular identities of SLO inducing LTi cells are well characterized, the identification of a defined cell type with a similar pivotal role/central function in HEV/TLS neogenesis remains elusive. Nonetheless, several cell types have been implicated in TLS formation in cancer and the evidence of their role in supporting HEV and TLS formation is briefly discussed below:

1.3.3.2.4.1 DCs

Besides serving as professional antigen presenting cells, DCs play functionally important roles in shaping the inflammatory microenvironment of the tumor. In this regard, DCs have demonstrated the ability to produce cytokines necessary for the formation of TLSs and their footprint within the TME positively correlates with TLS formation in several human cancers. In breast cancer, DC-LAMP$^+$ mature DCs were found to be the major producers of LTβ in the TME where LTβ$^+$ mature DC infiltration was strongly associated with increased HEV density and T and B-cell infiltration [243]. Mature DCs also organize such structures in renal cell carcinoma where an increased count of DC-LAMP$^+$ CD80$^+$ CD86$^+$ mature DCs was associated with increased HEV density and improved T-cell infiltration [244]. Separately, our group has previously demonstrated that *in situ* vaccination of MCA sarcomas and MC38 colon carcinomas with Tbet or IL-36γ expressing mature DCs also promotes the formation of HEVs and non-classical TLS within the TME [237]. Together, these observations implicate conditionally activated mature DCs as instigators in the formation and maintenance of HEVs and TLS in the cancer setting.

1.3.3.2.4.2 NK cells and effector T-cells

NK cells and T-cells are also implicated in HEV/TLS neogenesis. In elegant studies conducted by Peske *et al.*, NK cells and cytotoxic CD8$^+$ T-cells were shown to play separate but sometimes

redundant roles in TLS formation within different sites of cancer [245]. HEV induction was shown to be controlled strictly by $LT\alpha_3^+$ $CD8^+$ T-cells in intraperitoneal melanoma lesions whereas both, $IFN\gamma^+$ NK cells and $LT\alpha_3^+$ $CD8^+$ T-cells were determined as necessary for HEV formation in subcutaneous melanoma models [245]. While these results implicate NK cells and effector T-cells as relevant and important mediators of HEV/TLS neogenesis, they also highlight the possible mechanistic differences underlying the induction of these structures in solid tumors located in disparate anatomic locations within the body.

1.3.3.2.4.3 Removal of Tregs

Furthermore, immunosuppressive cells may be expected to inhibit HEV/TLS neogenesis by mitigating local tissue inflammation. In pre-clinical studies performed with MCA sarcomas, targeted depletion of Tregs (using $FoxP3^{DTR}$ mice) conferred superior tumor rejection in association with a significant improvement in tumor infiltration by $CD4^+$ and $CD8^+$ T-cells [236 238]. Hindley et al., postulated that changes to the tumor vasculature might underlie the robust lymphocytic infiltration observed upon depleting peripheral Tregs [238]. Indeed, tumors from Treg-deficient mice demonstrated a significant increase in, both, the density of intratumoral HEVs and the relative abundance of TLS inducing homeostatic chemokines and lymphotoxins within the TME vs control animals. Furthermore, in correlative analysis, HEV density was both inversely related to the tumor growth rate and directly related to the number of $TNF\alpha^+$ T-cell infiltrates, which were independently shown to maintain HEV morphology through feed-forward TNF signaling events [236]. This suggests that Tregs function as a rheostat for TLS formation and that their targeted depletion could also favor HEV and TLS neogenesis in the TME.

1.3.3.2.5 Strategies to induce HEVs and/or TLS in peripheral tissues

1.3.3.2.5.1 Lymphotoxins and LIGHT

TNF superfamily members including lymphotoxins and TNFSF14 (also known as LIGHT) play crucial roles in the induction of TLS [246]. The forced overexpression of LTα under the rat insulin promoter (RIPLTα) and its cognate expression in kidney and pancreatic tissues promoted the formation of lymphocytic aggregates enriched in T-cells, B-cells and APCs in association with extensive reprogramming of the tumor vasculature as evidenced by increased expression of VCAM, ICAM, MAdCAM and PNAd [247]. Furthermore, RIPLTα mice also demonstrated elevated levels of SLC (CCL21) and BLC (CXCL13) in renal and pancreatic tissues suggesting the central TLS-inducing potential of LTα [248]. However, the TLS promoting effects of LTα are amplified through the combined overexpression of LTβ (as RIPLTαβ mice demonstrate a significant increase in HEV abundance), infiltration of naïve lymphocytes and elevated expression of homeostatic chemokines compared to RIPLTα mice [249]. Seminal studies performed by Schrama *et al*. showed that the targeted overexpression of LTα also promotes HEV and TLS formation in cancer. Briefly, by administering a tumor antigen-specific GD2 scFv-LTα fusion protein in mice harboring B16.F10 melanoma, Schrama et al. demonstrated that therapy associated with LTα overexpression resulted in an increased intratumoral HEV density and the development of a diverse T-cell repertoire in association with the presence of TLS [250]. In addition, in breast cancer models, LTβ expression by intratumoral DCs correlated positively with HEV density, suggesting that LTβ also independently drives HEV/TLS neogenesis [243]. However, the biology of LTα and LTβ converges at the level of their cognate receptor, LTβR, whose central role in HEV/TLS neogenesis can be appreciated through the loss of such structures when Ltα/Ltβ is administered in combination with

competitive blocking LTβR-Ig proteins [251] [252]. Additionally, LT and TNF signaling axes demonstrate a certain degree of crosstalk since soluble $LT\alpha_3$ homotrimers also induce HEV development via activation through TNFRI rather than LTβR [236] [245].

Another related TNF family member protein, TNFSF14 (also known as LIGHT), also contributes to TLS formation in cancer. LIGHT produced by immune cells activates the surface receptor HVEM and to a lesser extent, LTβR, to induce early events in SLO organogenesis. Recent evidence suggests that it plays a similar role in TLS organogenesis. In a study carried out by Gantsev *et al.*, newly formed lymph nodes in freshly resected breast cancer tissue exhibited a significant increase in local expression of TNFSF14 vs. adjacent mature lymph nodes within the tumor tissue, suggesting that LIGHT serves as an early inducer of ectopic lymphoid organogenesis [253]. To further elucidate the role of LIGHT in TLS formation, Ganss et al. delivered LIGHT to blood vessels through a vascular targeting peptide and observed the *de novo* induction of classical TLS together with increased vascular normalization within the TME, thus suggesting that activation of HVEM and/or LTβR by LIGHT on VECs is sufficient to induce formation of cuboidal HEVs and classical TLS in solid cancers [233]. Additionally, forced expression of LIGHT in a murine model of fibrosarcoma led to the therapeutic rejection of tumors which occurred in association with an increased infiltration of naïve lymphocytes and increased local production of homeostatic chemokines [254] [255]. Together, the $LT\alpha_1\beta_2$/LIGHT-LTβR, $LT\alpha_3$-TNFRI and LIGHT-HVEM signaling axes represent key targets for the ectopic induction of local TLS formation in cancer lesions.

1.3.3.2.5.2 IFN-I

Type I interferons are another class of cytokines implicated in the formation of HEV/TLS. In studies analyzing the role of IFNβ in pulmonary GC formation in response to Influenza A Virus

(IAV) infection, a subset of PDGFRα^{+} lung fibroblasts were identified as major producers of CXCL13 in response to intranasal transfusion of IFNβ [256]. The *in vivo* activation of IFNAR in these fibroblasts enhanced intrapulmonary CXCR5^{+} B-cells^{+} TLS formation which collectively promoted the development of a more broadly neutralizing repertoire of antiviral antibodies capable of conferring cross-strain protection when compared to TLS-deficient animals exhibiting greater susceptibility when burdened with diverse strains of IAV [256 257]. Furthermore, IFN-I production by activated DCs was positively correlated with worsened clinical score, increased autoantibody production and TLS formation in a hydrocarbon (TMPD)-induced model of autoimmune SLE [258 259]. Additionally, IFN-I-IFNAR signaling sponsors HEV/TLS neogenesis by promoting the production of several known TLS nucleating factors such CXCL10/11 [260] and lymphotoxins [261] via feed-forward signaling loops. Therefore, the administration of IFN-I, either directly or indirectly by activating other signaling cascades, may condition the TME for local HEV and TLS neogenesis.

1.3.3.2.5.3 TNFR1 agonism

TNF receptors are expressed on endothelial cells and function as key signaling nodes for endothelial proliferation and function. In studies performed by Peske *et al.*, expression of TNFR1/2 receptors on endothelial cells was highlighted to be necessary for HEV neogenesis and the corollary infiltration of naïve T-cells into established melanoma tumors in pre-clinical models of melanoma [245]. Using WT, TNF$^{-/-}$ and TNFR1/2$^{-/-}$ mice, the authors demonstrated that only tumors grown in TNFR1/2 $^{-/-}$ hosts had significantly decreased expression of PNAd^{+} HEVs and infiltration of CD62L^{+} naïve lymphocytes [245]. Since HEV density and naïve lymphocyte infiltration was comparable in TNF$^{-/-}$ hosts as compared to WT control animals, the authors postulated that the agonistic interaction of the alternative TNFR ligand, LTα$_3$, and host TNFR1/2 was responsible for

HEV/TLS neogenesis. Accordingly, the adoptive transfer of either $TNF^{-/-}$ or $LTA^{-/-}$ $CD8^+$ T-cells into $Rag^{-/-}$ hosts revealed that HEV/TLS formation was negatively affected only in the cohort that received $LTA^{-/-}$ $CD8^+$ T-cells [245]. Finally, using WT -> $TNFR1/2^{-/-}$ and reciprocal bone marrow chimera experiments, it was shown that TNFR1/2 expression on host (endothelial) cells and its activation by $LT\alpha_3$ produced by endogenous/transferred TILs was ultimately necessary for successful HEV/TLS neogenesis in the setting of melanoma [245] and sarcoma [236].

These findings suggest that treatments inducing production of LTα in the hematopoietic compartment and/or the administration of agonistic antibodies targeting TNFR1/2 within the TME may favor conditional formation of tumor associated HEVs and TLS.

1.3.3.2.5.4 Ectopic expression of IL-36

We have previously shown that local overexpression of IL-1F9/IL-36γ within the TME induces local TLS formation. In untreated human colorectal cancer, IL-36γ is expressed by the tumor vasculature, and this expression correlated with an increase in the density of $CD20^+$ B-cells within TLS in tumors, indicating that local IL-36γ production may also play a role in maintaining TLS [262]. In pre-clinical studies, Chen *et al.* demonstrated that DCs engineered to overexpress Tbet (i.e., DC.Tbet) were particularly effective in sponsoring TLS development upon direct injection into tumor lesions [237]. This effect was strictly dependent on the production of IL-36γ (known to be transactivated by Tbet) by DC.Tbet cells, as both the therapeutic benefit and TLS formation were lost in $IL\text{-}36R^{-/-}$ mice receiving DC.Tbet treatment. Further experiments with DCs engineered to overexpress IL-36γ suggested that DC. IL36γ concomitantly upregulated the expression of Tbet, highlighting an operational positive feedback loop between IL-36γ and Tbet associated with the ability of these genetically modified DCs to induce TLS in a transplantable mouse model of colon cancer [237]. Several factors that are involved in TLS formation, including but not limited to LTα,

IFNγ, Tbet, CXCL9, and CXCL10, are upregulated by autocrine/paracrine activation of the IL-36R expressed by immune and stromal cells within the TME [263].

1.3.3.2.5.5 Homeostatic chemokines and HEV/TLS formation in the TME

Following the development of specialized vasculature, homeostatic chemokines play an important role in recruiting and organizing interactions between lymphocytes and APCs. In this context, ectopic expression of homeostatic chemokines has also been explored as a strategy to induce TLS formation.

CXCL13

The forced expression of B-lymphocyte chemoattractant (BLC)/CXCL13 under the influence of the rat insulin promoter (RIP) in β cells of the pancreas induced the formation of TLS containing B-cells, T-cells and MAdCAM1$^+$/PNAd$^+$ HEVs with a further elevated production of BLC in tissue immediately surrounding the observed follicles [248]. These changes were indeed dependent on the initial infiltration of B-cells and the activation of the LTαβ-LTβR signaling cascade in the pancreas [248]. In cancer, the local production of CXCL13 by T_{FH} [264] and tumor-associated fibroblasts [256] correlated positively with the presence of GCs containing CXCR5$^+$ B-cells suggesting that ectopic expression of CXCL13 could independently drive TLS neogenesis.

CCL19 and CCL21

Additionally, the overexpression of CCL19 and CCL21 may also induce TLS neogenesis by recruiting CCR7$^+$ naïve T/B-cells and APCs. In murine studies, Luther *et al.* demonstrated that the ectopic expression of CCL19 (RIP-CCL19) or CCL21 (RIP-CCL21) in murine pancreatic tissue sponsored the formation of TLS containing CD4$^+$ T-cells, B220$^+$ B-cells and CD11c$^+$ DCs surrounding HEVs [265]. In both instances, TLS formation was strictly dependent on the chemokine induced expression of LTα1β2, IL-4 and IL-7 by CD4$^+$ T-cells [265]. Further, the ectopic expression

of CCL21 in the thyroid gland (TGCCL21) also induced $CD3^+$ $B220^+$ TLS neogenesis which required, both, endogenous $CD4^+$ T-cells and local LTβR activation [266].

1.3.3.2.6 Prognostic value of HEVs and TLS in solid cancers

In this regard, supporting SLO reactions with immune priming in TLS may confer several therapeutic benefits. Firstly, canonical TLS, which typically form at the tumor margin (i.e., the interface between tumor and normal adjacent tissue), are thought to improve antitumor immune responses by facilitating T-cell activation proximal to sites of (neo)antigen load and active disease, thus limiting the inefficiencies associated with DC migration, distal T-cell induction and subsequent recruitment into the TME [240 267]. Secondly, the tumor stroma contains a high antigen load in addition to bearing rich APC infiltration, making it an attractive auxiliary site for the *de novo* priming of T and/or B-cells [268]. Lastly, TLS may also serve as a haven for immune activation and/or function. In human melanoma patients who received ICB treatments, TLS associated T-cells expressed greater levels of activation and co-stimulatory markers including CD25, CD44 and 4-1BB respectively when compared to disperse T-cell infiltrates [232]. Furthermore, TLS-associated B-cells also expressed elevated levels of Ki67 compared to non-TLS B-cell infiltrates which together suggests that TLS augment B- and T-cell functionality within the TME [232]. Weinstein *et al.* observed similar trends in murine models of colon cancer where TIL isolated from mice treated with therapeutic HEV/TLS-inducing DC.IL-36γ vaccines also collectively expressed lower levels of T-cell exhaustion markers CTLA-4, PD-L1 and Tim-3 [237].

Therefore, TLS, by virtue of approximating T and/or B-cells with stimulatory APCs in an antigen-rich environment, may promote superior (cross)priming and functionality of T and B-cells exhibiting unique, locally expanded (anti-tumor) repertoires.

1.3.4 STING agonists as anti-angiogenic agents

Given the phenotypic abnormality of tumor vessels and the requirement of functional blood vessels for successful T-cell access to the tumor, angiostatic agents hold significant therapeutic value in treating cancer by way of slowing angiogenesis to fortify dysfunctional tumor vasculature leading to enhanced T cell infiltration and the corollary local inflammation that ultimately facilitate tumor clearance. In this regard, recent cancer studies exploring the therapeutic relevance of small molecule agonists of the Stimulator of Interferon Genes (STING) suggest that intratumoral STING activation might be highly anti-angiogenic.

Although traditionally known for its role in virus detection, STING has recently garnered attention as a candidate immune adjuvant in the treatment of cancer thanks to its robust ability to drive type I immunity upon conditional activation. However, pre-clinical dose-escalation studies for STING agonists in cancer revealed that therapeutic failure at high, near-MTD doses of several STING agonists (5,6-dimethylxanthenone-4-acetic acid (DMXAA) and 10-carboxymethyl-9-acridanone (CMA)) was associated with overt apoptosis of both tumor blood vessels [269] and tumor infiltrating T-cells [270 271] resulting in an immunologically-cold, pro-tumoral TME. Therefore, given STING's potent angiostatic function, and in line with previously published vascular paradigms, STING agonists, when dosed appropriately, may be expected to have novel utility as vascular and immune reconditioning agents capable of enhancing the T-cell dependent surveillance of cancer.

The following section introduces a few fundamental concepts in STING biology and is followed by a section that presents mechanistic evidence for STING's proposed role in reconditioning the TME for improved immune cell delivery and therapeutic function.

1.3.4.1 What is STING?

STING (also known as N-Terminal **M**ethionine-**P**roline-T**y**rosine-**S**erine Plasma Membrane Tetraspanner (MYPS), Transmembrane Protein 173 (TMEM173)) is an intracellular ER-associated pattern recognition receptor which serves to detect cytosolic dsDNA in eukaryotes [272]. In mammals, the detection of cytosolic DNA is a multi-step process that involves several accessory proteins both upstream and downstream of STING. Interestingly, STING does not directly recognize dsDNA but rather recognizes cGAMP, the catalyzed dsDNA product released by the cytosolic enzyme cyclic GMP AMP synthase or cGAS [273]. Although the role of STING in viral surveillance has been well studied [274] [275], evidence of free-floating circulating tumor DNA (ctDNA) [276] [277] and tumor-derived cGAMP [278] within the TME has focused significant attention on the role of STING in therapeutic anti-tumor responses. Furthermore, STING activation might also logically extend therapeutic benefits in the cancer setting given that it enhances production of a number of inflammatory cytokines including, but not limited to, IFN-I which has previously exhibited independent therapeutic value in clinical trials [279]. STING therefore represents a relevant and attractive therapeutic target in the TME and the interest surrounding its role in cancer is evidenced by the emergence of several small-molecule agonists being tested in early phase clinical trials as outlined in **Table 3**.

	STING Agonist	Cancer Type	Route	Combination	Phase/Status	Phase	Identifier
1a	**E7766**	Urinary bladder neoplasm	intravesical	N/A	Recruiting	I	NCT04109092
1b	"	Lymphoma or advanced solid tumors	intratumor	N/A	Recruiting	I	NCT04144140
2	**GSK3745417**	Neoplasm	intravenous	Pembrolizumab	Recruiting	I	NCT03843359
3a	**ADU S-100**	Solid tumors or lymphomas	intratumor	PDR001	Active not recruiting	I	NCT03172936
3b	"	Advanced metastatic or solid tumors or lymphomas	intratumor	Ipilimumab	Active not recruiting	I	NCT02675439
3c	"	Metastatic HNSCC	intratumor	N/A	Recruiting	II	NCT03937141
4	**SNX281**	Advanced solid tumors or lymphomas	intravenous	Pembrolizumab	Recruiting	I	NCT04609579
5	**TAK-676**	Solid neoplasms	intravenous	Pembrolizumab	Recruiting	I	NCT04420884
6	**SB11285**	Melanoma or HNSCC or other solid tumors	intravenous	Atezolizumab	Recruiting	I	NCT04096638

Table 3: *STING agonists currently being investigated in clinical trials*

1.3.4.2 The STING signaling cascade

The STING signaling cascade is a well-regulated, multi-step process. First, STING activation by cGAMP leads to a series of conformational changes, both, within the STING molecule and in independent accessory proteins which interact with STING, that ultimately transduce downstream signals. The first step in STING activation involves the binding of cGAMP/synthetic CDNs to the pocket-like STING ligand binding domain [280]. While human STING ligand binding domain resembles an open binding pocket, murine STING ligand binding domain adopts a more closed configuration, and this critical interspecies difference underscored the early clinical challenges faced by flavonoid STING agonists like DMXAA, whose spatial properties could only activate smaller murine STING ligand binding domain [281-284]. These findings have led to the careful development of subsequent STING agonists which possess an ability to bind both, murine and human STING ligand binding domain, and several such multispecies STING agonists are listed in **Table 3**. Regardless, following successful activation, the STING ligand binding domain undergoes a 180-degree rotation with relation to its transmembrane domain [285] which subsequently also

induces a conformational change to the STING C terminal tail (CTT) [286 287]. Together, these two conformational changes allow STING to interact with its accessory downstream kinase, Tank Binding Kinase 1 (TBK1) [288]. TBK1 crucially licenses STING to interact with associated transcription factors, especially IRF3, by phosphorylating a conserved PLxIS domain on STING's CTT to create negatively charged moieties capable of attracting positively charged domains of IRF3 [289]. While STING does not possess any intrinsic kinase activity, it plays a pivotal role in this process by functioning as a scaffold protein to approximate TBK1 and IRF3 interactions for subsequent phosphorylation events. Remarkably, proteomic sequencing revealed that IRF3 also contains a PLxIS domain which serves as a second substrate for the kinase activity of TBK1 [290]. Phosphorylated IRF3 subsequently detaches from the STING CTT and dimerizes with a second pIRF3 monomer before translocating to the nucleus, where this homodimer induces the expression of STING/interferon-associated genes [291-295] (**summarized in Fig. 3**).

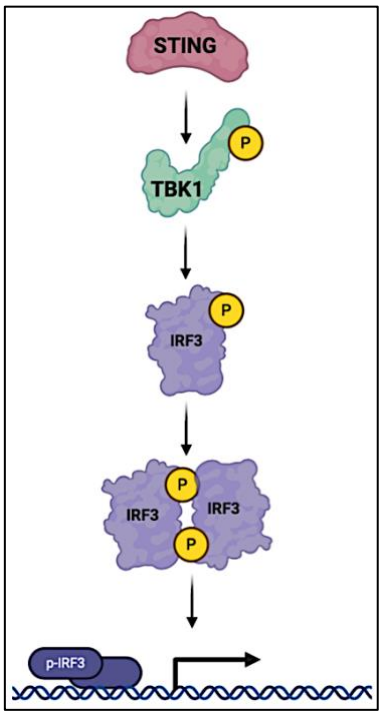

Figure 3: *The canonical STING signaling cascade*

*Graphical representation of the stepwise activation of the STING signaling cascade involving downstream phosphorylation of TBK1 and IRF3. *Shapes depicted are not representative of actual protein 3D structure.*

1.3.4.3 Dose-dependent characteristics of STING agonists – clues for the vasculature/immune normalization in the TME

Interestingly, although well-tolerated, dosing studies with STING agonists suggest that immune-dependent, durable anti-tumor responses to these agents are observed only at doses well below the maximum tolerable dose (MTD). Specifically, previous reports using first generation STING agonists (DMXAA and CMA) have elegantly demonstrated, i.e., cautioned, that the use of high, near-MTD doses of STING agonism yields poor immune protection in part by strongly inducing apoptosis in tumor infiltrating T-cells [270 271]. Separately, other flavonoid-based STING agonists (such as DMXAA) when administered at high, near MTD-doses also promote the selective ablation of tumor endothelial cells (i.e., vascular necrosis), but not endothelial cells of other peripheral organs, which, besides disrupting important physiological functions, may also

directly limit immune cell delivery into the TME [269] (**Fig. 4**). In contrast, recent studies using metronomic, sub-MTD dosing of STING agonists show profound tumor protection, wherein the therapeutic TME is characterized by enhanced infiltration of TILs with no observable T-cell toxicities [296] (**Fig. 4**). However, while such studies have shown remarkable success in slowing tumor growth with sub-MTD doses of STING agonists, they have stopped short of characterizing therapy-associated changes to the tumor vasculature which we expect will evolve on-treatment given STING's documented ability to modulate tumor angiogenesis.

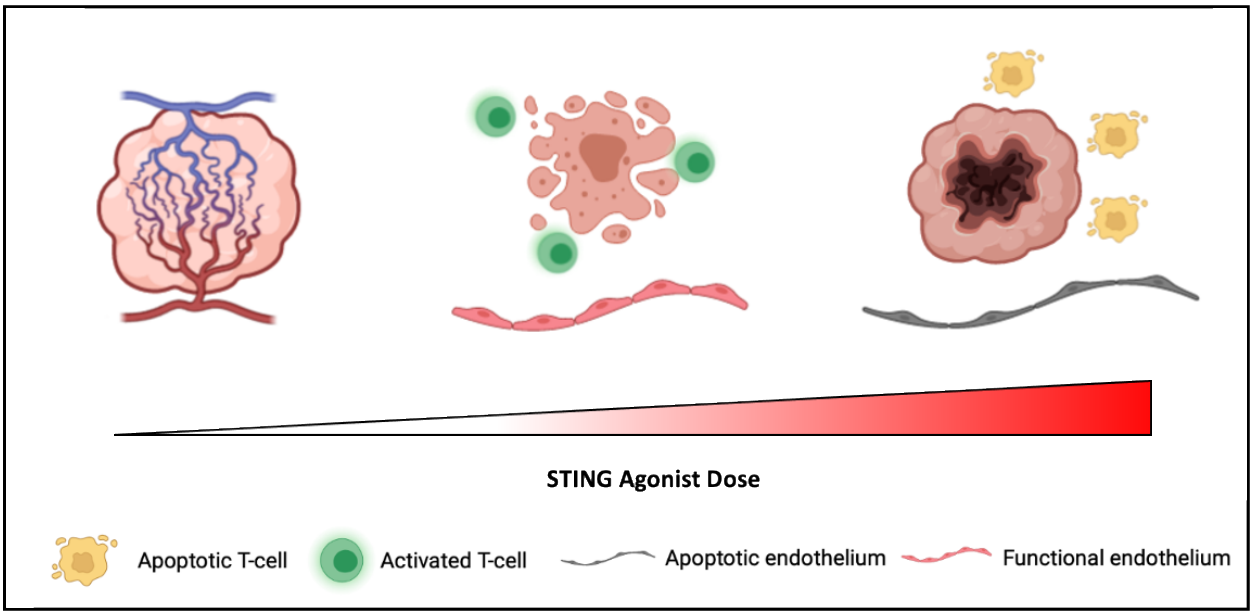

***Figure 4**: Dose-dependent effect of STING agonism in the treatment of cancer.*

High, near-MTD doses of STING agonist leads to the necrosis and acute ulceration of tumor tissue, apoptosis of infiltrating immune cells, especially effector T-cells, and ablation of tumor endothelial cells. Contrastingly, sub-MTD metronomic dosing of STING agonists promotes T-cell infiltration into the TME and inhibits tumor growth via apoptosis without operational vasoablation.

1.4 Statement of the problem

T-cells constitute a critical arm of the adaptive immune system that are responsible for mediating effective immunosurveillance and eradicating rapidly evolving cancers. However, cancer cells counter T-cell mediated tumor rejection by developing abnormal vascular networks

(via rapid angiogenesis) that effectively exclude T-cells from the TME. The pathologic growth of solid tumors is therefore often characterized by an immune cell- and inflammation-devoid, 'cold' TME. Thus, a key challenge facing the optimization of cancer immunotherapy approaches lies in appropriately conditioning the TME for enhanced recruitment of tumor-reactive T-cells capable of rejecting solid tumors. In this regard, appropriate dosing of anti-angiogenic therapies has been shown to therapeutically counteract the highly-angiogenic TME, leading to normalized tumor blood vessels that support improved T-cell infiltration into the tumors.

Early studies characterizing STING agonists have demonstrated their potent ability to ablate tumor vascular networks highlighting their anti-angiogenic potential within the TME. In this regard, pre-clinical studies have now shown that while tumor rejection at high, near-MTD doses of STING activation is associated with an immune-independent, vaso-ablative response, therapeutic tumor rejection at sub-MTD doses is characterized by a robust intratumoral infiltration of anti-tumor T-cells. However, whether this enhanced T-cell infiltration at sub-MTD doses is linked to therapeutic changes to the tumor vasculature remains underappreciated. Unraveling these vascular-centric therapeutic underpinnings for the anti-tumor activity of low-dose STING agonism will inform the development of more effective next-generation STING agonists-based immunotherapies for solid cancers.

1.5 Hypothesis

In line with previously proposed VN paradigms, I hypothesize that provision of sub-MTD, low-doses of STING agonist within the TME will achieve optimal T-cell infiltration by refurbishing and/or reprogramming the tumor vasculature (i.e., vascular normalization or VN), leading to a state of sustained cell- and cytokine-mediated type I inflammation capable of sponsoring HEV/TLS induction which together result in the "immune normalization" of the melanoma TME.

Indeed, the original research detailed in the following chapter of this thesis successfully identifies normalization of existing tumor vasculature and neovascularization of HEVs as two therapeutic vascular mechanisms through which sub-MTD doses of STING agonism potentiate T-cell infiltration and local HEV-related TLS formation in treated melanomas.

Briefly, I demonstrate that the therapy-associated response to sub-MTD doses of STING agonist ADU S-100 promotes robust infiltration of therapeutic T-cells into the TME by normalizing the existing tumor vasculature, inducing HEV formation, and ultimately the formation of non-classical TLS within the melanoma TME. Supporting the putative functions of normalized vessels and HEVs/TLS, we also observed quantitative and qualitative differences in the treatment-associated T-cell repertoire, as STING-treated tumors contained both increased numbers of peripherally expanded T-cell clonotypes (as a function of VN) and a unique TIL repertoire of $CD8^+$ T-cells expanded locally within the TME (in association with HEV/TLS neogenesis).

These findings suggest that the enhanced therapeutic T-cell response observed with low-dose STING agonism relies on therapeutic changes to the tumor vasculature which facilitate enhanced accumulation of tumor-reactive T-cells that are either recruited from the circulation after initial cross-priming in the periphery or that are recruited as naïve T-cells and locally cross-primed within the STING-activated TLS^+ melanoma TME.

2.0 STING Agonist-Based Treatment Promotes Vascular Normalization and Tertiary Lymphoid Structure Formation in the Therapeutic Melanoma Microenvironment

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2.1 Chapter synopsis

Background: The degree of immune infiltration in tumors, especially $CD8^+$ T cells, greatly impacts patient disease course and response to interventional immunotherapy. Enhancement of TIL is a critical element of efficacious therapy and one that may be achieved via administration of agents that promote tumor vascular normalization (VN) and induce the development of tertiary lymphoid structures (TLS) within the tumor microenvironment (TME).

Methods: Low-dose STING agonist ADU S-100 (5 μg/mouse) was delivered intratumorally to established s.c. B16.F10 melanomas on days 10, 14 and 17 post-tumor inoculation. Treated and control tumors were isolated at various time points to assess transcriptional changes associated with VN and TLS formation via qPCR, with corollary immune cell composition changes in isolated tissues determined using flow cytometry and immunofluorescence microscopy. *In vitro* assays were performed on $CD11c^+$ BMDCs treated with 2.5 μg/mL ADU S-100 or $CD11c^+$ DCs isolated from tumor digests and associated transcriptional changes analyzed via qPCR or profiled using DNA microarrays. For TCRβ-CDR3 analyses, T cell CDR3 was sequenced from gDNA isolated from splenocytes and enzymatically digested tumors.

Results: We report that activation of STING within the TME leads to slowed melanoma growth in association with increased production of anti-angiogenic factors including *Tnfsf15 (Vegi) and Cxcl10,* and TLS-inducing factors including *Ccl19, Ccl21, Lta, Ltb* and *Light.* Therapeutic responses resulting from intratumoral STING activation were characterized by improved VN, enhanced tumor infiltration by $CD8^+$ T cells and $CD11c^+$ DCs and local TLS neogenesis, all of which were dependent on host expression of STING. Consistent with a central role for DC in TLS formation, ADU S-100-activated $mCD11c^+$ DCs also exhibited upregulated expression of TLS promoting factors including lymphotoxin-α (LTA), IL-36, inflammatory chemokines and type I interferons *in vitro* and *in vivo*. TLS

formation in ADU S-100-treated mice was associated with the development of a highly oligoclonal TIL repertoire enriched in expanded T cell clonotypes unique to the TME and not detected in the periphery.

Conclusions: Our data support the premise that i.t. delivery of low-dose STING agonist promotes VN and a pro-inflammatory TME supportive of TLS formation, enrichment in the TIL repertoire and tumor growth control.

2.2 Background

Melanoma remains a significant health concern, representing the 5th most commonly diagnosed form of cancer in the US in 2020[1]. Many melanoma patients lack discernable tumor infiltrating lymphocytes (TIL), a harbinger of poor clinical prognosis and responsiveness to first-line immune checkpoint blockade[160]. This places a premium on development of interventional regimens that effectively promote a pro-inflammatory TME, which may then be combined with immune reinvigorating therapies such as checkpoint blockade to optimize objective clinical response rates amongst advanced stage melanoma patients with primary/acquired resistance to first-line intervention.

In this context, we and others have actively studied therapeutic VN as an interventional strategy to promote enhanced immune infiltration and a pro-inflammatory TME [205 297]. In the VN paradigm originally proposed by Jain et al.[176 298], provision of anti-angiogenic agents at low-moderate (sub-MTD) doses results in improved tumor vascular integrity and perfusion, leading to tissue normoxia, increased stromal production of pro-inflammatory chemokines and augmentation in levels of TIL[298 299]. One class of agents that concomitantly activates robust inflammatory immune responses includes agonists of STING, a cytosolic dsDNA sensor, which have demonstrated therapeutic potential in early phase clinical trials[300,301]. However, the mechanisms underlying effective treatment of cancer with STING agonists remain only partially resolved.

We now report that intralesional treatment of melanoma-bearing mice with STING agonist ADU S-100 promotes local production of anti-angiogenic factors and normalization of tumor associated vasculature. Additionally, local STING activation also upregulates the production of TLS-inducing chemokines/cytokines within the TME and the maturation of dendritic cells (DC) supporting increased pro-inflammatory immune infiltration and formation of non-classical TLS in association with controlled tumor growth. These therapeutic effects are strictly dependent on host, but not tumor cell, expression of STING. Furthermore, the STING therapy associated TIL TCR repertoire demonstrates greater clonality and population richness vs. TIL in control mice. This includes an expanded cohort of unique T cell clonotypes found only in the TME, supporting the concept of local cross-priming of T cells within the therapeutic TME.

Together, these findings further our translational understanding of STING agonist-based treatment regimens in the cancer setting and support a paradigm for VN and local TLS formation in the operational effectiveness of this class of immunotherapeutic agent.

2.3 Methods

2.3.1 Animal models and cell culture

Female C57BL/6J (Cat. No. 000664), STING^{KO} goldenticket (Cat. No. 017537) and BALB/C (Cat. No. 000651) mice aged between 6-8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed in a pathogen-free facility at the University of Pittsburgh and handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC). The B16.F10 (CRL-6475) and RENCA (CRL-2947) murine tumor cell lines were purchased from ATCC (Manassas, VA), maintained and passaged under sterile conditions. B16.F10 and RENCA cells were cultured in RPMI (Cat. No. 21870-076, Gibco) supplemented

with 10% heat-inactivated fetal bovine serum (Cat. No. F442, Sigma-Aldrich, St. Louis, MO), 100 μg/mL streptomycin, 100U/mL penicillin (Cat. No. 15140-22, Gibco) and 10 mmol/L L-glutamine (Cat. No. 25030-081, Gibco) in a humidified incubator under 5% CO_2 tension and 37°C. BPR20 ($BRAF^{V600E}PTEN^{-/-}$) melanoma cells were derived from the BP melanoma cell line[302](the kind gift of Dr. Jennifer Wargo, MD Anderson Cancer Center) under *in vitro* selection with 20 □M Dabrafenib in complete DMEM culture media. All cell lines were tested for, and confirmed to be free of, mycoplasma contamination.

2.3.2 Animal experiments

Mice received subcutaneous (s.c.) injections of 10^5 syngeneic B16.F10, BPR or RENCA tumor cells in 100 μL of PBS on the right flank (or in both flanks for bilateral model experiments). Ten days after inoculation, tumors were measured, and mice were randomized to obtain cohorts with comparable mean tumor sizes. Mice were then injected intratumorally (right flank) with sterile PBS or 5 μg of endotoxin free ADU S-100 (Cat.No: HY-12885B, MedChemExpress) resuspended in sterile PBS. Repeat injections were administered on days 14 and 17 post-tumor inoculation. Tumor growth was monitored daily and measured (two dimensions; long axis and short axis) every two days using a Vernier caliper. Tumor growth is reported as tumor area (in $mm^2 \pm$ SD) based on the product of orthogonal measurements of the long and short axes of the palpable tumor. For studies characterizing the tumor vasculature, mice received an i.v. injection of 200 μL of 1 mg/mL of Alexa Fluor 488-conjugated Lycopersicon Esculentum (a.k.a. lectin) (Cat. No. DL-1174-1, Vector Laboratories) diluted in sterile PBS just prior to euthanasia. All mice were monitored, treated and euthanized according to IACUC approved protocols and the University of Pittsburgh's Division of Laboratory Animal Resources (DLAR) recommended guidelines.

2.3.3 Bone marrow harvest and dendritic cell culture

Bone marrow (BM) isolated from C57BL/6J mice was treated with ACK lysis buffer to remove contaminating RBCs. Purified bone marrow cells were plated in a 6-well plate at a density of 2 x 10^6 cells/5 mL of DC culture media (complete RPMI + rmGM-CSF (1000U/mL, Peprotech) + rmIL-4 (1000U/mL, Peprotech)) in a humidified incubator at 37°C and 5% CO_2. BM culture was supplemented with fresh DC culture media on day 3, with cells harvested by scraping on day 5. $CD11c^+$ DC were isolated using STEMCELL magnetic $CD11c^+$ negative selection kits per the manufacturer's protocol. For *in vitro* experiments, 2.5 μg/mL of ADU S-100 was added to $CD11c^+$ DCs in culture for 16h at 37°C. For TBK1 inhibition experiments, $CD11c^+$ DCs were pre-treated with 150 μg/mL Amlexanox (InvivoGen) for 1h at 37°C, prior to addition of 2.5 μg/mL of ADU S-100.

2.3.4 Western blotting

Cells for western blotting were collected and washed twice using cold PBS. Cell pellets were lysed using a lysis buffer containing protease inhibitor cOmplete Mini (Cat. No. 11836170001, Roche) and phosphatase inhibitor, phosSTOP (Cat. No. 4906837001, Roche) and incubated at 4°C for 30 minutes. Protein containing supernatants were isolated following high-speed centrifugation at 4°C. Purified proteins were boiled and separated on SDS PAGE gels in reducing conditions. Post separation, the proteins were blotted on to PVDF membranes, blocked using 5% non-fat dry milk in PBS + 0.1% Tween-20 or 5% BSA solution in TBS + 0.1% Tween-20 for 1 hour at RT. Appropriate primary antibodies (listed in **Table 4**) in 2% NFDM in PBST or in 2% BSA in TBST were incubated for 16-18h at 4°C. Appropriate HRP conjugated secondary antibodies (1:10000 in 2% NFDM) were incubated for 1 hour at room temperature. SuperSignal West Femto (Cat. No: 34095, Thermo) chemiluminescence substrate was used to visualize resulting protein bands.

2.3.5 Tumor tissue processing

Tumors were resected on the day of euthanasia and were digested using a cocktail of enzymes [RPMI containing DNAse I (Sigma D5025 @ 20U/mL), Collagenase IA (Sigma C5894 @ 0.5 mg/mL), Collagenase II (C1764 @ 0.5 mg/mL) and Collagenase IV (Sigma C1889 @ 0.5 mg/mL)] for 30 minutes at 37^{o}C on a shaker. Tumor digests were then dissociated through a 70 μm filter and washed twice using PBS. Tumor-derived single cell suspensions were then analyzed.

2.3.6 Flow cytometry

Purified cell populations and tumor digests were washed twice with PBS prior to flow staining. Tumor digests were blocked with FcR block (BD Pharmingen, Cat. No: 553142) prior to staining for flow cytometry. Cells were then incubated with appropriate primary antibodies in FACS buffer for 30 minutes at 4^{o}C prior to flow cytometry analysis performed using either BD LSR II or BD Fortessa machines within the Unified Flow Cytometry Core at the University of Pittsburgh. Flow cytometry data was acquired using BD FACSDiva software and analyzed using FlowJo version 10.

2.3.7 Immunofluorescence microscopy

Tumor tissues were processed and stained using protocols published by the University of Pittsburgh's Center for Biological Imaging (CBI, https://www.cbi.pitt.edu). Probes used are listed in **Table S1**. Fluorescence images were acquired using Olympus Provis or Nikon 90i microscopes. Quantitation of fluorescent probes were performed on the Nikon Elements AR or ImageJ software. Post-acquisition statistical analyses on fluorescent images were performed on GraphPad Prism 8.

2.3.8 Real Time PCR

mRNA from $CD11c^+$ DCs or enzymatically dissociated tumors was isolated using the RNEasy Micro Plus Kit (Cat. No. 74034, Qiagen) according to manufacturer's protocol. Isolated mRNA was converted to cDNA using a high-capacity RNA to cDNA kit (Cat. No. 4387406, Applied Biosystems). Quantitative PCR was performed on cDNA using the Fast SYBR Green Master Mix (Cat. No. 4385612, Applied Biosystems). PCR reactions were quantitated on the StepOnePlus thermocycler (Applied Biosystems). Gene expression was normalized to mHPRT1 (Cat. No. QT00166768, Qiagen) and fold changes were calculated using $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in **Table 5**.

2.3.9 Tumor apoptosis assay

Cultured B16.F10 cells were treated with PBS or 2.5 μg/mL of ADU S-100 for 30 hours or 0.5 μM staurosporine (Cat. No: S1421, Selleckchem) for 5 hours. Following incubation with respective drugs, tumor cells were harvested by trypsinization. Induction of apoptosis was quantified using flow cytometric analysis of Annexin V (Cat. No: V13246, Invitrogen) and LIVE/DEAD Fixable Aqua staining (Cat. No: L34957, Invitrogen).

2.3.10 TCRβ-CDR3 sequencing

gDNA was isolated from day 18 tumor digests (processed as detailed above) and spleen digests (mechanically disrupted, ACK lysed). Following gDNA isolation, TCRβ CDR3 gene regions were amplified using proprietary primers designed by Adaptive Biotechnologies (Seattle, WA). Amplified TCRβ-CDR3 regions were then sequenced at a survey depth using the Illumina HiSeq platform. gDNA isolation, CDR3 library preparation and CDR3 sequencing were all performed on a fee-per-service basis by Adaptive Biotechnologies. Analysis of TCR sequencing data was

performed using the ImmunoSEQ Analyzer, a proprietary TCRseq analysis software created by Adaptive Biotechnologies.

2.3.11 Statistical tests

Comparisons between two groups were performed using two-tailed Student's t-tests while comparisons between multiple groups were performed using (one-way or two-way) analysis of variance (ANOVA) with Tukey's post-hoc analysis. p-values < 0.05 were considered significant. Prism 8 (GraphPad Software Inc., La Jolla, CA) was used to generate graphs and perform statistical tests.

2.4 Results

2.4.1 STING agonist ADU S-100 slows tumor growth, promotes VN and enhances immune cell infiltration into the TME

Previous reports have highlighted the immune-independent tumor rejection and dose-dependent toxicities of STING agonist-based therapy[296 303 304]. To mitigate such adverse effects, we evaluated the anti-tumor potential of STING activation in the TME by administering low-doses of the small-molecule STING agonist ADU S-100 intratumorally (i.t.) in transplantable subcutaneous (s.c.) murine B16.F10 melanoma models. In order to avoid vasoablation and T cell apoptosis observed with high, near-MTD doses of STING agonists[270 305 306], and based on preliminary findings for tumor ulceration necessitating euthanasia at doses > 5 μg/tumor (data not shown), we adopted the use of a low-dose (5 μg/tumor; i.e. ~100-fold lower than conventional dosing) of ADU S-100 for i.t. injections administered on days 10, 14 and 17 post-tumor inoculation (**Fig. 5A**). Under these treatment conditions, ADU S-100 injections resulted in slowed tumor growth (**Fig. 5B**) and prolonged survival (**Fig. 5C**) vs. mice treated with PBS. Similar anti-tumor effects for this interventional therapy were observed in two unrelated s.c. tumor

models; BPR ($BRAF^{V600E}PTEN^{-/-}$) melanoma in C57BL/6 hosts (**Fig. 6a**) and RENCA renal carcinoma in BALB/c hosts (**Fig. 6b**).

Since the first-generation murine STING agonist, DMXAA, was developed as an anti-angiogenic agent[304], we sought to determine whether delivery of low-dose ADU S-100 would support VN via a paradigm originally proposed by Jain et al.[6], leading to increased production of anti-angiogenic factors within the treated TME. To test this hypothesis, RNA was isolated from PBS control- or ADU S-100-treated tumors and analyzed by qPCR for expression of anti-angiogenic factors[307 308]. Compared to control tumors, ADU S-100 treated tumors coordinately expressed: i.) significantly elevated levels of transcripts encoding anti-angiogenic factors *Tnfsf15 (Vegi)* and *Cxcl10,* and ii.) significantly reduced expression of hypoxia-associated transcripts *Hif1a* and *Hif2a* (**Fig 5D**) and hypoxia-responsive cancer stem cell markers CD133 and Jarid1b[204] (**Fig 5E**), which together supported possible VN in the TME on-treatment with STING agonist.

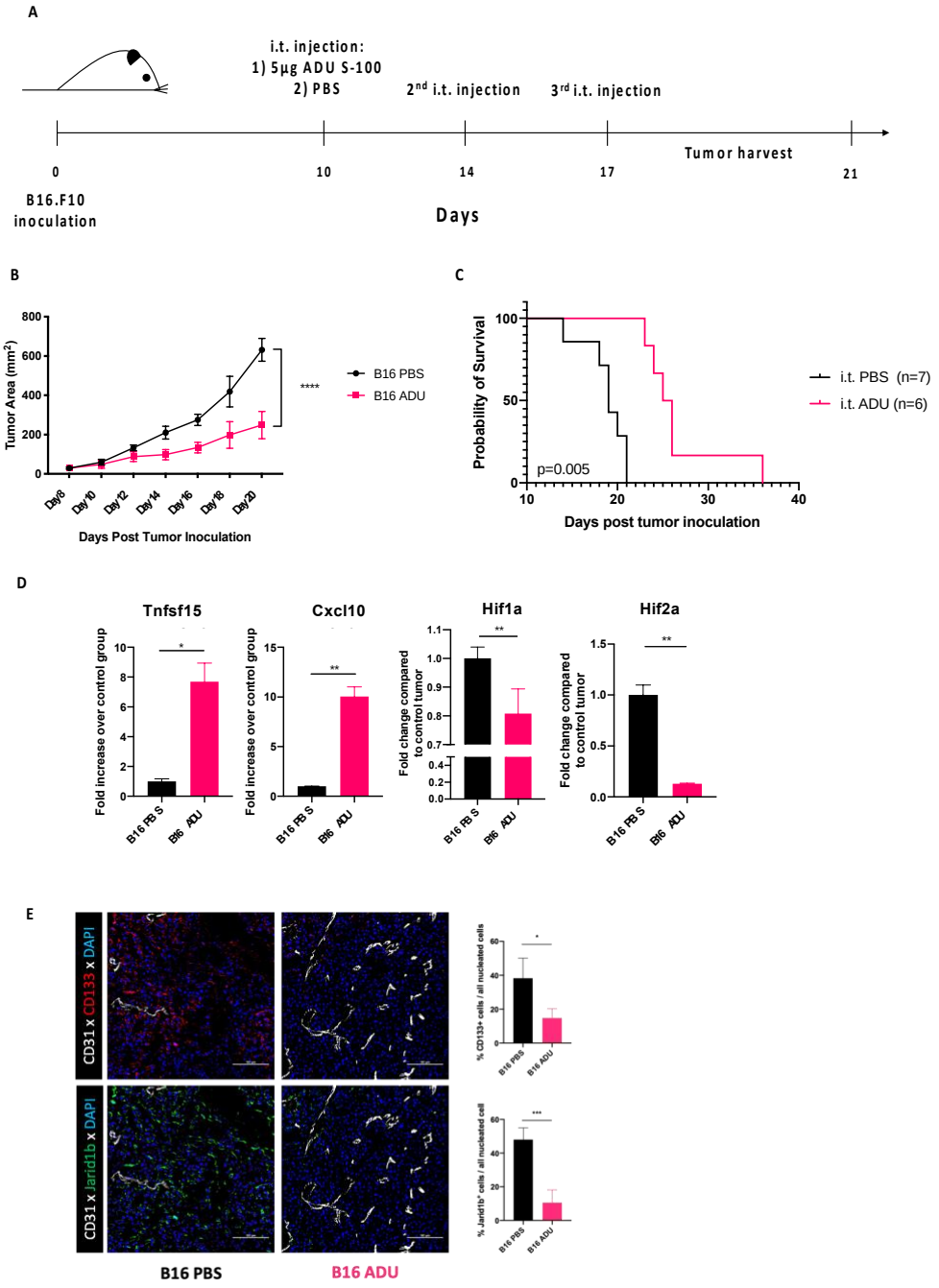

***Figure 5**: Intratumoral STING activation slows melanoma growth in mice.*

A. *Schematic depiction of our in vivo experimental design. C57BL/6J mice bearing subcutaneous B16.F10 tumors received three intratumoral injections of 5µg ADU S-100 over the span of a week. (n=5/group)* ***B.*** *representative tumor growth curves from cohorts of B16.F10 melanoma showing significantly slower tumor growth kinetics when mice were treated with ADU S-100 intratumorally. Tumor measurements represented as total tumor area (calculated as small axis X large axis) ****p < 0.0001, two-way ANOVA* ***C.*** *Representative Kaplan-Meier survival plot*

*depicting improved survival in mice treated with ADU S-100 vs control mice. *p = 0.005, MantelCox log RANK test.* ***D.*** *Post-treatment tumor digests obtained on day 18 show transcriptional signatures associated with vascular normalization such as with increased anti-angiogenic factors (Tnfsf15/Vegi, Cxcl10) and decreased tissue hypoxia (using Hif1a and Hif2a as biomarkers) in ADU S-100 treated tumors. *p < 0.05; **p < 0.002.* ***E.*** *Immunofluorescence staining and image quantitation showing reduced expression of hypoxia-responsive cancer stem cell markers CD133 and JARID1B in ADU S-100-treated B16.F10. *p< 0.05; ***p < 0.0002. Data are representative of three independent experiments. ANOVA, analysis of variance; i.t, intratumorally*

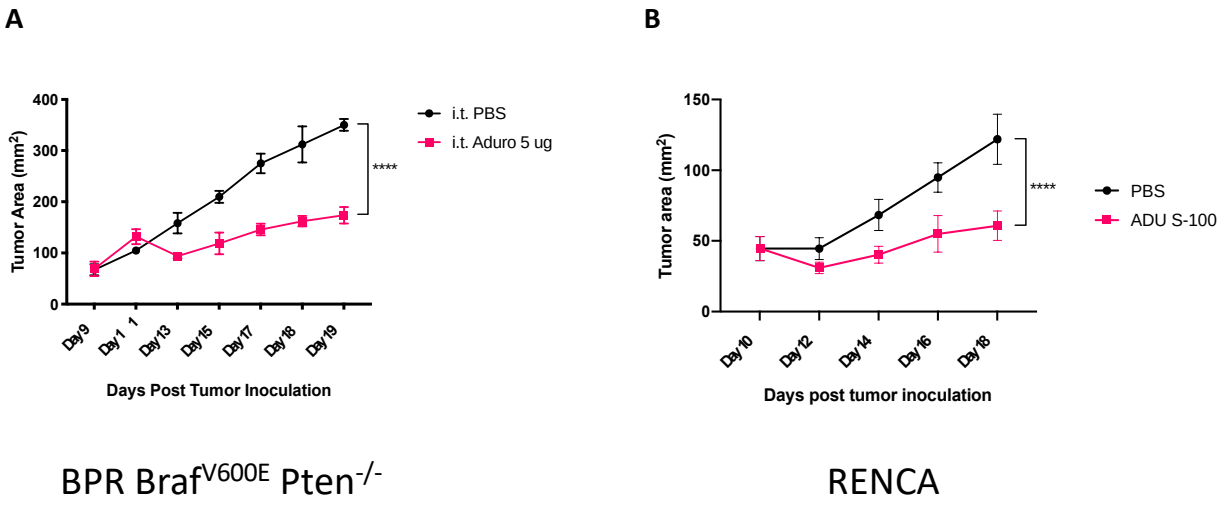

Figure 6: *STING agonist ADU S-100 slows growth of BPR Melanoma and RENCA renal cell carcinomas in syngeneic immunocompetent hosts.*

*Representative tumor growth curves from treated cohorts (n = 5/group) of C57BL/6 mice bearing established BPR melanomas (**A**) or BALB/c mice bearing established RENCA renal cell carcinomas (**B**) as described in **Fig. 5** and Materials and Methods. Note significantly slower tumor growth kinetics when mice were treated with STING agonist ADU S-100 intratumorally. Tumors were measured using calipers and sizes are represented as total tumor area (calculated as small axis x large axis). *p < 0.05, Two-Way ANOVA. Representative tumor growth curves from three independent experiments.*

We next performed immunofluorescence microscopy (IFM) studies on isolated tumor sections from control vs. ADU S-100-treated mice to discern therapy impact on indices of VN including vessel perfusion and tight pericyte coverage of blood vessels. Analysis of tumor specimens isolated from mice after i.v. injection of a fluorescently labeled vascular binding lectin revealed that blood vessels in

tumors from mice treated with ADU S-100 displayed improved perfusion vs. tumors isolated from PBS-treated control mice (**Fig. 7A-B**). Furthermore, IFM analyses confirmed improved PDGFRβ$^+$ pericyte coverage of CD31$^+$ VECs in ADU S-100 treated vs. control B16 tumors (**Fig. 7C-D, Fig. 8**) with the tight approximation of the CD31- and PDGFRβ-associated fluorescence signals on the abluminal vascular surface, consistent with the ability of low-dose ADU S-100 to promote therapeutic VN [298 309]. We also observed that CD31$^+$ VECs in tumors treated with ADU S-100 exhibited increased expression of VCAM1, an endothelial cell marker known to be upregulated in response to improved oxygenation[310] and inflammation[187 188], and which facilitates tissue recruitment of circulating VLA-4$^+$ T effector cells (**Fig. 7G-H**).

Another specialized vasculature in the TME is the lymphatic endothelial network. Lymphatics serve as draining conduits to lymph nodes (LN), permitting transport of APCs for adaptive immune cell priming in conventional secondary LNs[311]. Lymphatic vessels drain interstitial fluid from the TME, thus reducing tumor interstitial fluid pressure (TIFP) and facilitating influx of immune cell populations from the circulation[176 312]. Therapeutic lowering of TIFP in the TME via enhanced development of lymphatic vessels represents an additional index of VN[176] and has also recently been identified as a positive prognostic indicator in human melanoma[313]. In this regard, murine melanomas treated with low-dose STING agonist ADU S-100 exhibited significant increases in the density of Lyve-1$^+$ lymphatic endothelial cells (LEC) vs. PBS-treated control tumors (**Fig. 7E-F**).

Importantly, the presence of normalized and activated vascular networks in the therapeutic TME was associated with robust improvement in tumor infiltration by CD45$^+$ immune cells (**Fig. 7I**), CD8$^+$ T cells and CD11c$^+$ DCs (**Fig. 7J**) after i.t. administration of low-dose ADU S-100.

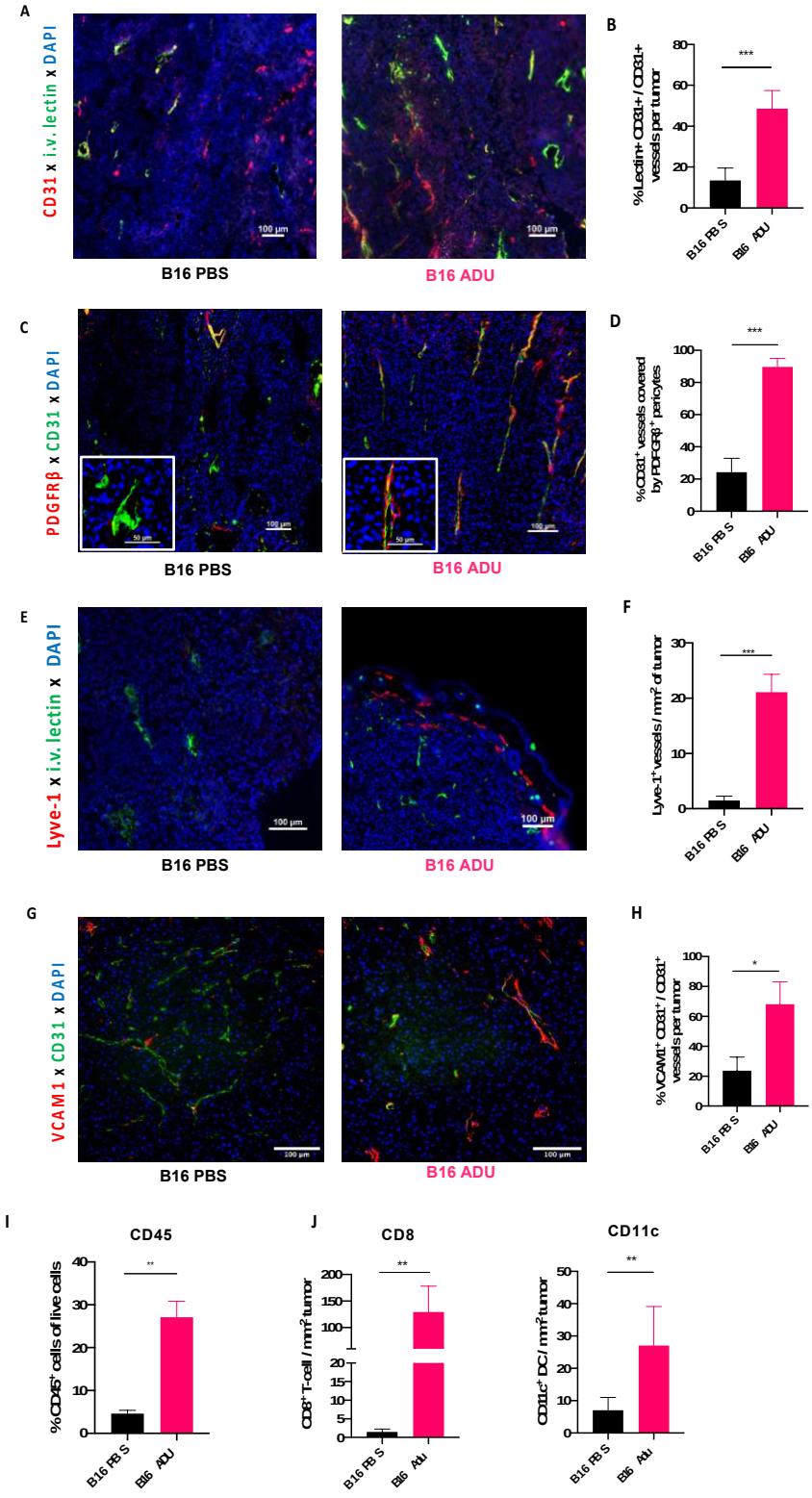

Figure 7: *Delivery of low-dose STING agonist into the TME promotes vascular normalization (VN), lymphangiogenesis and improved immune cell recruitment.*

A. *Representative images of lectin perfused functional vessels in PBS or ADU S-100 treated B16.F10 melanoma resected 18 days post-tumor inoculation.* ***B.*** *Quantitation of vessel perfusion in PBS or ADU S-100 treated tumors shown as a function of percent CD31+ VECs containing luminal lectin-AF488.* ***C.*** *Representative images depicting PDGFRβ+ pericyte coverage on tumor VECs in PBS or ADU S-100 treated B16.F10 tumors resected 18 days post inoculation (inset scale bar=50μm).* ***D.*** *Quantitation of the percentage of CD31+ VECs with tightly-approximated (covering) PDGFRβ+ pericytes based on overlapping fluorescence signals at the abluminal VEC surface-pericyte interface.* ***E.*** *Representative images showing increased abundance of Lyve-1+ lymphatic endothelial cells in ADU S-100 treated B16.F10 tumors.* ***F.*** *Quantitation of Lyve-1+ LEC density per unit area tumor.* ***G.*** *Representative images showing VCAM-1 expression on tumor VECs in PBS or ADU S-100 treated B16.F10 melanoma* ***H.*** *quantitation of VCAM-1 expression on CD31+ VECs.* ***I.*** *Percent quantitation of live CD45+ cells in resected B16.F10 melanoma treated with PBS or ADU S-100.* ***J.*** *Quantitation of CD8+ T cell and CD11c+ DC infiltrates in ADU S-100 treated or control B16.F10 tumors. Data are representative of three independent experiments. *p < 0.05; ** p < 0.002; ***p < 0.0002. scale bar=100μm. LEC, lymphatic endothelial cells; TME, tumor microenvironment.*

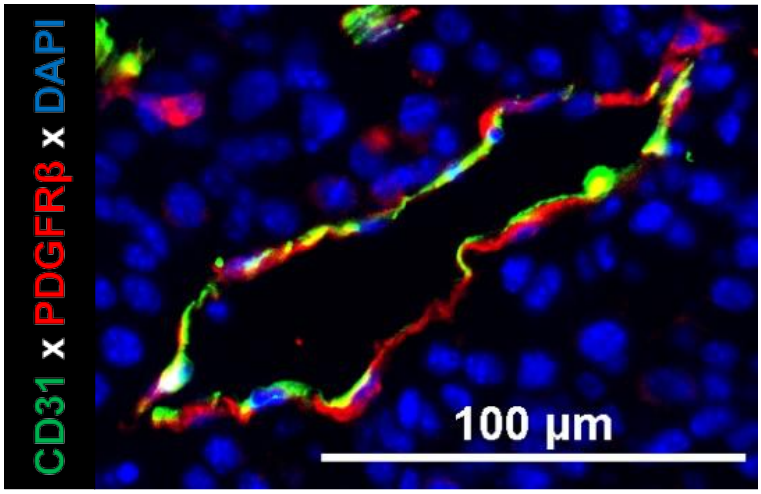

Figure 8: *Representative high-magnification immunofluorescence image demonstrating separate spatial stacking of PDGFRβ$^+$ pericytes and CD31$^+$ VEC in normalized blood vessels found in B16 melanomas treated with i.t. ADU S-100.*

Note yellow (overlap of red/green signals) pseudo-coloring of the abluminal VEC cell surfaces with tightly approximated pericyte cell surfaces. Scale bar = 100μm

2.4.2 STING-activated CD11c$^+$ DCs develop VN- and TLS-inducing properties

Having observed an increase in CD11c$^+$ DC infiltration within the ADU S-100 treated TME, and given the pivotal roles played by tumor-associated DCs in cross-priming therapeutic anti-tumor

immune responses, we next sought to characterize the direct impact of STING agonism on DCs. To address this, mCD11c^{+} BMDCs were treated with PBS or with 2.5 μg/mL ADU S-100 *in vitro* for 16h, after which mRNA expression was profiled using gene chip microarrays. We identified and analyzed ~1300 annotated gene products that were significantly up/down-regulated in ADU S-100-treated CD11c^{+} DCs [$|\log_2 FC| > 1$ and adjusted p-value < 0.05] and observed that their selective expression corresponded with several anti-tumor Gene Ontology phenotypes (**Fig. 9A, Fig. 10A, Fig. 10B**). Remarkably, a GSEA on Ingenuity Pathway Analysis (IPA, Qiagen) revealed that ADU S-100-activated DCs strongly upregulated expression of gene transcripts associated with the inhibition of angiogenesis (**Fig. 11A**) and the organogenesis/development and maintenance of lymphoid tissues (**Fig. 9B**).

To expand on these findings, we assessed the expression of targets reported in an *ad-hoc* biomarker panel for TLS formation[314], in addition to other validated DC-centric, pro-TLS factors including lymphotoxins, IL-36β and TNFα[237 239 246 315-317]. We observed that STING-activated DCs coordinately upregulate several factors within the *ad-hoc* panel in addition to *Lta, Tnfa and Il36b* when compared to control PBS-treated DCs (**Fig. 9C**). We validated the gene array expression findings at the transcriptional level using qPCR analyses performed on BMDCs treated with ADU S-100 vs. PBS *in vitro* (**Fig. 9D**) and on CD11c^{+} DCs isolated from digests of tumors treated with ADU S-100 vs. PBS *in vivo* (**Fig. 9E**). We further corroborated that the production of these TLS associated factors by DCs relied on an IFNAR-independent, but STING-TBK1-IRF3-dependent signaling cascade (**Fig. 12**).

Consistent with recent literature linking DC maturation to TLS presence/maintenance in tumors[244 318], microarray findings further suggested that STING activation promotes CD11c^{+} DC maturation (**Fig. 9F**) leading to the development of a CD54^{hi+}CD86^{hi+}CCR7^{hi+}PD-L1^{+} mature phenotype

confirmed by flow cytometry (**Fig. 9G**). In sum, our data suggests that STING-activated DCs might serve as sponsors for TLS formation within the TME.

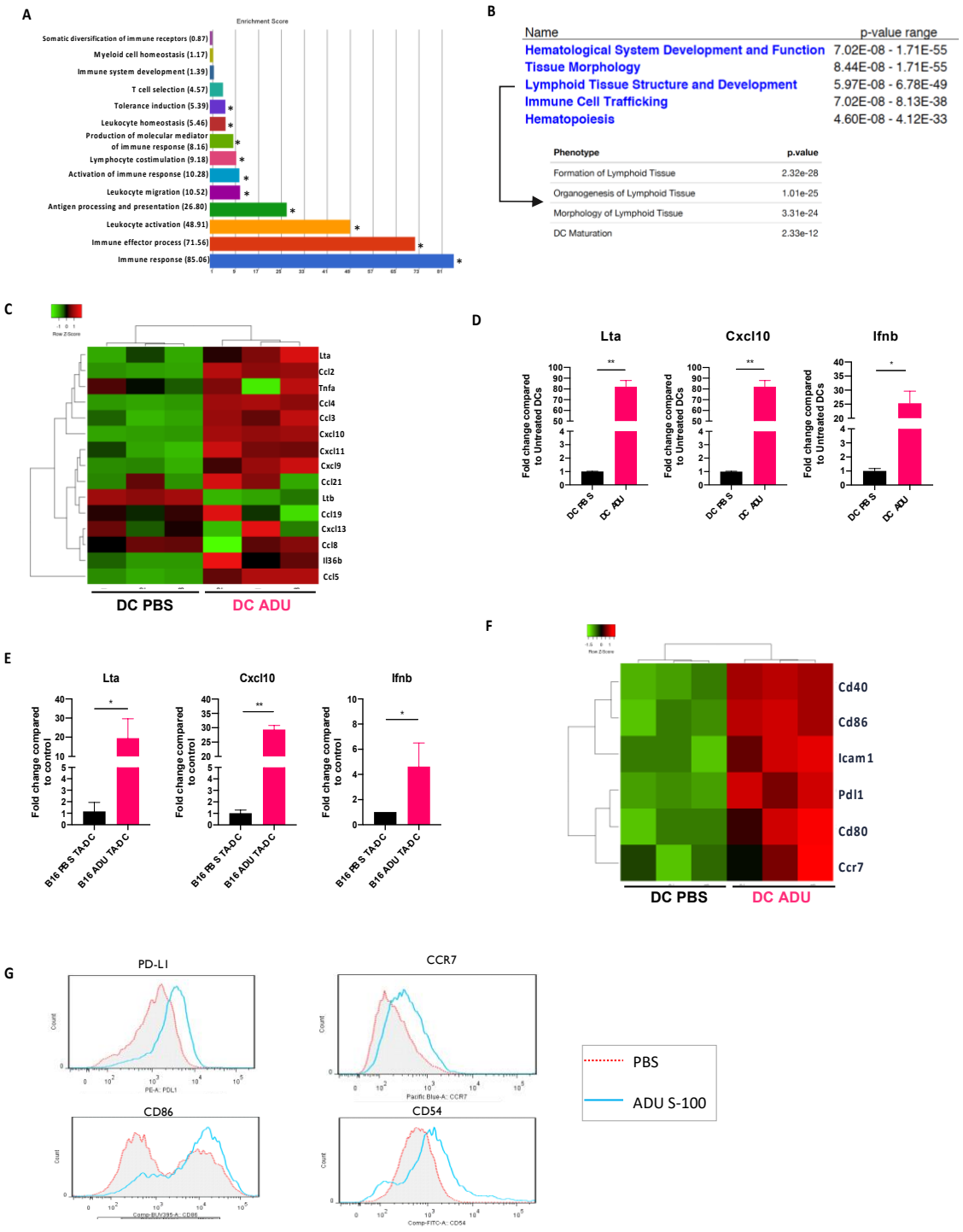

Figure 9: *STING activated DCs exhibit TLS inducing characteristics.*

A. *Visualization of Biological Processes Gene Ontology terms associated with differentially expressed genes (DEG) in sting activated CD11c+ DCs. Go analysis performed using Partek genomics suite, *p < 0.05, one-way ANOVA* ***B.*** *annotated microarray probes cross-referenced with ingenuity pathway analysis (Qiagen) implicates Deg gene expression of sting activated DCs*

in promoting their maturation and in the formation, structure and development of lymphoid tissues. ***C.*** *STING activated DCs upregulate factors associated with TLS formation.20* ***D.*** *Quantitative rtPCR validation of TLS inducing factors highlighted by microarray analysis.* ***E.*** *Quantitative rtPCR validation showing increased TLS-associated analyte production by CD11c+ DCs directly isolated from digests of tumors treated with ADU S-100 vs PBS in vivo.* ***F.*** *STING activated DCs demonstrate a more mature phenotype as evidenced by increased transcript levels of DC maturation markers.* ***G.*** *Flow cytometric validation of DC maturation on STING activation. Data representative of three independent experiments *p < 0.05; **p < 0.002. ANOVA, analysis of variance; DCs, dendritic cells; IFN, interferon TLS; tertiary lymphoid structures.*

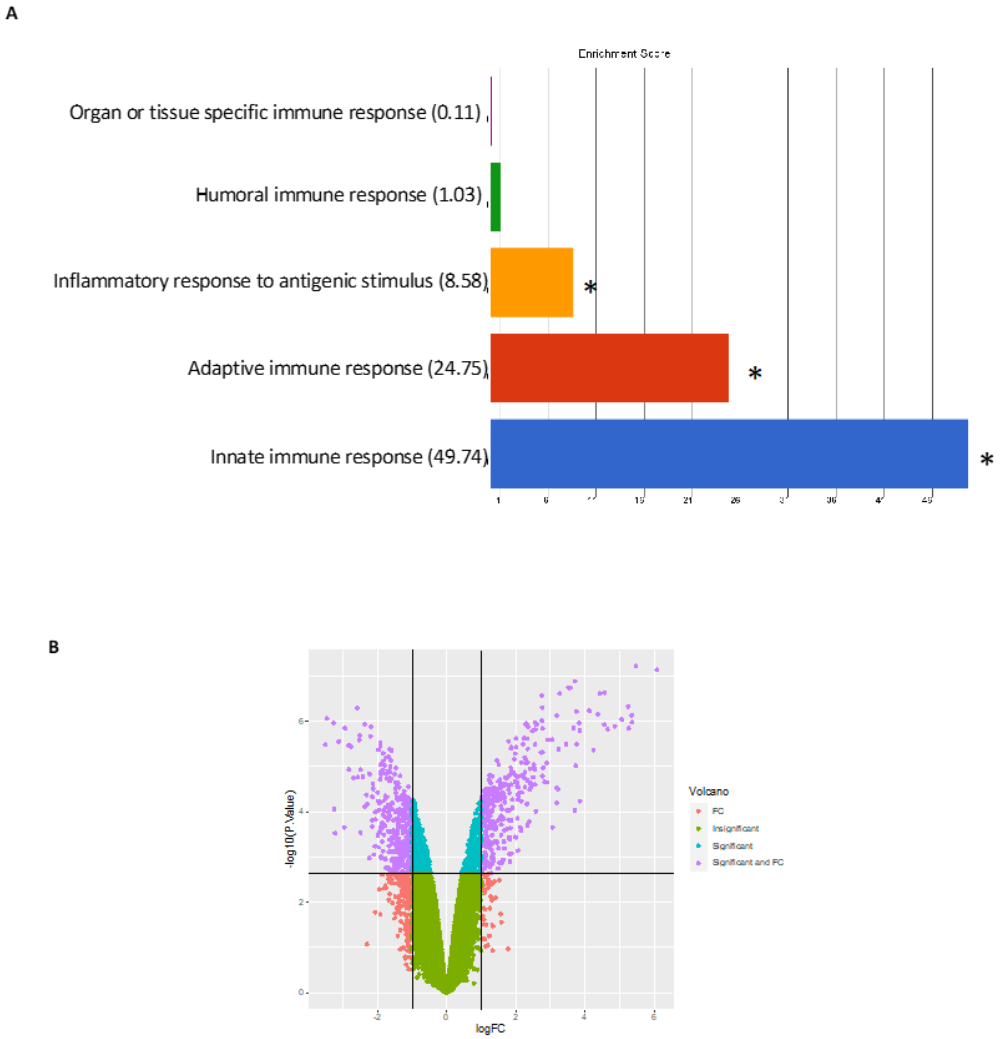

Figure 10: *Transcriptional profiling and pathway analysis of CD11c^{+} DC treated with ADU S-100 vs control media.*

A. *Biological processes associated with top GO term, immune response (GO:0006955), enriched in ADU S-100-treated CD11c^{+} DCs. *p-value < 0.05, One-way ANOVA.* ***B****. Volcano plot of CD11c^{+} DC genes analyzed via microarray. ~1300 genes (shown in purple) were found to be differentially expressed (|log2FC|>1 & adjusted p-value < 0.05) and were used for pathway and GO enrichment.*

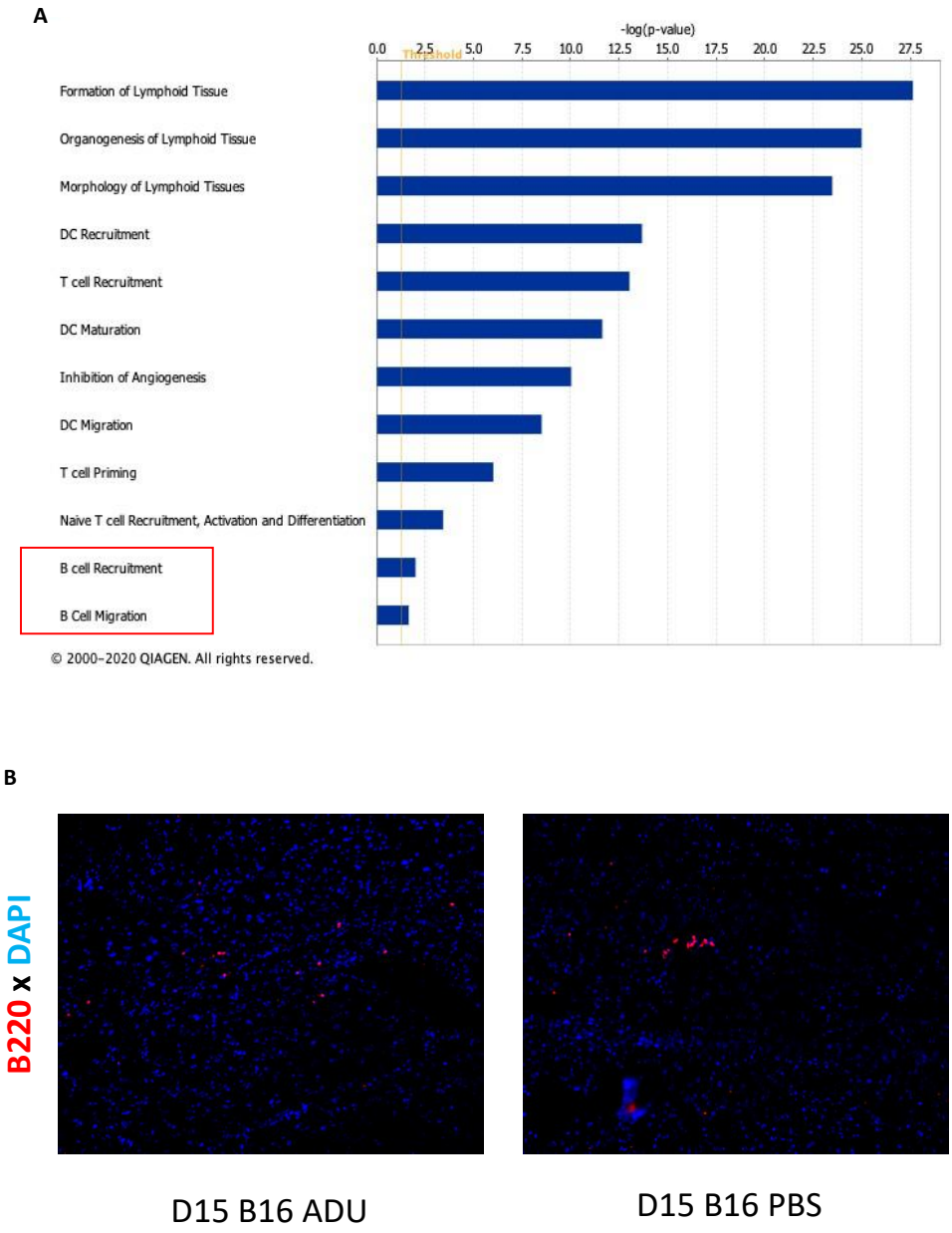

Figure 11: *STING activation does not improve B220+ B cell recruitment to s.c. B16.F10 melanomas.*

A*. Gene-set enrichment of STING activated CD11c^{+} DC gene expression showing significant TLS nucleating, DC recruiting and T cell recruiting signatures, but poor B cell recruiting signatures. Threshold = -log(p-value) of 1.3 or p-value of 0.05.* ***B****. Representative immunofluorescence image showing no observable differences in B cell infiltration with ADU S-100 vs. PBS treatment of B16.F10 melanomas in vivo.*

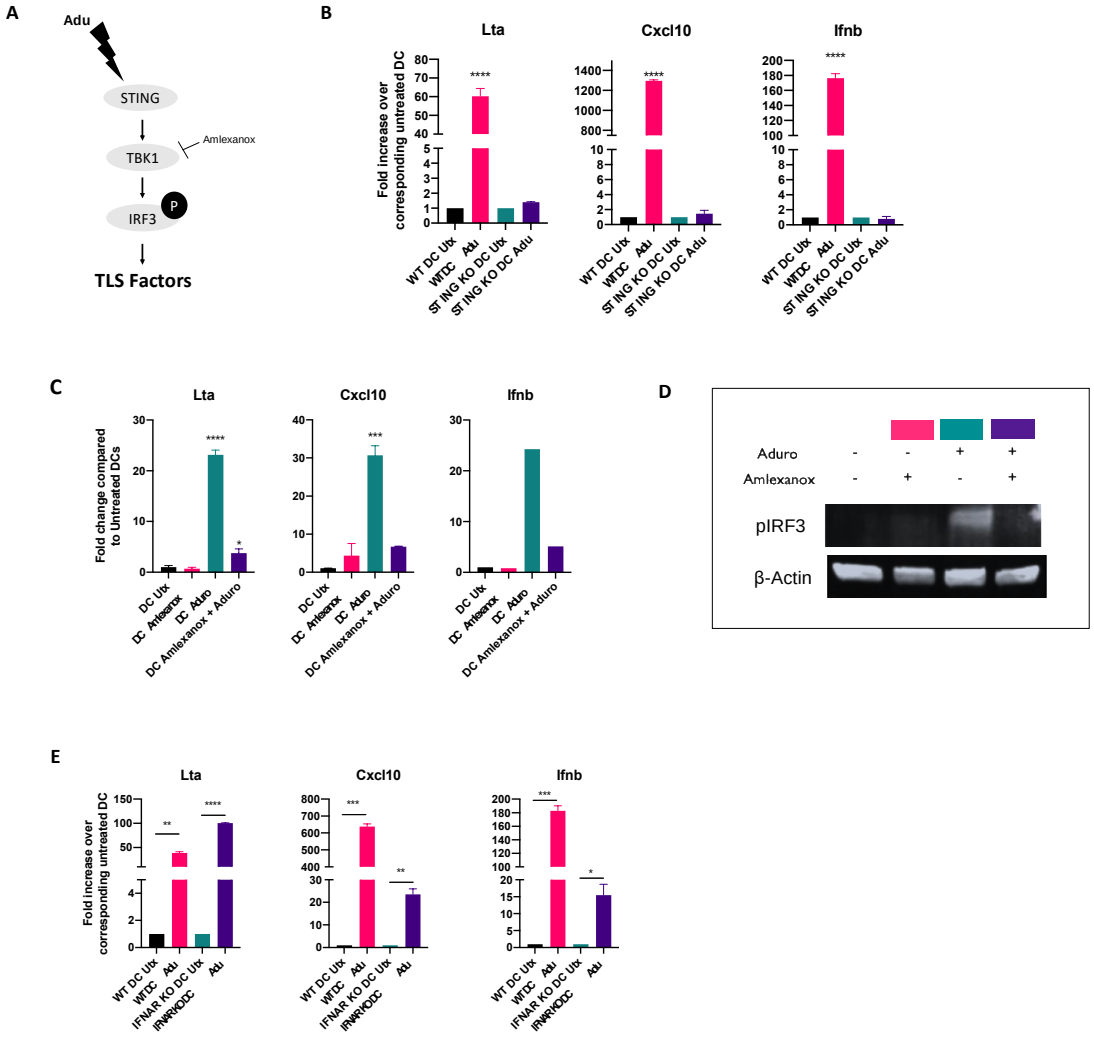

Figure 12: *STING activation is sufficient to promote production of TLS factors through a STING-TBK1-IRF3 signaling cascade.*

A*. Schematic representing canonical STING signaling cascade involving TBK1 and IRF3.* ***B****. STING activation significantly increases transcript levels of TLS factors in CD11c$^+$ DCs from WT hosts which is absent in DCs from STING KO hosts. *p-value < 0.05, One-Way ANOVA.* ***C****. Inhibition of TBK1 using Amlexanox prior to STING activation ablates production of STING associated TLS factors. *p-value < 0.05, One-Way ANOVA.* ***D****. Immunoblotting confirms upregulation of TLS associated transcripts in CD11c$^+$ DCs (in panel* ***C****) occurs only with IRF3 activation/phosphorylation (S396).* ***E.*** *IFNAR KO DC retain ability to produce TLS factors upon STING activation. *p-value < 0.05, unpaired t-test.*

2.4.3 Treatment with low-dose STING agonist promotes formation of non-classical TLS in the therapeutic TME.

We next investigated whether these STING-driven inflammatory events translated into the development of a pro-TLS TME. Tumors harvested on day 18 were analyzed by qPCR for expression of homeostatic chemokines and lymphotoxin genes associated with development of secondary/tertiary lymphoid tissues[230]. When compared to control tumors, ADU S-100-treated tumors expressed elevated levels of homeostatic chemokines *Ccl19* and *Ccl21* (but not *Cxcl13*), and the LTβR agonists *Lta*, *Ltb* and *Tnfsf14/Light* (**Fig. 13A**), suggesting that intratumoral STING activation promotes a TME favoring TLS neogenesis.

To determine whether this treatment regimen resulted in the formation of observable TLS, ADU S-100 treated B16.F10 tumors were resected at various time points on-treatment, with tumor sections analyzed by IFM for the presence of TLS. $CD45^+$ immune clusters surrounding $PNAd^+$ HEVs resembling *bona-fide* TLS[319] were identified as early as 5 days after initiating treatment with ADU S-100 (**Fig. 13B**). These therapy-induced TLS were richly-infiltrated with $CD11c^+$ DCs and $CD3^+$ T cells, resembling previously reported "non-classical" TLS[320] (**Fig. 13C**). IFM analyses did not however reveal significant B cell infiltrates in our specimens (**Fig. 11B**); consistent with our observed lack of *Cxcl13* expression in the TME of ADU S-100 treated animals (**Fig. 13A**). TLS were further quantified using $PNAd^+$ HEV as a canonical biomarker[231 321], revealing that ADU S-100-treated tumors contained more HEVs per unit area of tumor vs. control PBS-treated tumor specimens (**Fig. 13D**). ADU S-100 treated tumors were also characterized by a marked increase in the number of physical contacts made between $CD11c^+$ DCs and $CD8^+$ T cells within the TME (**Fig. 13E**). Hence, treatment with STING agonist ADU S-100 appears to primarily promote non-classical TLS formation within the TME.

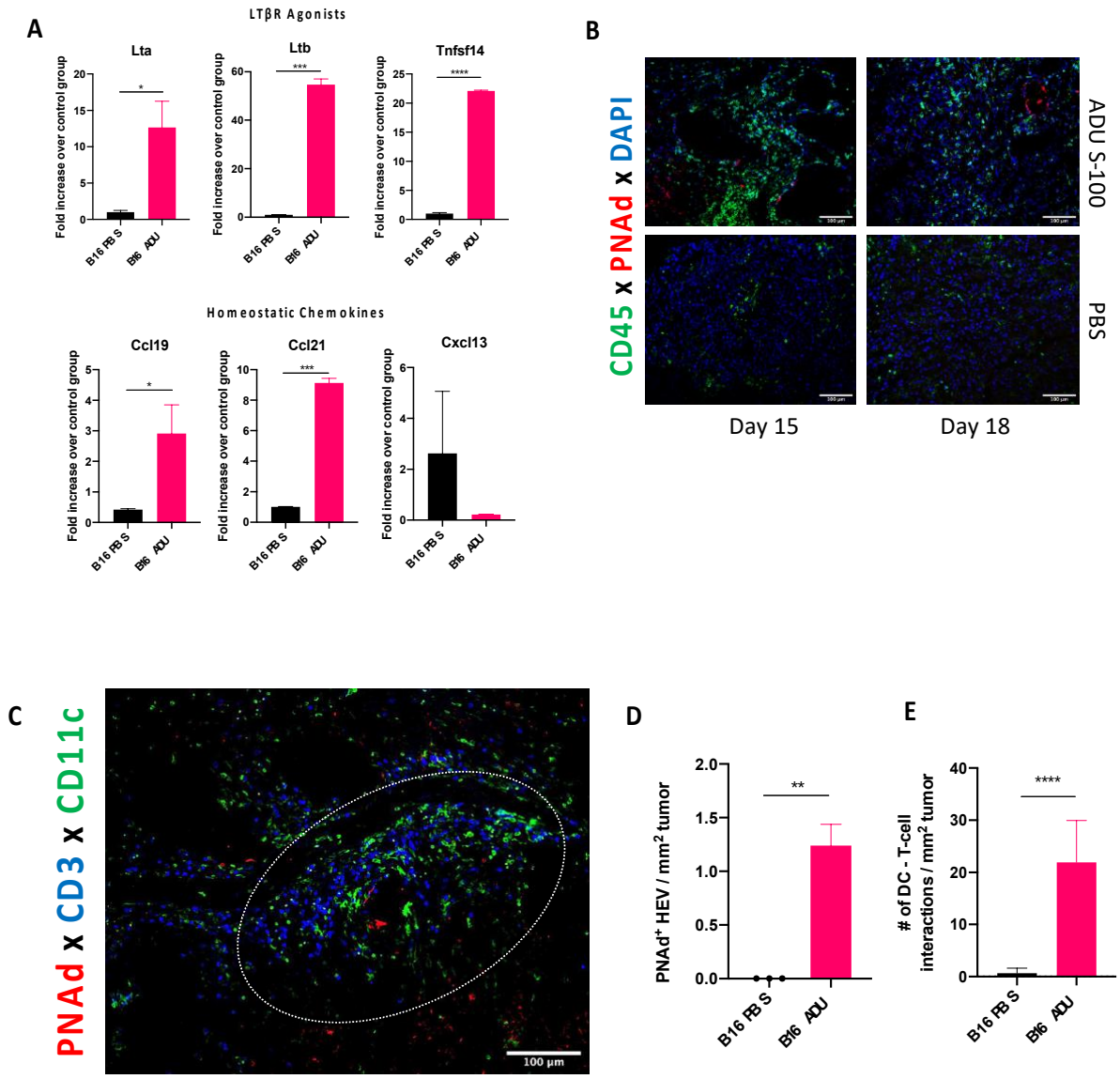

***Figure 13**: Low dose STING activation induces non-classical TLS formation in the therapeutic TME.*

A. *Post-treatment tumor digests obtained on day 18 show increased transcript levels of TLS inducing homeostatic chemokines (Ccl19 and Ccl21) and TLS inducing LTβR agonists (Lta, Ltb and Tnfsf14/Light).* ***B.*** *Representative immunofluorescent images showing TLS in ADU S-100 treated B16.F10 tumors resected on day 15 (2 injections completed) or on day 18 (3 injections completed) in comparison to PBS treated B16.F10 tumors lacking TLS.* ***C.*** *Representative image of ADU S-100 treated B16.F10 tumor resected on day 18 showing sting induced non-classical TLS composed of CD11c+ DCs and CD3+ T cells surrounding PNAd+ HEV.* ***D.*** *TLS formation quantitated using PNAd+ HEV density in PBS or ADU S-100 treated B16.F10 tumors. Data representative of three independent experiments.* ***E.*** *ADU S-100-treated vs control B16.F10 tumors demonstrate marked increase in number of physical contacts between infiltrating CD11c+ DCs and CD3+ T cells. *p < 0.05; **p < 0.002; ***p < 0.0002; ****p < 0.0001. Scale*

bar=100µm. DCs, dendritic cells; TLS, tertiary lymphoid structures; TMS, tumor microenvironment; TNF, tumor necrosis factor.

2.4.4 Host cell but not melanoma STING signaling drives the anti-tumor response, VN and TLS formation in the TME

In addition to innate immune cells, B16.F10 melanoma cells intrinsically express STING (data not shown) and could theoretically respond directly to ADU S-100. To parse out the role of tumor-intrinsic STING activation in the observed therapeutic responses, we administered low-dose ADU S-100 to WT or STING KO ($Tmem173^{gt}$) mice bearing established $STING^{+}$ B16.F10 melanomas (**Fig. 14A**). Interestingly, despite tumor-intrinsic expression of STING in both models, ADU S-100-based therapy failed to effectively treat (**Fig. 14B**), promote the development of TLS-associated $PNAd^{+}$ HEVs in the TME (**Fig. 14C**) or promote VN (**Fig. 14D**) in B16.F10-bearing STING KO mice (vs WT mice).

In extended studies, we determined that treatment of B16.F10 melanoma cells *in vitro* with ADU S-100 failed to promote tumor cell apoptosis (**Fig. 14E)** or tumor cell production of TLS promoting factors or canonical STING pathway gene activation (**Fig. 14F**). These data emphasize: i.) the selective importance of STING activation in host cells for the observed therapeutic effects of ADU S-100 administered into the TME and ii.) an apparent intrinsic defect in STING signaling in B16.F10 cells in response to ADU S-100.

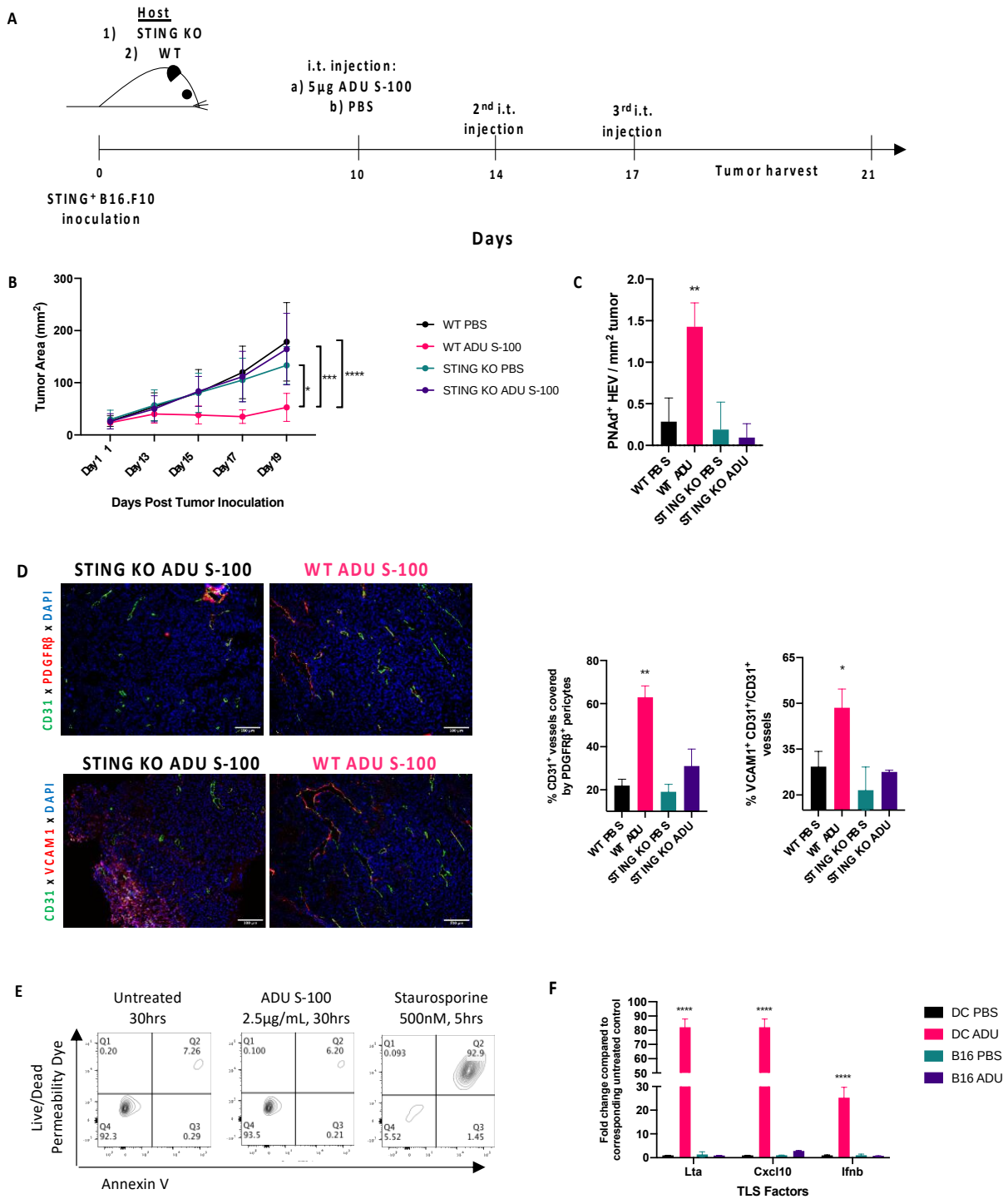

Figure 14: *Host STING expression is required for therapeutic VN, TLS neogenesis and treatment benefit.*

A. *Schematic representation of animal experiments performed using WT and sting KO (Tmem173gt) mice. Treatment timelines for PBS or ADU S-100 were identical as in previous experiments. All mice received S.C. injections of STING+ B16.F10 tumors. (n=5/group)* ***B.*** *Tumor*

*growth curves of WT and sting KO mice showing observed therapeutic effect in only the ADU S-100 treated WT host group. *p < 0.05, ***p < 0.0002; ****p < 0.0001, two-way ANOVA.* ***C.*** *Quantitation of HEVs in WT host or sting KO host receiving ADU S-100 or PBS **p < 0.002, one-way ANOVA.* ***D.*** *Representative images showing VN as a function of pericyte coverage and VEC activation in tumors resected from WT hosts, but not from sting KO hosts, treated with ADU S-100. *p < 0.05; **p < 0.002, one-way ANOVA.* ***E.*** *Representative flow cytometric plots from apoptosis assay on cultured B16.F10 cells confirming sting agonism is not directly tumoricidal.* ***F.*** *Quantitative rtPCR validation of the lack of response to sting activation in B16.F10 cells (as compared with responsive CD11c+ DCs). ****p < 0.0001, one-way ANOVA. Scale bar=100μm. ANOVA, analysis of variance; DC, dendritic cell; i.t, intratumorally; rtPCR, real time PCR; TLS, tertiary lymphoid structures; VN, vascular normalization.*

2.4.5 Therapeutic STING activation expands a TIL repertoire unique to the TME

ADU S-100 treated tumors exhibited increased infiltration by $CD3^+/CD8^+$ T cells **(Fig. 15A)**. To distinguish how therapy impacted the day 18 TIL repertoire, we performed TCRseq analyses of the TCRβ-CDR3 repertoires of TILs and animal-matched splenocytes isolated from STING agonist-treated vs. control-treated tumor-bearing mice.

Quantitative TCRseq comparisons demonstrated an increase in the ratio of T cells (i.e. TIL) per nucleated cell sequenced within ADU S100-treated tumor samples consistent with our flow cytometry data **(Fig. 15B)**. This metric also normalizes quantitative sequencing bias across all samples. We next compared productive TCR rearrangements, indicative of the number of distinct T cell clonotypes (as an index of population richness) within TIL and observed a significant increase in the number of productive TCR rearrangements within the ADU S-100 treated TILs when compared to control TILs **(Fig. 15C)**. This suggested that the STING-activated TME supports improved infiltration of divergent T cell clonotypes when compared to control tumors. To parse out the source of the therapy-associated repertoire observed within ADU S-100 TILs, we compared frequencies of clonotypes in ADU S-100 treated or control TILs with animal-matched splenocytes **(Fig. 15D)**. We hypothesized that local STING activation, by virtue of its induction of VN and TLS formation, would not only increase recruitment of clonotypes cross-primed in the periphery (as indexed in spleen), but also initiate *de novo*

expansion of unique clonotypes based on local T cell cross-priming within the TME. When expanded clonotypes (clonal count $\geq$ 10) were compared between animal-matched spleen and tumor specimens, we indeed observed significant increases in TIL clonotypes shared with spleen in ADU S-100 treated tumors vs control tumors, supportive of VN-enhanced recruitment of peripherally-expanded T cells (**Fig. 15E**). Further, when compared to control mice, we also observed a significant increase in the number of expanded clonotypes unique to the TME (vs. spleen) after treatment with ADU S-100 (**Fig. 15F**). These quantitative and compartmental changes in T cell clonotypes in ADU S-100- vs. control-treated animals resulted in an overall increase in oligoclonality of the therapeutic TIL TCR repertoire (**Fig. 15G**). These data support the notion of independent evolution of the therapeutic T cell repertoire in both the periphery and in the TLS^+ TME of ADU S-100 treated mice.

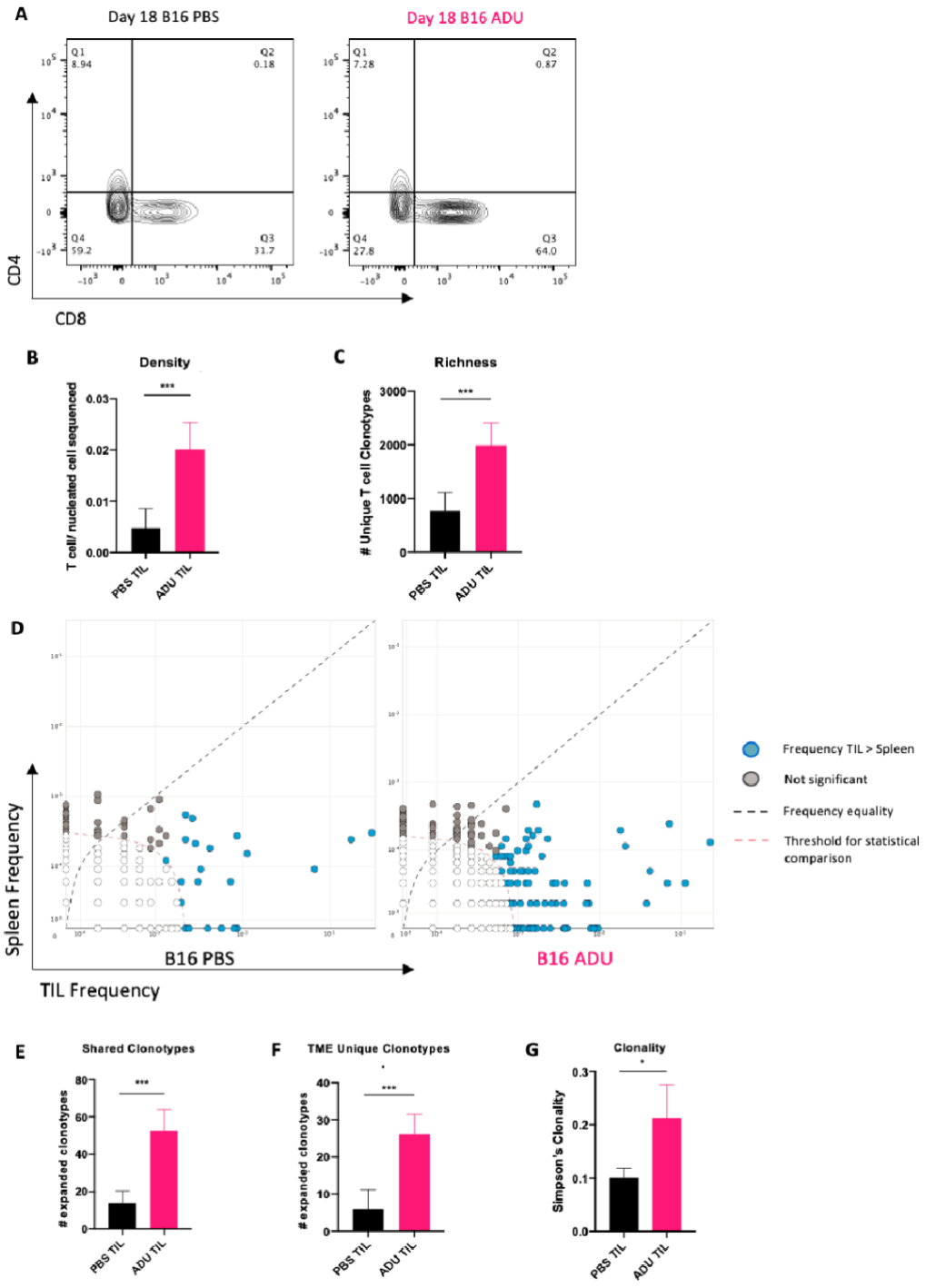

Figure 15: *Therapeutic STING activation expands a TIL repertoire unique to the TLS^{+} TME.*

A. *Representative flow cytometry plots from day 18 ADU S-100 treated or control tumors showing increased infiltration of CD8+ T cells post-STING activation.* ***B.*** *TCRseq analysis confirming increased T cell presence in ADU S-100 treated bulk tumor samples sequenced.* ***C.*** *TILs in ADU S-100 treated tumors characterized by increased populational richness (greater number of*

divergent clonotypes/sample). ***D.*** *differential abundance plots comparing relative frequencies of expanded clonotypes (using cut-off clonal count >10) between matched TILs and splenocytes.* ***E.*** *ADU S-100 treated tumors (vs control tumors) exhibit expansion in T cell clonotypes common to peripheral tissues (ie, spleen).* ***F.*** *ADU S-100 TILs (vs control TILs) contain expanded T cell clonotypes unique to the Tme.* ***G.*** *TILs in ADU S-100 treated tumors demonstrate increased clonality (more oligoclonal) compared with TILs from PBS-treated tumors. n=5/cohort. TCRseq differential abundance calculated using non-parametric two-tailed t-tests (bH ***< 0.0002; ****p<0.0001).*

2.5 Discussion

Novel findings presented in our report include the ability of low-dose STING agonist ADU S-100 to mediate therapeutic inhibition of melanoma growth by coordinately: i.) promoting tumor VN and lymphangiogenesis, ii.) stimulating $CD11c^+$ DC maturation and local production of VN- and TLS-promoting factors, iii.) facilitating enhanced immune cell infiltration and the induction of non-classical TLS formation (devoid of organized B cell regions, i.e. germinal centers) in the TME, and iv.) enhancing the quantity and richness of the TIL repertoire within the therapeutic TME of TLS^+ melanomas. The observed *in vivo* therapeutic benefits associated with ADU S-100 treatment required STING expression in host cells and were independent of intrinsic STING signaling in tumor cells. Indeed, STING signaling in the B16 and BPR murine tumor cell lines appears dysfunctional (Chelvanambi *et al., manuscript in preparation*), a finding consistent with recent published analyses of human colon carcinomas and melanomas[322].

Our data suggest a mechanism in which low-dose STING activation reprograms several aspects of the melanoma vasculature to confer immunotherapeutic benefit. First, by increasing local production of anti-angiogenic factors, STING activation helps normalize the melanoma vasculature to restore tissue normoxia and the functionality of these major conduits for recruitment of circulating immune cells. Our findings in melanoma models parallel observations by Yang and colleagues in lung carcinoma models for the ability of STING agonists to serve as conditioning agents to promote VN

and to synergize with therapeutic checkpoint blockade[323]. Second, STING activation promotes the local production of homeostatic chemokines and LTβR agonists to sponsor the formation of HEVs/tertiary lymphoid structures within the TME, where local (cross)priming of naïve/central memory T cells may take place. We show that both cultured BMDCs treated with ADU S-100 and $CD11c^+$ DCs isolated from the ADU S-100-treated TME exhibit improved maturation and enhanced production of factors supportive of TLS/HEV neogenesis, without excluding the possible additional contributions of other STING responsive (non-tumor) cell types found within the TME. Future studies using scRNAseq are expected to shed light on the selective/relative contributions of other cell types (macrophages, VECs, fibroblasts, stromal cells, etc.) for their roles in promoting VN and HEV/TLS formation.

Biophysically, VN and lymphangiogenesis together serve to reduce tumor interstitial fluid pressure, permitting improved trans-endothelial diffusion and convection of luminal contents including small molecule drugs and immune effectors into the TME[175]. Together with improved endothelial cell activation, STING agonist-treated vessels are expected to actively recruit and shuttle immune effectors into the tumor interstitium. Through HEV neogenesis and by virtue of approximating (DC-mediated) antigen cross-priming at source sites of antigen load, TLS are expected to improve TIL infiltration and local T cell cross-priming, leading to an expanded, diversified anti-tumor T cell repertoire.

Indeed, through analysis of the TIL TCRβ-CDR3 sequences, we observed an increase in the richness, clonality and uniqueness of the TIL repertoire of STING agonist-treated tumors vs control tumors, suggesting therapeutic benefits likely result from the participation of both peripherally and locally expanded TIL clonotypes. This finding may explain the widely observed improvement in efficacy of ICB when combined with STING agonists, wherein pre-conditioning tumors with STING activation may facilitate TLS formation and the development of locally expanded and diversified T cell

repertoire best adapted to react to antigenically heterogeneous tumor clonotypes[160 324-326]. Clonotypic comparisons of TILs with animal-matched splenic T cells revealed that TILs from ADU S-100 treated mice were significantly enriched with, both, peripherally shared clonotypes (primed within SLOs) and TME-unique clonotypes which qualitatively support the likely therapeutic relevance of VN and TLS neogenesis on-treatment with STING agonists. While our study, and a majority of TLS studies, cannot conclusively demonstrate that the detected TME-unique T cell clonotypes were cross-primed within therapy-induced TLS, our findings are consistent with other studies linking TLS and local T cell cross-priming[250 327] and the diversification of disease-relevant T cell repertoires via an epitope spreading paradigm[328]. Future studies characterizing the tumor antigen specificity of engineered T cells bearing the TME/TLS-restricted CDR3 sequences as found within ADU S-100 treated tumors may provide additional support for their therapeutic relevance and contribute to the design of novel adoptive cell therapy approaches in the melanoma/cancer setting.

Recently, B cells have been reported to represent a positive prognostic biomarker in human solid cancers by virtue of their production of anti-tumor antibodies and their ability to serve as tumor-resident APCs[329-333]. B cells have also been reported to promote a pro-TLS tissue microenvironment based on their robust production of LIGHT/TNFSF14[334]. However, in our studies, we did not observe significant B cell infiltration, germinal center (GC) formation or the development of classical TLS **(Fig. 11B)**. Consistent with this finding, neither our GSEA of ADU S-100-treated DCs nor transcriptional analyses of tumor specimens identified pathways relevant to B cell recruitment/infiltration or the initiation of humoral responses **(Fig. 10A, Fig. 11A).** Furthermore, we found no evidence for increased local production of the B cell homeostatic chemokine CXCL13 post-treatment with ADU S-100 in DCs (**Fig. 9G**) or tumors (**Fig. 13A**). Given these findings, treatment strategies combining STING agonists with agents that promote B cell, follicular DC (FDC) and/or T_{FH} recruitment into the TME might lead to the

more effective formation of classical mature TLS in the TME, resulting in enhanced treatment benefit. Candidate agents that activate TLR7/9 on DCs[335 336] or that block DNMT1 (decitabine) in the TME to promote enhanced CXCL13 production could be considered for use in such combination protocols[337]. However, one should also carefully consider previous reports linking B cells with tumor progression[338-340,341]. Regardless of the ultimate role for B cells in a therapeutic TLS paradigm, it is noteworthy that in humans, the presence of either classical/mature or non-classical (i.e. B-deficient) TLS in the TME correlates with improved patient outcome when compared to patients with tumors that fail to exhibit TLS[320].

While our studies were not specifically designed to identify mechanisms underlying resistance to i.t.-delivered STING agonist-based monotherapy that may have led to modest clinical activity in early-phase clinical trials, we observed that the treatment of DCs with ADU S-100 resulted in the compensatory upregulation of several known regulatory molecules that would be expected to mediate anti-inflammatory activity and thereby limit the therapeutic anti-tumor immune response. Notably, we observed that PD-L1, Ptgs2/COX2, Ptges and Arg2 expression were strongly upregulated on STING-activated DCs, suggesting these APCs may not mediate optimal/sustained immunostimulatory activity *in vivo* (**Fig. 16**)[342]. Other pre-clinical studies have indeed demonstrated therapeutic synergy when combining STING agonists with checkpoint inhibitors[324-326] or COX-2 inhibitors[305] *in vivo*, suggesting that antagonism of immunoregulatory pathways induced by STING activation might prove crucial for successful treatment of multifocal, advanced-stage disease. We are currently investigating the therapeutic impact of combined treatment with STING agonists + anti-PD-L1 and/or inhibitors of PTGES, PTGS2/COX-2 and ARG2 to determine impact on VN, TLS formation, TIL repertoire and tumor growth in our murine melanoma models.

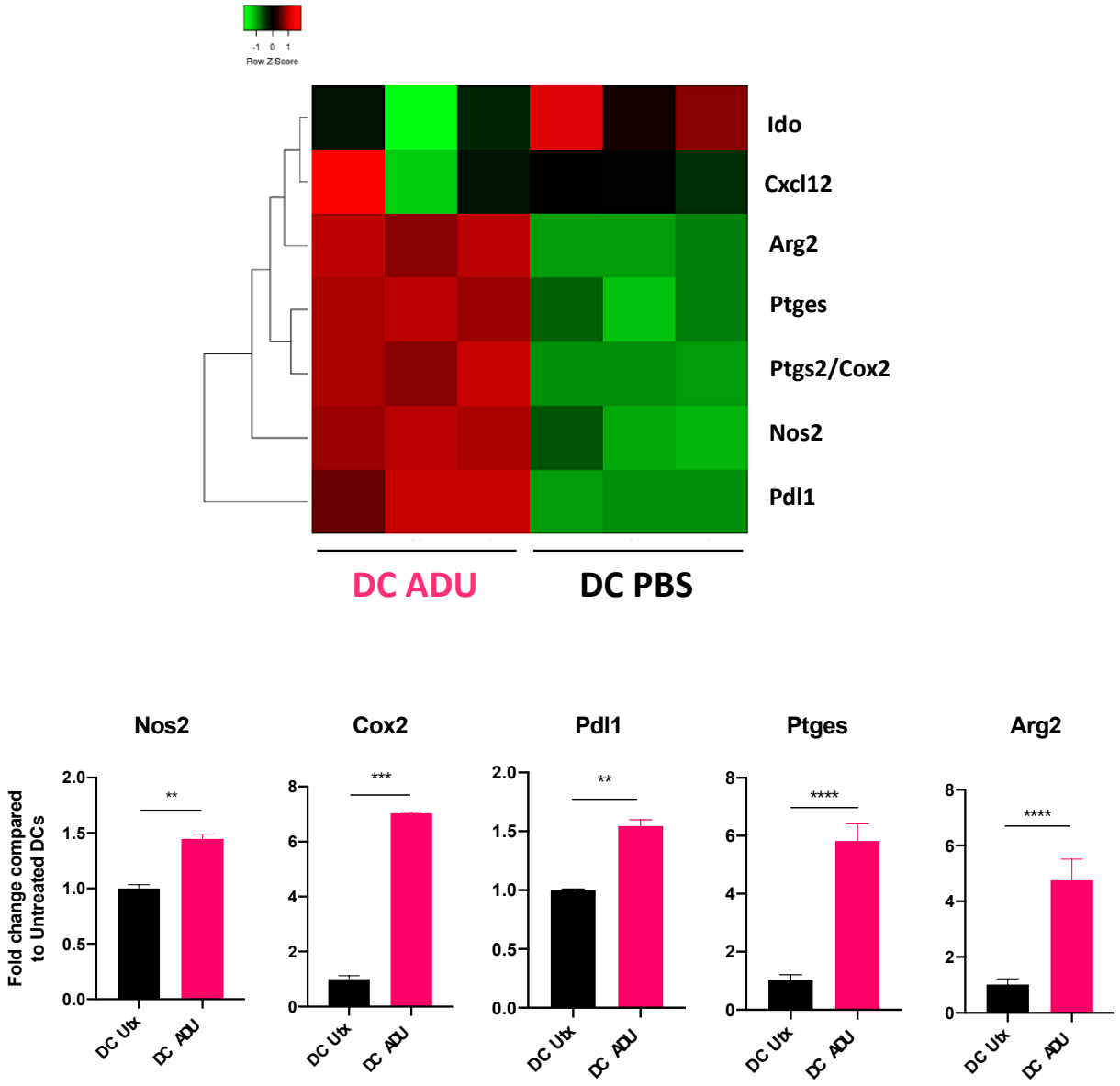

Figure* 16*: *STING mediated inflammation concomitantly upregulates expression of immune regulatory molecules by CD11c+ DC.*

A*. Transcriptional profiling of isolated CD11c^{+} DC treated with ADU S-100 vs. control media for immunoregulatory gene products including Arg2, Nos2, Pdl1, Ptges and Ptgs2/COX-2. Specific transcript levels determined by qRT-PCR as in* ***Fig. 3****. *p-value < 0.05, unpaired t-test.*

Finally, a number of studies, including ours, have highlighted the local therapeutic benefits of intratumoral STING activation using second-generation agents (i.e. MIW-815/ADU S-100, E7766),

but these interventions lead to only modest therapeutic impact on distal, untreated lesions in multifocal disease models in which only one tumor is treated (**Fig. 17**), Furthermore, systemic (i.p.) delivery of ADU S-100 fails to effectively treat s.c. B16.F10 tumors *in vivo* (data not shown). Given these logistic limitations for second-generation STING agonists, there is significant enthusiasm for future investigation of next-generation small molecule STING agonists designed for systemic delivery that have entered evaluation in early-phase clinical trials (i.e. SB11285 and GSK3745417). These agents will enable further testing of our proposed therapeutic paradigm in models of multifocal, disseminated melanoma treated i.v./i.p. with low doses of STING agonists.

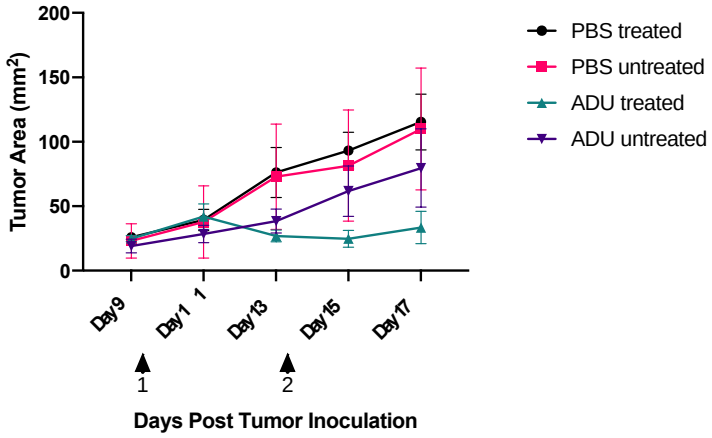

Figure 17: *Lack of prolonged systemic response with ADU S-100 in bilateral B16.F10 models.*

B16.F10 tumor growth curves from pilot trials showing lack of extended therapy in un-injected left flank tumors of mice receiving 5μg ADU S-100 (i.t.) in right-flank tumors.

Antigen	Clone	Vendor	Concentration
CD3 Alexa Fluor 647	17A2	BioLegend	1:50
CD45 Alexa Fluor 488	30-F-11	BioLegend	1:100
CD11c Alexa Fluor 488	N418	BioLegend	1:100
PNAd Purified	MECA 79	BD Pharmingen	1:100
CD31 Alexa Fluor 647	MEC 13.3	BioLegend	1:50
B220 FITC	RA3-6B2	Pharmingen	1:100
Lyve-1 Purified	ALY7	Invitrogen	1:100
PRGFRβ PE	APB5	Invitrogen	1:100
VCAM1 Purified	AF643	R&D Systems	1:100
Lectin Alexa Fluor 488	DL-1174	Vector Labs	200 µg/mouse
CD133	N/A	Santa Cruz Bio	1:100
Jarid1b	N/A	Abcam	1:500
pIRF3	CST 29047S	CST	1:1000
β-Actin	ab6276	Abcam	1:10000

Table 4: *List of antibodies and corresponding concentrations used for immunoblotting and immunofluorescence experiments*

Target	Forward Primer	Reverse Primer
Ifnb	TGGGAGATGTCCTCAACTGC	CCAGGCGTAGCTGTTGTACT
Lta	GCCCATCCACTCCCTCAGAA	TGCTGGGGTACCCAACAAGG
Ltb	GGACGTCGGGTTGAGAAGAT	ACGGTTTGCTGTCATCCAGT
Cxcl10	ATGACGGGCCAGTGAGAATG	TCGTGGCAATGATCTCAACAC
Ptgs2/Cox2	GGGCCCTTCCTCCCGTAGA	TGAGCCTTGGGGGTCAGGGA
Nos2	TCCTGGACATTACGACCCCT	CTCTGAGGGCTGACACAAGG
Pdl1	TCACTTGCTACGGGCGTTTA	ATCGTGACGTTGCTGCCATA
Ccl19	CCTGGGAACATCGTGAAAGC	TAGTGTGGTGAACACAACAGC
Ccl21	GTGATGGAGGGGGTCAGGA	GGGATGGGACAGCCTAAACT
Cxcl13	TCTCCAGGCCACGGTATTCT	GGGGCGTAACTTGAATCCGA
Tnfsf15	GACTGTATGCTTCGGGCCAT	ATTGTCAGGTGTGCTCTCGG
Arg2	ATCGGCTGATTGGCAAAAGG	AATCCCCCTACAACAGGGGT
Ptges	GCTGCGGAAGAAGGCTTTTG	GCTCCACATCTGGGTCACTC

Table 5*: List of qPCR primers used*

2.6 Acknowledgements

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Conflict of Interest: The authors report no competing interests.

Data Availability: All data published in this report is available upon reasonable request.

Ethics Approval: All animal experiments were performed in accordance with the guidelines approved by the University of Pittsburgh Division of Laboratory Animal Resources (DLAR) and Institutional Animal Care and Use Committee (IACUC).

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3.0 General Discussion

3.1 Thesis summary

T-cells are extremely efficient in the surveillance and rejection of transformed cancer cells due to their ability to mount specific and potent cytotoxic anti-tumor responses. Given this, improved T-cell infiltration into the TME is a strong indicator of functional tumor immunosurveillance *in situ* and desirable clinical outcomes [138 343 344]. However, cancers evolve cellular and molecular mechanisms (both intrinsic and conferred to stromal cells) to limit interaction with and recognition by T-cells, as discussed in detail in the introduction to this thesis. Importantly, T-cell access to the tumor relies upon vascular networks which are known to be both structurally and functionally abnormal in solid cancers thus implicating the aberrant tumor vasculature as a major culprit for T-cell exclusion. Therefore, strategies that disrupt pathologic angiogenesis within the TME are expected to 'normalize' the tumor vasculature to promote T-cell infiltration into immunologically cold tumors and several such strategies are also discussed in the introduction to this thesis. In this regard, agents that activate STING, an innate immune sensor for cytosolic DNA, have recently demonstrated strong angiostatic potential as evidenced by significant vaso-ablation with non-therapeutic near-MTD dosing. Subsequent dosing studies have instead shown remarkable T-cell mediated tumor protection with sub-MTD doses of STING agonists but therapeutic changes to the tumor vasculature with such modified dosing regimen have not yet been characterized. Therefore, given the strong angiostatic potential of STING activation, we hypothesized that treatment with low, sub-MTD doses of STING agonism will lead to 'normalization' of the tumor vasculature and of the resultant therapeutic immune landscape, leading to sustained local inflammation necessary for successful T-cell mediated tumor clearance.

Indeed, in this thesis, I have shown that provision of metronomic low doses of STING agonists promote anti-tumor responses by significantly improving the TIL footprint within murine melanoma lesions. I considered two important observations to design my first hypothesis to explain the observed improvement in T-cell infiltration on-treatment. First, melanomagenesis is driven by rapid neoangiogenesis involving the integration of structurally- and functionally defective blood vessels. Second, tumor endothelial cells demonstrate a heightened sensitivity to STING-associated inflammation, with near-MTD doses of STING agonists ablating endothelial cells [269 306 345] found within the TME. Considering that T-cells depend on blood vessels to enter the TME, and in light of the therapeutic vascular paradigms proposed by Rakesh Jain and colleagues[175 176], I hypothesized that poor baseline T-cell infiltration in melanoma can be attributed to the dysfunctional vessels and that the functional normalization of these conduits with low-dose anti-angiogenic STING agonists will underlie the enhanced T-cell infiltration achieved on-treatment. In support of this hypothesis, I first observed that intratumoral activation of STING led to increased transcript levels of anti-angiogenic factors with no noticeable changes in pro-angiogenic factors within the TME, suggesting that low-dose STING agonism sponsored a generally angiostatic tumor microenvironment. I next probed for structural and functional biomarkers of vascular normalization (as proposed by Jain et al.) such as vessel perfusion and pericyte coverage of tumor vasculature[175 176] and indeed observed that tumor vasculature on-treatment with ADU S-100, developed vasculature that had, both, significantly improved luminal perfusion and enhanced abluminal coverage of endothelial cells by mural pericytes. Further since TNFα and type-I IFN were upregulated with STING activated TME, and given their independent roles as activators of endothelial cells [346-348], I probed for markers of vessel activation and found a significant increase in VCAM-1^{+} activated endothelia on-treatment. In addition to these functional

improvements in the blood vasculature, I also observed an increase in density of Lyve-1$^+$ lymphatic vessels on-treatment which further normalizes the TME by draining pooled interstitial plasma to relieve high tissue pressure [176]. These findings suggest that STING activation results in an overall reprogramming and activation of tumor vascular networks. Consistent with occurrence of such reprogrammed tumor vasculature on-treatment, I observed a significant reduction in tissue hypoxia as evidenced by decreased transcript levels of Hif1α and Hif2α and decreased surface expression of hypoxia responsive cancer stem cell markers Jarid1b and CD133 within the ADU S-100 treated TME [204]. Together, STING agonism normalizes the TME by improving vessel functionality and reducing tissue hypoxia which may synergistically improve T-cell infiltration and/or function within the TME.

Interestingly, in addition to improved T-cell infiltration, I also observed a significant increase in intratumoral CD11c$^+$ DC in STING activated tumors. Given the contextual ability of CD11c$^+$ DCs to skew inflammatory responses [349], I next characterized the transcriptional changes associated with STING activated DCs to further investigate the nature of the local immune response induced by such STING conditioned tumor infiltrating APCs. Using DNA microarray analyses, I found that STING activation in CD11c$^+$ DCs promoted their acute activation and maturation as previously reported in the literature. But most remarkably, GSEA performed on the transcriptional dataset predicted STING activated DCs to be involved in the formation and maintenance of lymphoid tissues. Such ectopic lymphoid aggregates in peripheral tissue sites are referred to as tertiary lymphoid structures (TLS) which provide a site for lymphocytes to interact with APCs for enhanced local (re)priming [230 240]. This suggested that intratumoral STING activation, at least in part by conditionally activating infiltrating DCs, could promote the formation of TLS within the TME. In line with observing no increase (and possibly a decrease) in the local

production of CXCL13 with STING agonism, I postulated that any induced TLS with ADU S-100 would be of the non-classical type i.e., that they would lack germinal center B-cells. Indeed, through histological characterization, I observed non-classical TLS in ADU S-100 treated tumors that were characterized by the aggregation of T-cells and DCs, but not B-cells, around $PNAd^{+}$ HEVs within the STING-activated TME. These changes were strictly dependent on the activation of host STING since tumor bearing STING KO hosts resembled control WT hosts with regards to all therapeutic biomarkers considered (tumor growth, VN and HEV/TLS neogenesis).

Since STING activation promoted, both, the normalization of existing tumor vasculature and induction of lymph node-like vasculature, I proposed that these changes would importantly impart a qualitative difference to the TIL repertoire in addition to the previously observed quantitative differences. Based on this working understanding, I next hypothesized that the TIL repertoire on-treatment with ADU S-100 would be characterized by 1) An improved infiltration of peripherally shared clonotypes as a result of normalizing tumor vasculature 2) An enhanced expansion of a TME unique repertoire as a result of promoting local priming via HEV/TLS neogenesis. To test this hypothesis, I profiled the clonotypic identities (qualitative) and associated quantities of each TIL clonotype with regards to its distribution in the periphery (indexed in spleen) by sequencing TCRβ-CDR3 identities in each sample. The data from these sequencing assays supported my hypothesis by demonstrating, both, quantitative and qualitative differences in TIL repertoires. Briefly, I observed 1) a significant increase in the number of expanded TIL clonotypes that were also indexed in animal matched, paired spleens suggesting that therapy with STING agonists promoted enhanced infiltration of T-cells from the circulation 2) a significant increase in the number of expanded TIL clonotypes that were unique to the TME suggesting an independent evolution of a TIL repertoire unique to the STING activated TME. Associated with these

quantitative and qualitative differences, STING activated TIL repertoires demonstrated increased population richness and increased oligoclonality suggesting that therapy with ADU S-100 was associated with the participation, local expansion and independent evolution of divergent T-cell clonotypes in the TLS^+ TME.

Overall, the work presented in my thesis proposes that therapeutic T-cell responses observed with low-dose STING agonism in melanoma can be attributed to fundamental changes to the existing tumor vasculature as well as induction of a specialized lymph node-like neovasculature (HEVs). This creates an actionable TME for the enhanced infiltration and local function of immune cells; especially $CD8^+$ T-cells. Further, local activation of host cells, including but not limited to $CD11c^+$ DCs, changes the local inflammatory landscape to support the formation of non-classical, B-cell devoid, TLS within the TME where newly infiltrating T-cells may encounter novel epitopes/antigens presented by tumor associated APCs. These observations crucially offer novel insights for STING-based therapeutic regimen by highlighting the therapeutic involvement of reprogrammed vasculature that sponsor the function of both, peripherally- and locally expanded clonotypes within the STING-activated TLS^+ melanoma TME (summarized in **Appendix fig. 1**).

3.2 B-cells and germinal centers in TLS

While we did not expect nor observe significant changes in B-cell infiltrates in our model, recent evidence suggests B-cells, especially when organized in germinal centers within TLS, might be beneficial for the control of tumor progression. In a hallmark study, Helmink *et al.* demonstrated that B-cell signatures correlated positively with clinical outcome in metastatic melanoma and colorectal carcinoma patients receiving ipilimumab and nivolumab combination treatments. In these patients, histological examination of tumor biopsies showed B-cell aggregates that closely resembled the germinal centers found in SLOs [232]. B-cells in these tumor-resident germinal center

(GC) demonstrated an increased clonal expansion, increased total BCR diversity, enhanced proliferation (as a function of Ki67 expression) and evidence of class switching from IgM expressing naïve B-cells to IgG expressing class-switched B-cells when compared to non-GC B-cells found within the TME [232]. T-cells in TLS^+ melanoma patients also benefitted from associating with GC B-cells as was evidenced by the elevated signatures of T-cell activation markers CD44, GZMB and 4-1BB when compared to disperse T-cell infiltrates found outside of GC B-cell zones [232]. This suggests that B-cells within TLS retain an active role in the ongoing anti-tumor response but the extent of their involvement i.e., through direct 'helper-like' cellular responses or through indirect systemic humoral responses, is yet to be characterized. In most solid cancers, attributing tumor reactivity and specificity of such TLS B-cells is made complicated by the challenges in associating BCR identities to the three-dimensional, non-contiguous epitopes that B-cells typically recognize on tumor antigens.

However, the unique epidemiology of head and neck squamous cell carcinoma (HNSCC) provides an excellent model to study the functional role of antigen specific TLS B-cells since the human papilloma virus (HPV) serves as an etiological agent for a small cohort of HNSCC patients. Besides inducing an inflammatory TME for the neogenesis of TLS, HPV driven cancers contain viral antigens, whose identities are better characterized, against which anti-tumoral B-cell and T-cell responses can be monitored. Indeed, TLS with GC B-cells are found more frequently in HPV^+ HNSCC patients than in HPV- HNSCC patients [234 350]. In HPV^+ patients, GC^+ TLS correlate with better clinical prognoses and B-cells found within the TLS differentiate to produce large quantities of antibody (hence called antibody secreting cells or ASCs)[234], which suggests that antibody promoting responses within the TME could be a harbinger of good clinical response[351]. In extension of these findings, antibodies sequenced from TLS^+ patients showed evidence of having

undergone extensive somatic hypermutation[352] and class switching from IgM/IgD to IgG1 and IgG2/3 [234] and demonstrated an enhanced ability to recognize non-structural HPV proteins E2, E6 and E7. Together, these observations suggest that B-cell priming against HPV antigens within the TME (presumably within such classical TLS) leads to the development of a protective systemic humoral response[234]. It is therefore within reason to expect the TLS generated humoral responses to confer systemic immunity through neutralization of pro-tumoral proteins, induction of phagocytosis[353 354], ADCC [355 356] and complement formation [357-360] to support tumor clearance. Intriguingly, TLS B-cells selectively produce IgG1 antibodies which capably induces type-1 inflammatory responses via the activation of FcGR1A on NK cells and macrophages for ADCC and phagocytosis respectively [361-363]. In addition, B-cells found within such TLS could also serve as APCs to effector T-cells, provide antigen-independent help with T-cell activation [364] and/or promote local DC functionality through enhanced expression of CD40L [365-367]. Collectively, these studies present compelling evidence in favor of promoting the recruitment of B-cells to the TME to improve current cancer therapies.

Although the above instances highlight the role of GC B-cell aggregates and classical (mature) TLS in cancer, non-classical (immature) forms of TLS characterized by different participating immune cell types have been observed in cancer [244 368-370] where such B-cell devoid TLS, quantified by $PNAd^+$ HEV density and/or TLS associated cytokine signatures[238 239], also correlate with better clinical outcomes. Consistent with the putative functional role of either type of TLS, my work demonstrates the development of a unique TIL repertoire within the TLS^+ ADU S-100 treated TME which was not evidenced in the periphery. We would thus propose that combination regimens, like with the inclusion of TLR7 agonists [335 336 371], that promote the local expression of

CXCL13 may be required to achieve classical B-cell^{+} TLS formation in therapeutic regimens using STING agonists.

3.3 Lack of systemic clinical benefit with local STING agonism

While STING activation significantly improved local therapeutic responses in the injected lesion, an important question for locally delivered drugs, such as ADU S-100, in metastatic disease lies in whether therapy confers systemic protection against metastatic growth or in the setting of multi-focal disease. This is particularly important for clinical feasibility and translatability since distal lesions are often found in inaccessible sites, such as the brain and lungs. To address if intratumoral ADU S-100 injections conferred systemic protection, we performed pilot studies on mice harboring established B16 melanomas, treating only the right flank tumor with i.t. PBS or ADU S-100 (5 μg/tumor on days 10 and 14). Although the contralateral un-injected tumors in ADU S-100 treated animals grew at a slightly slower rate vs. PBS control-treated mice up to day 13 ($p=0.06$), these tumors eventually progressed after treatment discontinuation on day 14 and paralleled the growth of control PBS tumors on both flanks (**Fig. 17**). This suggests that although early immune responses arising from intratumoral STING activation results in slowed tumor growth of distal lesions, the prolonged efficacy of the anti-tumoral immune response appears to be limited. It may be possible that innate immune activation of NK cells and macrophages may be responsible for this acute control. However, we believe such promising early responses may be further extended temporally and/or accentuated via combination immunotherapy approaches integrating local or systemic STING agonists, checkpoint blockade to reinvigorate activated T-cells and/or other immune-potentiating agents that are upregulated in response to STING activation. Interestingly, since other groups have demonstrated efficacious control of bilateral tumors of breast and colon cancer origins with single lesion intratumoral injection of ADU S-100,

it may be possible that interlesional heterogeneity in melanoma might represent a significantly greater hurdle than that observed in other tumors. One may speculate that inherent properties of melanomagenesis, including aggressive growth, loss of PTEN and/or dysfunctional angiogenesis, could be candidate causative factors but further validation with systemic STING agonists are necessary to address these challenges.

3.4 Intrinsic STING signaling defects in melanoma cells

Comparison of the tumor growth kinetics in STING KO and WT hosts receiving ADU S-100 conclusively demonstrated that STING activation in the host was sufficient and necessary for the anti-tumor responses observed with ADU S-100 (**Fig. 14B**). Contrastingly, this also suggested that B16.F10 tumors might be refractory to STING activation since STING KO hosts receiving ADU S-100 completely failed to develop a therapeutic response. Based on follow-up pilot data and findings already presented in the published literature, we propose that tumor intrinsic STING signaling defects might be caused by one or more of the following reasons.

3.4.1 STING or TBK1 degradation

Studies analyzing anti-viral responses highlight several pathways that regulate STING protein expression to limit the development of irAEs. These maybe extended to the tumor setting as possible axes of resistance. First, P62/SQSTM1 is a protein involved in the STING signaling cascade that is also phosphorylated by TBK1 but serves to chaperone ubiquitinated STING to the autophagosome for degradation[372]. Accordingly, stimulation of P62 deficient cells with extracellular DNA shows defective shuttling of STING into autophagosomes resulting in chronic STING signaling and an associated increase in IFN-I secretion in vitro[372]. Considering this evidence, gain of function mutations in tumor associated P62 could be one causative factor for

poor STING response in melanoma. Second, STING signaling in melanoma may be attenuated downstream of STING and at the level of TBK1 through the increased expression of proteins implicated in TBK1 degradation such as SOCS3 [373-375], DDX19[376] and TRAF3IP3[377]. Of these proteins, SOCS3[378 379] and TRAF3IP3[380] have been previously found to be highly expressed in melanoma and may be responsible for STING dysfunction. If true, these proteins factor as novel targets for therapeutic intervention to reconstitute tumor intrinsic STING signals.

3.4.2 Defects in IRF3 phosphorylation

While comparing B16.F10 melanoma cells to syngeneic bone marrow-derived dendritic cells (DC), we found that although both cell types express sufficient levels of STING, TBK1 and IRF3 proteins, only DCs stimulated with ADU S-100 were competent to induce the activation/phosphorylation of IRF3, suggesting that melanoma cells exhibit a signaling defect downstream of STING but upstream of IRF3 activation (**Appendix fig. 2**). Signaling downstream of STING could theoretically be silenced by i.) structural mutations in STING that preclude its effectiveness in serving as a scaffold for TBK1-IRF3 interaction necessary for the phosphorylation of IRF3[288] or ii.) overexpression of inactivating protein tyrosine phosphatases (PTPs), such as PTPN1/PTP1B, PTPN2, PTPN11/SHP2, Cdc25A, MAPK phosphatase 5/DUSP10, each of which has been reported to interact with STING, TBK1 and/or IRF3[381-385]

3.4.3 Defects in transportation and degradation of DNA/CDN

It is also entirely possible that events independent of and unrelated to the STING signaling cascade could dictate tumor STING dysfunction. Two potential hypotheses to test this idea include: i) melanoma cells contain/acquire loss of function defects at the level of DNA/CDN membrane transporters necessary for adequate cellular uptake of STING agonists and ii) melanomagenesis is associated with gain of function mutations at the level of proteins involved in

the intracellular degradation of DNA. Firstly, previous reports have suggested that aggressive melanoma growth is accompanied by a change in surface expression of membrane transporters that support tumor progression[386]. While it was clear that extracellular CDNs possessed the ability to be taken up by cells, the exact identity of the membrane transporter responsible for their uptake remained largely unknown. In a recently published study using CRISPR knockdown libraries, SLC19A1 was identified as the transporter that is primarily responsible for the intracellular uptake of both, cGAMP and CDNs[387]. SLC19A1 deficient THP-1 monocytic cells with intact STING, TBK1 and IRF3 lose all downstream signaling and IFN-I production in response to extracellularly administered CDNs[387]. Considering this, it would be of interest to evaluate the expression and/or functional status of SLC19A1 in melanoma to address if functional defects with transporting CDNs is a causative factor for STING dysfunction. Alternatively, connexons play important roles in transporting cGAMP/CDNs between cells via the gap junctions they polymerize to form [388]. If mutations/functional changes in these proteins are indeed the drivers of STING dysfunction in melanoma, targeted genetic correction/rescue can be proposed to coerce melanoma cells to respond to STING agonists to ultimately improve the efficacy of STING agonists for the clinical control of cancer.

Second, exonucleases are responsible for the timely degradation of genetic material in order to avoid overactivation of DNA repair and DNA sensing pathways [389]. Overexpression of DNA exonucleases could lead to a reduced half-life of second messengers cGAMP and CDNs thus placing temporal restraints on events preceding STING activation. In this sense, Trex1 is one such exonuclease that is overexpressed in melanoma[390] and therefore represents one of many possible candidates to be tested through this hypothesis. While it is unclear if CDNs, like dsDNA, are susceptible to the catabolic activity of exonucleases, it would be of great scientific/therapeutic

interest to determine if Trex1 overexpression in melanoma correlates with STING dysfunction and/or failed clinical response.

3.4.4 Epigenetic silencing of STING responsive elements

A final possibility lies at the level of epigenetic modifications at IRF3/7-responsive promoter regions. In human colorectal carcinoma, dysfunction in intrinsic STING signaling was observed to mainly involve the suppression of STING expression via epigenetic regulation, with mutation or deletion events in these genes only rarely observed [322] [391]. Although STING transcripts have been reported to be reduced in ~50% of human melanoma cell lines tested [392] as in the case of our report, Mulé and colleagues [393] determined that ADU S-100 can fail to activate $STING^{+}$ human melanoma cell lines via an as yet unknown mechanism. However, using a general DNA demethylation approach by treating mice harboring B16.F10 tumors with ADU S-100 and a DNMT1 inhibitor, Decitabine, we observed a slightly synergistic reduction in the growth rates of the tumor in pilot studies (**Appendix fig. 3**). In vitro profiling of B16.F10 cancer cells showed elevated expression of IFN-I and CXCL10 in ADU S-100 + Decitabine treated cancer cells suggesting B16.F10 cells broadly possess epigenetic roadblocks downstream of the STING signaling cascade (**Appendix fig. 3**). Regardless, epigenetic modifications possibly represent only one part of the bigger challenge with activating STING in melanoma since ADU S-100 + Decitabine treated melanoma cells still fail to upregulate several STING associated cytokines such as lymphotoxin alpha.

While the data presented in this thesis has analyzed this phenotype in detail for B16.F10 melanoma, the primary tumor model used in my dissertation research, scope-expanding pilot studies have observed a similar STING signaling defect in 2 unrelated $BRAF^{V600E}PTEN^{-/-}$ melanoma cell lines (BPR, YUMM3.3) after treatment with ADU S-100 *in vitro*, suggesting

STING dysfunction may represent a general paradigm amongst murine melanomas. Current research in our lab is investigating each of these possibilities in our 3 murine melanoma cell lines including the functional assessment of specific gene knock-down/overexpression and/or pharmacologic inhibition experiments and plan to develop and publish our mature results in a future report.

Regardless, the confounding nature of STING biology in host cells vs tumors does not diminish the therapeutic value of ADU S-100/STING agonism since these treatments still sufficiently promote VN and HEV/TLS formation in a manner that does not require the involvement of melanoma STING. In these circumstances, clearly STING expression in host tumor-associated stromal cells appears to be responsible for the therapeutic alterations observed in the melanoma TME. At present, we cannot implicate specific host cell subpopulations which would require development and implementation of targeted STING-KO mouse models and/or scRNAseq explorations which are planned for future investigations.

4.0 Future Perspectives

4.1 Future of STING agonists

STING agonists have become attractive agents for the treatment of cancer, yet the modest success or intratumoral delivery approaches in the clinic has energized efforts to develop improvements in targeted drug design for enhanced tolerability and efficacy in the disseminated disease setting [394]. Successful development of the first-generation STING agonist DMXAA enabled pre-clinical studies designed to test the role of STING activation as a paradigm for effective anti-tumor therapy [304]. Intriguingly DMXAA demonstrated only modest efficacy in human studies due to its inability to bind human STING variants which possess a different conformation than murine STING [281 282 395]. Further, DMXAA and natural second messenger cGAMP show poor bioavailability given their increased susceptibility to the phosphodiesterases found in the circulating blood thus limiting their systemic distribution[396-398]. ADU S-100, a CDN derivative used in all the studies presented in this thesis, addresses these shortcomings by robustly binding both human and murine STING and demonstrating a significantly prolonged half-life in systemic circulation [303]. However, results from early clinical trials suggest that although these improvements are sufficient to induce moderate responses in patients, ADU S-100 remains limited to approaches involving local delivery via direct intratumoral injection (NCT03937141) and other second-generation STING agonists such as E7766 share a similar fate of being restricted to local delivery applications (NCT04144140). These delivery restrictions have also limited use of ADU S-100 and E7766 to trials enrolling patients with at least one treatable cutaneous lesion which excludes a variety of solid cancers where primary lesions are often surgically inaccessible. Novel STING agonists such as Spring Bank Pharmaceuticals' SB11285 and Glaxo Smith Kline's GSK3745417 have demonstrated improved stability in circulation in pre-clinical models where

intratumoral, intraperitoneal and/or intravenous injections confer protective immune responses against multiple transplantable models of solid cancer. Successful early phase clinical trials using these systemic STING agonists would significantly improve adoptability of STING agonism in stand-alone or combination immunotherapy trials. Ultimately, these agents will enhance the testing of the proposed therapeutic paradigms discussed in this thesis especially as they pertain to multifocal/metastatic disease.

4.2 Targetable immunoregulatory pathways with STING activation

Through our GSEA, we observed the upregulation of several compensatory immunoregulatory molecules including COX2, PDL1, IDO, ARG2 and PTGES with STING activation and propose that the targeted inhibition of these axes using commercially available inhibitors in subsequent studies would further enhance the therapeutic response currently observed with single agent STING activation [399]. Indeed, several groups are investigating the use and efficacy of such combination regimen in improving therapy.

4.2.1 Combination targets in trials

4.2.1.1 PD-L1

Combining ICB antibodies with STING activation has emerged as an attractive combination strategy especially due to the significant increase in TIL population that is achieved on-treatment [399]. In an ID8^{-}Trp53$^{-/-}$ model of murine ovarian cancer, Ghaffari *et al*. observed that therapy with STING activation was associated with a significant increase in intratumoral infiltration of PD-1^{+} CD8^{+} T-cells and systemic increase in PD-L1 expressing myeloid MDSC and macrophages [400] where PD-1/PD-L1 expression may be driven by i) the enhanced production of IFNγ by activated TILs, STING and type-I IFN responsive DCs/macrophages/NK cells on-treatment [400] or ii) type-I

IFN associated activation of JAK/STAT pathways [120]. Indeed, combination of STING agonism with ICB in a prostate cancer model showed synergistic efficacy in controlling tumor growth when compared to either single treatment modality [324]. Encouraging results from such pre-clinical combination efforts has led to the inclusion of STING agonists in ICB based clinical trials such as those highlighted in **Table 3**.

4.2.1.2 COX2

Separately, we also found that bone marrow DCs upregulated Ptgs2/Cycloxygenase-2 or COX2 in response to STING activation. COX2 contributes to local immunosuppression through the catabolic reduction of arachidonic acid into PGH2; the pro-form of the active immunosuppressant PGE2 [401]. Consistent with our observation of enhanced COX2 production with STING activation, a recent report by Lemos *et al*. found that CDA administration to murine LLC tumors resulted in the concomitant upregulation of COX2 in the LLC TME [305]. Since murine LLCs eventually relapse despite receiving continued administration of STING agonists, the authors hypothesized that targeted inhibition of the key immunosuppressive axis coordinated by COX2 would improve STING-based therapy by extending survival and preventing tumor relapse. Indeed, the combined administration of Celecoxib, a selective COX2 inhibitor, with CDA significantly reduced tumor growth rates, prevented relapse and extended survival when compared to CDA monotherapy [305]. Findings from our studies and from Lemos *et al*. suggest that COX2 inhibition holds significant promise as a co-therapy in future combination STING-based interventional regimens.

4.2.1.3 IDO

In a separate study by Lemos *et al*. the authors observed that relapse with STING activation in LLC was accompanied by an enhanced infiltration of immunosuppressive MDSCs[402]. Analysis of the differential gene expression profiles of STING activated tumors from WT mice with their

counterparts from STING KO mice revealed that indoleamine 2,3-dioxygenase (IDO) was significantly overexpressed in the recurrent tumors from WT hosts [402]. Extending this observation, they observed that therapeutic administration of IDO inhibitors significantly delayed tumor growth in WT hosts receiving STING agonists suggesting that IDO production actively limits therapeutic efficacy of STING agonism. Mechanistically, since IDO enzymatically converts tryptophan to kynurenine [403], upregulation of IDO within the TME can be expected to deplete local tryptophan thereby inducing metabolic dysfunction of tumor infiltrating T-cells [404]. Further, an enhanced local generation of kynurenine limits anti-tumor immune responses by promoting Treg induction [405] and skewing DC differentiation towards more tolerogenic phenotypes [406] suggesting that IDO inhibition could also factor as an important combination strategy integrating STING agonist-based treatments in the future.

4.2.2 Novel immunoregulatory targets:

While PD1/PDL1, COX2 and IDO inhibitors are already being evaluated in combination with STING agonists, our analyses highlight Arg2 and Ptges as two additional STING associated immunoregulatory molecules whose inhibition via commercially available inhibitors may improve current therapeutic responses with STING agonism.

4.2.2.1 ARG2

Although the therapeutic anti-tumor role of DCs and T-cells with STING agonism has been the focal point of the work presented in this thesis, several other studies have suggested that macrophages are also heavily involved in STING based anti-tumor responses. In a recent report, Ohkuri *et al.* demonstrated that therapy with intratumoral injections of cGAMP in CT26 colon carcinoma, B16 melanoma and 4T1 breast cancer is associated with an increased infiltration of inflammatory macrophages [407]. Tumor infiltrating macrophages exhibit a significant degree of

phenotypic plasticity and the phenotypic polarization and/or maintenance of a pro-inflammatory M1 phenotype or an anti-inflammatory M2 phenotype is largely dependent on the tissue cytokine milieu [408]. In this context, a significant body of evidence suggests that Arg2 skews macrophages to acquire more M2-like phenotypes [409 410]. GSEA data from DCs treated with ADU S-100 suggest that STING activation indeed upregulates Arg2 production. While we currently have not profiled the tumor infiltrating macrophages in our therapeutic model, our data suggests that anti-tumor responses with STING agonists could be directly or indirectly dampened via secondary promotion/accumulation of M2-like macrophages within the TME. Therefore, the data presented in this thesis would recommend the novel inclusion of Arg2 inhibitors with STING-based treatments to further enhance therapeutic responses by reinforcing the promotion of M1 macrophages which independently correlate positively with TLS formation/presence in human colorectal cancers [262].

4.2.2.2 PTGES

Furthermore, we observed that STING-activated DCs, in addition to upregulating their expression of COX2, also upregulate their expression of PTGES/mPGES1, an enzyme responsible for the final conversion of PGH2 to bioactive PGE2. By binding to its ubiquitous cognate receptor EP4, PGE2 suppresses local inflammation and promotes cancer progression through a variety of mechanisms [401]. In T-cells, PGE2 downregulates the production of IL-2 and the IL-2 receptor thus theoretically weakening Th1 polarization, T-cell activation and T-cell proliferation within the STING-activated TME [411]. Activation of EP4 receptors on monocytes and DCs by PGE2 results in the upregulation of IL-12p40, a competitive inhibitor of the IL-12 receptor [411], and the downregulation of key TLS-associated lymphocyte recruiting chemokine CCL19 [412] which can further significantly: i.) affect nodal priming of naïve T-cells by APCs, ii.) limit formation of TLS

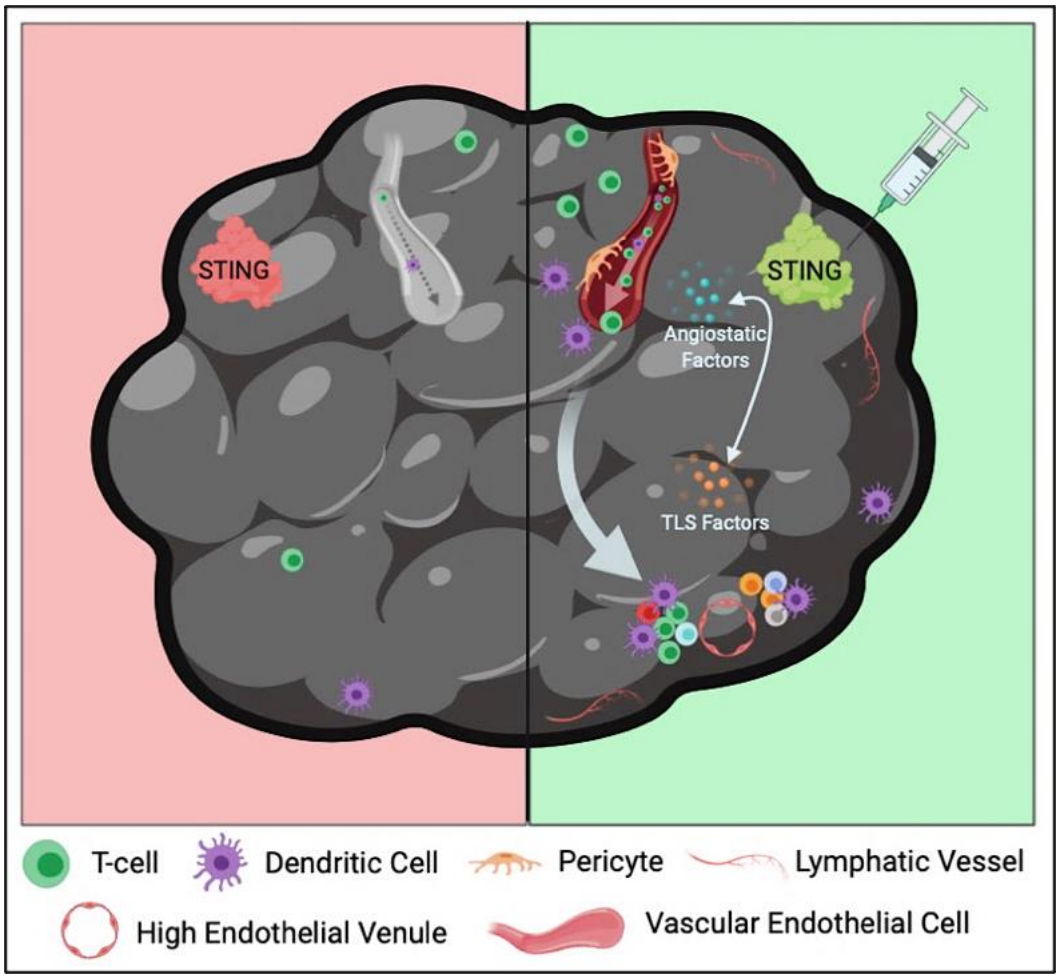
within the STING activated TME. Moreover, PGE2 can dampen the STING-based anti-tumor immune response by downregulating the expression of common gamma chain receptors on cytotoxic NK cells [413] and promoting tumor-associated macrophage polarization towards the M2 subtype [414]. PGE2 may also contribute to tumor progression via transactivation of PI3K/Akt [415], Wnt [415], MAPK [416] pathways. Considering the multi-functional, pro-tumoral role of PGE2, our data suggests that, in addition to COX2 inhibition, PTGES inhibition/blockade (such as using Cay10526 or Cay10678) should also be tested in combination with STING agonists in future investigations.

4.3 Oncolytic viruses at the confluence of STING activation and TLS neogenesis

The work detailed in this thesis also extends interesting and novel therapeutic outcomes/readouts for other forms of immunotherapy that may introduce foreign/self dsDNA into the TME. Oncolytic viruses provide an exciting platform to treat cancers due to i) their specific targeted lytic activity in tumor cells and ii) their ability to carry additional therapeutic payloads as viral vectors [417]. Several oncolytic products are now in trials and include vaccinia based oncolytic product, Pexa-vec, and herpes simplex based oncolytic products, T-vec and IMLYGIC[418-420]. Due to their dsDNA genome, it is reasonable to expect these oncolytic viruses to activate STING during the course of infection[418]. Recent literature suggests that oncolytic viruses indeed require and activate host STING to mount successful anti-tumor immune responses [391]. Interestingly, as was the case for my studies using ADU S-100, tumor-intrinsic STING activation does not appear central to the success of oncolytic viruses and in fact, may limit oncolytic activity by preventing successful and repeated oncolytic infection and inducing immunosuppressive axes [421]. Indeed, colorectal cancers that harbor loss-of-function mutations in cGAS and STING demonstrate the greatest degree of susceptibility to oncolytic viruses (with dsDNA backbones)[391]. Thus, tumors

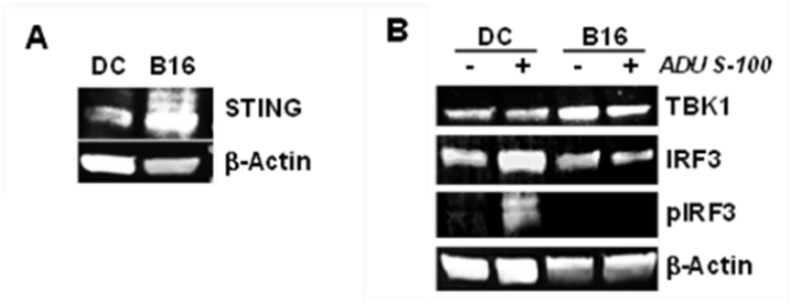
with silenced/mutated STING might serve as compelling candidates for oncolytic virus-based intervention and in this setting, host STING-mediated response to oncolytic viruses might be orchestrated by the reprogramming/normalization of the tumor vasculature and local TLS neogenesis in the TME as outlined by studies presented in this thesis and by the work of other groups studying TLS-induced immune reactions in non-oncolytic HPV^+ human cancers[234 350]. Given the growing body of evidence demonstrating an independent ability of several inflammatory factors to induce TLS, it may also be proposed that oncolytic viruses can, in addition to activating STING, be engineered to induce secretion of cytokines/homeostatic chemokines to further potentiate TLS neogenesis within the infected TME. In this light, preliminary studies are underway in our group to evaluate the therapeutic and TLS inducing potential of a recombinant oncolytic vaccinia virus designed to produce high local levels of TNFSF14/LIGHT, CCL19 and CCL21 within the TME.

Appendix



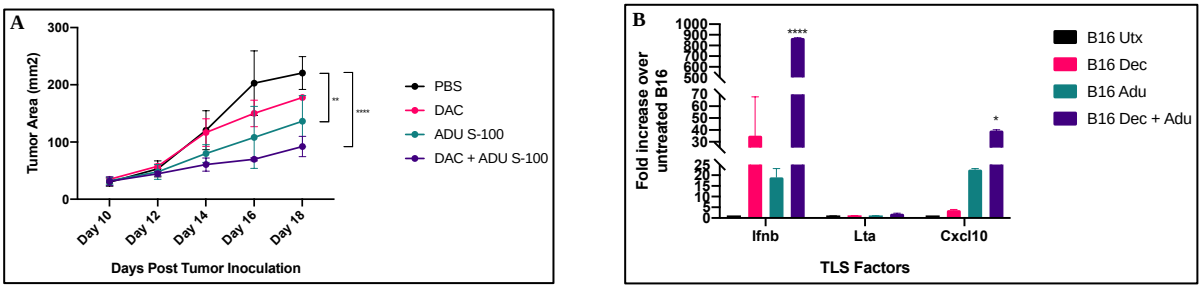
Appendix figure 1: *Summary of work presented in this thesis*

The activation of host STING leads to the normalization of existing tumor vasculature and HEV/TLS neogenesis where such therapy-associated vascular networks enhance local T-cell infiltration associated with slowed tumor growth and extended survival. This enhanced TIL footprint in STING activated tumors is qualitatively characterized by both, enhanced quantities of peripherally primed T-cells (function of VN) and the existence of a unique repertoire of T-cells expanded within the TME (function of HEV/TLS neogenesis) that together drive robust anti-tumor responses.



Appendix figure 2: *Tumor-intrinsic dysfunction in STING signaling despite normal expression of key pathway components*

A. *Immunoblotting confirms expression of STING in CD11c^{+} DCs and B16.F10 melanoma cells.* ***B.*** *Immunoblotting confirms equivalent expression of STING cascade proteins TBK1 and IRF3 but activation (phosphorylation) of IRF3 in DCs but not B16.F10 melanoma. Cell lysates for western blotting experiments obtained 60 minutes post-stimulation with ADU S-100.*



Appendix figure 3: *DNMT1 inhibition synergizes with STING activation for therapy*

A. *Mice harboring s.c. B16.F10 melanoma show synergistic tumor growth control when intratumoral ADU S-100 treatments were combined with daily intraperitoneal administration of DNMT1 inhibitor Decitabine (DAC).* ***B.*** *In vitro, B16.F10 melanoma cells show enhanced production of inflammatory cytokines when tumor cells are pre-treated with DAC prior to STING activation. * $p<0.05$, Two-way ANOVA for tumor growth curve and One-way ANOVA for transcriptional data.*

Bibliography

1. Society AC. ACS Facts and Figures 2020. 2020

2. Apalla Z, Nashan D, Weller RB, et al. Skin Cancer: Epidemiology, Disease Burden, Pathophysiology, Diagnosis, and Therapeutic Approaches. *Dermatol Ther (Heidelb)* 2017;7(Suppl 1):5-19. doi: 10.1007/s13555-016-0165-y [published Online First: 2017/02/06]

3. Elwood JM, Jopson J. Melanoma and sun exposure: an overview of published studies. *Int J Cancer* 1997;73(2):198-203. doi: 10.1002/(sici)1097-0215(19971009)73:2<198::aid-ijc6>3.0.co;2-r [published Online First: 1997/10/23 22:33]

4. Gandini S, Sera F, Cattaruzza MS, et al. Meta-analysis of risk factors for cutaneous melanoma: II. Sun exposure. *Eur J Cancer* 2005;41(1):45-60. doi: 10.1016/j.ejca.2004.10.016 [published Online First: 2004/12/25]

5. Rigel DS. Epidemiology of melanoma. *Semin Cutan Med Surg* 2010;29(4):204-9. doi: 10.1016/j.sder.2010.10.005 [published Online First: 2011/02/01]

6. Caini S, Gandini S, Sera F, et al. Meta-analysis of risk factors for cutaneous melanoma according to anatomical site and clinico-pathological variant. *Eur J Cancer* 2009;45(17):3054-63. doi: 10.1016/j.ejca.2009.05.009 [published Online First: 2009/06/24]

7. Meyle KD, Guldberg P. Genetic risk factors for melanoma. *Hum Genet* 2009;126(4):499-510. doi: 10.1007/s00439-009-0715-9 [published Online First: 2009/07/09]

8. Rastrelli M, Tropea S, Rossi CR, et al. Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. *In Vivo* 2014;28(6):1005-11. [published Online First: 2014/11/16]

9. Holick MF. Resurrection of vitamin D deficiency and rickets. *J Clin Invest* 2006;116(8):2062-72. doi: 10.1172/JCI29449 [published Online First: 2006/08/04]

10. D'Orazio J, Jarrett S, Amaro-Ortiz A, et al. UV radiation and the skin. *Int J Mol Sci* 2013;14(6):12222-48. doi: 10.3390/ijms140612222 [published Online First: 2013/06/12]

11. Morgan AM, Lo J, Fisher DE. How does pheomelanin synthesis contribute to melanomagenesis?: Two distinct mechanisms could explain the carcinogenicity of pheomelanin synthesis. *Bioessays* 2013;35(8):672-6. doi: 10.1002/bies.201300020 [published Online First: 2013/05/08]

12. Duval C, Smit NP, Kolb AM, et al. Keratinocytes control the pheo/eumelanin ratio in cultured normal human melanocytes. *Pigment Cell Res* 2002;15(6):440-6. doi: 10.1034/j.1600-0749.2002.02055.x [published Online First: 2002/11/28]

13. Pleasance ED, Cheetham RK, Stephens PJ, et al. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* 2010;463(7278):191-6. doi: 10.1038/nature08658 [published Online First: 2009/12/18]

14. Cooper KL, Yager JW, Hudson LG. Melanocytes and keratinocytes have distinct and shared responses to ultraviolet radiation and arsenic. *Toxicol Lett* 2014;224(3):407-15. doi: 10.1016/j.toxlet.2013.11.010 [published Online First: 2013/11/26]

15. Hodis E, Watson IR, Kryukov GV, et al. A landscape of driver mutations in melanoma. *Cell* 2012;150(2):251-63. doi: 10.1016/j.cell.2012.06.024 [published Online First: 2012/07/24]

16. Assefa Z, Garmyn M, Bouillon R, et al. Differential stimulation of ERK and JNK activities by ultraviolet B irradiation and epidermal growth factor in human keratinocytes. *J Invest Dermatol* 1997;108(6):886-91. doi: 10.1111/1523-1747.ep12292595 [published Online First: 1997/06/01]

17. Zhang J, Bowden GT. Activation of p38 MAP kinase and JNK pathways by UVA irradiation. *Photochem Photobiol Sci* 2012;11(1):54-61. doi: 10.1039/c1pp05133d [published Online First: 2011/08/23]

18. Schaffer JV. Update on melanocytic nevi in children. *Clin Dermatol* 2015;33(3):368-86. doi: 10.1016/j.clindermatol.2014.12.015 [published Online First: 2015/04/19]

19. Bevona C, Goggins W, Quinn T, et al. Cutaneous melanomas associated with nevi. *Arch Dermatol* 2003;139(12):1620-4; discussion 24. doi: 10.1001/archderm.139.12.1620 [published Online First: 2003/12/17]

20. Lin WM, Luo S, Muzikansky A, et al. Outcome of patients with de novo versus nevus-associated melanoma. *J Am Acad Dermatol* 2015;72(1):54-8. doi: 10.1016/j.jaad.2014.09.028 [published Online First: 2014/12/03]

21. Haenssle HA, Mograby N, Ngassa A, et al. Association of Patient Risk Factors and Frequency of Nevus-Associated Cutaneous Melanomas. *JAMA Dermatol* 2016;152(3):291-8. doi: 10.1001/jamadermatol.2015.3775 [published Online First: 2015/11/05]

22. Damsky WE, Bosenberg M. Melanocytic nevi and melanoma: unraveling a complex relationship. *Oncogene* 2017;36(42):5771-92. doi: 10.1038/onc.2017.189 [published Online First: 2017/06/13]

23. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet* 2003;33(1):19-20. doi: 10.1038/ng1054 [published Online First: 2002/11/26]

24. Roh MR, Eliades P, Gupta S, et al. Genetics of melanocytic nevi. *Pigment Cell Melanoma Res* 2015;28(6):661-72. doi: 10.1111/pcmr.12412 [published Online First: 2015/08/25]

25. Ranade K, Hussussian CJ, Sikorski RS, et al. Mutations associated with familial melanoma impair p16INK4 function. *Nat Genet* 1995;10(1):114-6. doi: 10.1038/ng0595-114 [published Online First: 1995/05/01]

26. Lesueur F, de Lichy M, Barrois M, et al. The contribution of large genomic deletions at the CDKN2A locus to the burden of familial melanoma. *Br J Cancer* 2008;99(2):364-70. doi: 10.1038/sj.bjc.6604470 [published Online First: 2008/07/10]

27. Potrony M, Badenas C, Aguilera P, et al. Update in genetic susceptibility in melanoma. *Ann Transl Med* 2015;3(15):210. doi: 10.3978/j.issn.2305-5839.2015.08.11 [published Online First: 2015/10/22]

28. Chudnovsky Y, Khavari PA, Adams AE. Melanoma genetics and the development of rational therapeutics. *J Clin Invest* 2005;115(4):813-24. doi: 10.1172/JCI24808 [published Online First: 2005/04/21]

29. Haferkamp S, Becker TM, Scurr LL, et al. p16INK4a-induced senescence is disabled by melanoma-associated mutations. *Aging Cell* 2008;7(5):733-45. doi: 10.1111/j.1474-9726.2008.00422.x [published Online First: 2008/10/10]

30. Sherr CJ. Cancer cell cycles. *Science* 1996;274(5293):1672-7. doi: 10.1126/science.274.5293.1672 [published Online First: 1996/12/06]

31. Rizos H, Darmanian AP, Holland EA, et al. Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF. *J Biol Chem* 2001;276(44):41424-34. doi: 10.1074/jbc.M105299200 [published Online First: 2001/08/24]

32. Zaman A, Wu W, Bivona TG. Targeting Oncogenic BRAF: Past, Present, and Future. *Cancers (Basel)* 2019;11(8) doi: 10.3390/cancers11081197 [published Online First: 2019/08/21]

33. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417(6892):949-54. doi: 10.1038/nature00766 [published Online First: 2002/06/18]

34. Wu H, Goel V, Haluska FG. PTEN signaling pathways in melanoma. *Oncogene* 2003;22(20):3113-22. doi: 10.1038/sj.onc.1206451 [published Online First: 2003/06/06]

35. Dankort D, Curley DP, Cartlidge RA, et al. Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. *Nat Genet* 2009;41(5):544-52. doi: 10.1038/ng.356 [published Online First: 2009/03/14]

36. Duncan LM. The classification of cutaneous melanoma. *Hematol Oncol Clin North Am* 2009;23(3):501-13, ix. doi: 10.1016/j.hoc.2009.03.013 [published Online First: 2009/05/26]

37. Markovic SN, Erickson LA, Rao RD, et al. Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. *Mayo Clin Proc* 2007;82(3):364-80. doi: 10.4065/82.3.364 [published Online First: 2007/03/14]

38. Bhatia S, Tykodi SS, Thompson JA. Treatment of metastatic melanoma: an overview. *Oncology (Williston Park)* 2009;23(6):488-96. [published Online First: 2009/06/24]

39. Hamm C, Verma S, Petrella T, et al. Biochemotherapy for the treatment of metastatic malignant melanoma: a systematic review. *Cancer Treat Rev* 2008;34(2):145-56. doi: 10.1016/j.ctrv.2007.10.003 [published Online First: 2007/12/14]

40. Meer L, Janzer RC, Kleihues P, et al. In vivo metabolism and reaction with DNA of the cytostatic agent, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC). *Biochem Pharmacol* 1986;35(19):3243-7. doi: 10.1016/0006-2952(86)90419-3 [published Online First: 1986/10/01]

41. Mizuno NS, Decker RW. Alteration of DNA by 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (NSC-407347). *Biochem Pharmacol* 1976;25(23):2643-7. doi: 10.1016/0006-2952(76)90521-9 [published Online First: 1976/12/01]

42. Eggermont AM, Kirkwood JM. Re-evaluating the role of dacarbazine in metastatic melanoma: what have we learned in 30 years? *Eur J Cancer* 2004;40(12):1825-36. doi: 10.1016/j.ejca.2004.04.030 [published Online First: 2004/08/04]

43. Pasquali S, Hadjinicolaou AV, Chiarion Sileni V, et al. Systemic treatments for metastatic cutaneous melanoma. *Cochrane Database Syst Rev* 2018;2:CD011123. doi: 10.1002/14651858.CD011123.pub2 [published Online First: 2018/02/07]

44. Lui P, Cashin R, Machado M, et al. Treatments for metastatic melanoma: synthesis of evidence from randomized trials. *Cancer Treat Rev* 2007;33(8):665-80. doi: 10.1016/j.ctrv.2007.06.004 [published Online First: 2007/10/09]

45. Propper DJ, Braybrooke JP, Levitt NC, et al. Phase II study of second-line therapy with DTIC, BCNU, cisplatin and tamoxifen (Dartmouth regimen) chemotherapy in patients with

malignant melanoma previously treated with dacarbazine. *Br J Cancer* 2000;82(11):1759-63. doi: 10.1054/bjoc.2000.1141 [published Online First: 2000/06/06]

46. Chapman PB, Einhorn LH, Meyers ML, et al. Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. *J Clin Oncol* 1999;17(9):2745-51. doi: 10.1200/JCO.1999.17.9.2745 [published Online First: 1999/11/24]

47. Middleton MR, Grob JJ, Aaronson N, et al. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *J Clin Oncol* 2000;18(1):158-66. doi: 10.1200/JCO.2000.18.1.158 [published Online First: 2000/01/07]

48. Patel PM, Suciu S, Mortier L, et al. Extended schedule, escalated dose temozolomide versus dacarbazine in stage IV melanoma: final results of a randomised phase III study (EORTC 18032). *Eur J Cancer* 2011;47(10):1476-83. doi: 10.1016/j.ejca.2011.04.030 [published Online First: 2011/05/24]

49. Grossman D, Altieri DC. Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. *Cancer Metastasis Rev* 2001;20(1-2):3-11. doi: 10.1023/a:1013123532723 [published Online First: 2002/02/08]

50. Schadendorf D, Worm M, Algermissen B, et al. Chemosensitivity testing of human malignant melanoma. A retrospective analysis of clinical response and in vitro drug sensitivity. *Cancer* 1994;73(1):103-8. doi: 10.1002/1097-0142(19940101)73:1<103::aid-cncr2820730119>3.0.co;2-k [published Online First: 1994/01/01]

51. Osieka R. Studies on drug resistance in a human melanoma xenograft system. *Cancer Treat Rev* 1984;11 Suppl A:85-98. doi: 10.1016/0305-7372(84)90047-1 [published Online First: 1984/03/01]

52. Swiecki M, Colonna M. Type I interferons: diversity of sources, production pathways and effects on immune responses. *Curr Opin Virol* 2011;1(6):463-75. doi: 10.1016/j.coviro.2011.10.026 [published Online First: 2012/03/24]

53. MacMicking JD. Interferon-inducible effector mechanisms in cell-autonomous immunity. *Nat Rev Immunol* 2012;12(5):367-82. doi: 10.1038/nri3210 [published Online First: 2012/04/26]

54. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* 2004;202:8-32. doi: 10.1111/j.0105-2896.2004.00204.x [published Online First: 2004/11/18]

55. Fuertes MB, Woo SR, Burnett B, et al. Type I interferon response and innate immune sensing of cancer. *Trends Immunol* 2013;34(2):67-73. doi: 10.1016/j.it.2012.10.004 [published Online First: 2012/11/06]

56. Harding SM, Benci JL, Irianto J, et al. Mitotic progression following DNA damage enables pattern recognition within micronuclei. *Nature* 2017;548(7668):466-70. doi: 10.1038/nature23470 [published Online First: 2017/08/02]

57. Mackenzie KJ, Carroll P, Martin CA, et al. cGAS surveillance of micronuclei links genome instability to innate immunity. *Nature* 2017;548(7668):461-65. doi: 10.1038/nature23449 [published Online First: 2017/07/25]

58. Kang TH, Mao CP, Kim YS, et al. TLR9 acts as a sensor for tumor-released DNA to modulate anti-tumor immunity after chemotherapy. *J Immunother Cancer* 2019;7(1):260. doi: 10.1186/s40425-019-0738-2 [published Online First: 2019/10/18]

59. Lorenzi S, Mattei F, Sistigu A, et al. Type I IFNs control antigen retention and survival of CD8alpha(+) dendritic cells after uptake of tumor apoptotic cells leading to cross-priming. *J Immunol* 2011;186(9):5142-50. doi: 10.4049/jimmunol.1004163 [published Online First: 2011/03/29]

60. Yang I, Kremen TJ, Giovannone AJ, et al. Modulation of major histocompatibility complex Class I molecules and major histocompatibility complex-bound immunogenic peptides induced by interferon-alpha and interferon-gamma treatment of human glioblastoma multiforme. *J Neurosurg* 2004;100(2):310-9. doi: 10.3171/jns.2004.100.2.0310 [published Online First: 2004/04/17]

61. Creagan ET, Ahmann DL, Frytak S, et al. Phase II trials of recombinant leukocyte A interferon in disseminated malignant melanoma: results in 96 patients. *Cancer Treat Rep* 1986;70(5):619-24. [published Online First: 1986/05/01]

62. Dummer R, Garbe C, Thompson JA, et al. Randomized dose-escalation study evaluating peginterferon alfa-2a in patients with metastatic malignant melanoma. *J Clin Oncol* 2006;24(7):1188-94. doi: 10.1200/JCO.2005.04.3216 [published Online First: 2006/03/01]

63. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 1973;137(5):1142-62. doi: 10.1084/jem.137.5.1142 [published Online First: 1973/05/01]

64. Steinman RM, Witmer MD. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* 1978;75(10):5132-6. doi: 10.1073/pnas.75.10.5132 [published Online First: 1978/10/01]

65. Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. *Annu Rev Immunol* 2000;18:767-811. doi: 10.1146/annurev.immunol.18.1.767 [published Online First: 2000/06/03]

66. Steinman RM, Hawiger D, Liu K, et al. Dendritic cell function in vivo during the steady state: a role in peripheral tolerance. *Ann N Y Acad Sci* 2003;987:15-25. doi: 10.1111/j.1749-6632.2003.tb06029.x [published Online First: 2003/05/03]

67. Cerboni S, Gentili M, Manel N. Diversity of pathogen sensors in dendritic cells. *Adv Immunol* 2013;120:211-37. doi: 10.1016/B978-0-12-417028-5.00008-9 [published Online First: 2013/09/28]

68. Patente TA, Pinho MP, Oliveira AA, et al. Human Dendritic Cells: Their Heterogeneity and Clinical Application Potential in Cancer Immunotherapy. *Front Immunol* 2018;9:3176. doi: 10.3389/fimmu.2018.03176 [published Online First: 2019/02/06]

69. Reis e Sousa C. Dendritic cells in a mature age. *Nat Rev Immunol* 2006;6(6):476-83. doi: 10.1038/nri1845 [published Online First: 2006/05/13]

70. Cintolo JA, Datta J, Mathew SJ, et al. Dendritic cell-based vaccines: barriers and opportunities. *Future Oncol* 2012;8(10):1273-99. doi: 10.2217/fon.12.125 [published Online First: 2012/11/08]

71. Dudek AM, Martin S, Garg AD, et al. Immature, Semi-Mature, and Fully Mature Dendritic Cells: Toward a DC-Cancer Cells Interface That Augments Anticancer Immunity. *Front Immunol* 2013;4:438. doi: 10.3389/fimmu.2013.00438 [published Online First: 2014/01/01]

72. Butterfield LH, Comin-Anduix B, Vujanovic L, et al. Adenovirus MART-1-engineered autologous dendritic cell vaccine for metastatic melanoma. *J Immunother* 2008;31(3):294-309. doi: 10.1097/CJI.0b013e31816a8910 [published Online First: 2008/03/05]

73. Blalock LT, Landsberg J, Messmer M, et al. Human dendritic cells adenovirally-engineered to express three defined tumor antigens promote broad adaptive and innate immunity. *Oncoimmunology* 2012;1(3):287-357. doi: 10.4161/onci.18628 [published Online First: 2012/06/28]

74. Bodey B, Bodey B, Jr., Siegel SE, et al. Failure of cancer vaccines: the significant limitations of this approach to immunotherapy. *Anticancer Res* 2000;20(4):2665-76. [published Online First: 2000/08/23]

75. Engell-Noerregaard L, Hansen TH, Andersen MH, et al. Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation

between clinical response and vaccine parameters. *Cancer Immunol Immunother* 2009;58(1):1-14. doi: 10.1007/s00262-008-0568-4 [published Online First: 2008/08/23]

76. Ridolfi L, Petrini M, Fiammenghi L, et al. Unexpected high response rate to traditional therapy after dendritic cell-based vaccine in advanced melanoma: update of clinical outcome and subgroup analysis. *Clin Dev Immunol* 2010;2010:504979. doi: 10.1155/2010/504979 [published Online First: 2010/10/12]

77. Dudley ME, Yang JC, Sherry R, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 2008;26(32):5233-9. doi: 10.1200/JCO.2008.16.5449 [published Online First: 2008/09/24]

78. Bernatchez C, Radvanyi LG, Hwu P. Advances in the treatment of metastatic melanoma: adoptive T-cell therapy. *Semin Oncol* 2012;39(2):215-26. doi: 10.1053/j.seminoncol.2012.01.006 [published Online First: 2012/04/10]

79. El-Mallawany NK, Geller L, Bollard CM, et al. Long-term remission in a child with refractory EBV(+) hydroa vacciniforme-like T-cell lymphoma through sequential matched EBV(+)-related allogeneic hematopoietic SCT followed by donor-derived EBV-specific cytotoxic T-lymphocyte immunotherapy. *Bone Marrow Transplant* 2011;46(5):759-61. doi: 10.1038/bmt.2010.176 [published Online First: 2010/07/28]

80. Moosmann A, Bigalke I, Tischer J, et al. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. *Blood* 2010;115(14):2960-70. doi: 10.1182/blood-2009-08-236356 [published Online First: 2010/01/28]

81. Danilova L, Anagnostou V, Caushi JX, et al. The Mutation-Associated Neoantigen Functional Expansion of Specific T Cells (MANAFEST) Assay: A Sensitive Platform for Monitoring Antitumor Immunity. *Cancer Immunol Res* 2018;6(8):888-99. doi: 10.1158/2326-6066.CIR-18-0129 [published Online First: 2018/06/14]

82. Hutchison S, Pritchard AL. Identifying neoantigens for use in immunotherapy. *Mamm Genome* 2018;29(11-12):714-30. doi: 10.1007/s00335-018-9771-6 [published Online First: 2018/09/01]

83. Buonaguro L, Petrizzo A, Tornesello ML, et al. Translating tumor antigens into cancer vaccines. *Clin Vaccine Immunol* 2011;18(1):23-34. doi: 10.1128/CVI.00286-10 [published Online First: 2010/11/05]

84. Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* 2008;358(25):2698-703. doi: 10.1056/NEJMoa0800251 [published Online First: 2008/06/21]

85. Benlalam H, Vignard V, Khammari A, et al. Infusion of Melan-A/Mart-1 specific tumor-infiltrating lymphocytes enhanced relapse-free survival of melanoma patients. *Cancer Immunol Immunother* 2007;56(4):515-26. doi: 10.1007/s00262-006-0204-0 [published Online First: 2006/07/29]

86. Kawakami Y, Eliyahu S, Jennings C, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *J Immunol* 1995;154(8):3961-8. [published Online First: 1995/04/15]

87. Gilboa E. The makings of a tumor rejection antigen. *Immunity* 1999;11(3):263-70. doi: 10.1016/s1074-7613(00)80101-6 [published Online First: 1999/10/08]

88. Prehn RT, Main JM. Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* 1957;18(6):769-78. [published Online First: 1957/06/01]

89. Klein G, Sjogren HO, Klein E, et al. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res* 1960;20:1561-72. [published Online First: 1960/12/01]

90. Kripke ML. Antigenicity of murine skin tumors induced by ultraviolet light. *J Natl Cancer Inst* 1974;53(5):1333-6. doi: 10.1093/jnci/53.5.1333 [published Online First: 1974/11/01]

91. Lennerz V, Fatho M, Gentilini C, et al. The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. *Proc Natl Acad Sci U S A* 2005;102(44):16013-8. doi: 10.1073/pnas.0500090102 [published Online First: 2005/10/26]

92. Prehn RT. Analysis of antigenic heterogeneity within individual 3-methylcholanthrene-induced mouse sarcomas. *J Natl Cancer Inst* 1970;45(5):1039-45. [published Online First: 1970/11/01]

93. Wortzel RD, Philipps C, Schreiber H. Multiple tumour-specific antigens expressed on a single tumour cell. *Nature* 1983;304(5922):165-7. doi: 10.1038/304165a0 [published Online First: 1983/07/14]

94. Klebanoff CA, Gattinoni L, Torabi-Parizi P, et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci U S A* 2005;102(27):9571-6. doi: 10.1073/pnas.0503726102 [published Online First: 2005/06/28]

95. Kodumudi KN, Weber A, Sarnaik AA, et al. Blockade of myeloid-derived suppressor cells after induction of lymphopenia improves adoptive T cell therapy in a murine model of

melanoma. *J Immunol* 2012;189(11):5147-54. doi: 10.4049/jimmunol.1200274 [published Online First: 2012/10/27]

96. Muranski P, Boni A, Wrzesinski C, et al. Increased intensity lymphodepletion and adoptive immunotherapy--how far can we go? *Nat Clin Pract Oncol* 2006;3(12):668-81. doi: 10.1038/ncponc0666 [published Online First: 2006/12/02]

97. Dwyer CJ, Knochelmann HM, Smith AS, et al. Fueling Cancer Immunotherapy With Common Gamma Chain Cytokines. *Front Immunol* 2019;10:263. doi: 10.3389/fimmu.2019.00263 [published Online First: 2019/03/08]

98. Falk K, Rotzschke O, Stevanovic S, et al. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991;351(6324):290-6. doi: 10.1038/351290a0 [published Online First: 1991/05/23]

99. Steentoft C, Migliorini D, King TR, et al. Glycan-directed CAR-T cells. *Glycobiology* 2018;28(9):656-69. doi: 10.1093/glycob/cwy008 [published Online First: 2018/01/26]

100. Sadelain M, Riviere I, Riddell S. Therapeutic T cell engineering. *Nature* 2017;545(7655):423-31. doi: 10.1038/nature22395 [published Online First: 2017/05/26]

101. Zhao L, Cao YJ. Engineered T Cell Therapy for Cancer in the Clinic. *Front Immunol* 2019;10:2250. doi: 10.3389/fimmu.2019.02250 [published Online First: 2019/11/05]

102. Morgan RA, Yang JC, Kitano M, et al. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010;18(4):843-51. doi: 10.1038/mt.2010.24 [published Online First: 2010/02/25]

103. Parker KR, Migliorini D, Perkey E, et al. Single-Cell Analyses Identify Brain Mural Cells Expressing CD19 as Potential Off-Tumor Targets for CAR-T Immunotherapies. *Cell* 2020;183(1):126-42 e17. doi: 10.1016/j.cell.2020.08.022 [published Online First: 2020/09/23]

104. Simon B, Uslu U. CAR-T cell therapy in melanoma: A future success story? *Exp Dermatol* 2018;27(12):1315-21. doi: 10.1111/exd.13792 [published Online First: 2018/10/06]

105. Barrett DM, Grupp SA, June CH. Chimeric Antigen Receptor- and TCR-Modified T Cells Enter Main Street and Wall Street. *J Immunol* 2015;195(3):755-61. doi: 10.4049/jimmunol.1500751 [published Online First: 2015/07/19]

106. Morgan RA, Chinnasamy N, Abate-Daga D, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* 2013;36(2):133-51. doi: 10.1097/CJI.0b013e3182829903 [published Online First: 2013/02/05]

107. Linette GP, Stadtmauer EA, Maus MV, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood* 2013;122(6):863-71. doi: 10.1182/blood-2013-03-490565 [published Online First: 2013/06/19]

108. Cameron BJ, Gerry AB, Dukes J, et al. Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. *Sci Transl Med* 2013;5(197):197ra03. doi: 10.1126/scitranslmed.3006034 [published Online First: 2013/08/09]

109. Brunner MC, Chambers CA, Chan FK, et al. CTLA-4-Mediated inhibition of early events of T cell proliferation. *J Immunol* 1999;162(10):5813-20. [published Online First: 1999/05/07]

110. Walunas TL, Lenschow DJ, Bakker CY, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994;1(5):405-13. doi: 10.1016/1074-7613(94)90071-x [published Online First: 1994/08/01]

111. Collins M, Ling V, Carreno BM. The B7 family of immune-regulatory ligands. *Genome Biol* 2005;6(6):223. doi: 10.1186/gb-2005-6-6-223 [published Online First: 2005/06/18]

112. Linsley PS, Greene JL, Brady W, et al. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* 1994;1(9):793-801. doi: 10.1016/s1074-7613(94)80021-9 [published Online First: 1994/12/01]

113. Linsley PS, Brady W, Urnes M, et al. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med* 1991;174(3):561-9. doi: 10.1084/jem.174.3.561 [published Online First: 1991/09/01]

114. van der Merwe PA, Bodian DL, Daenke S, et al. CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J Exp Med* 1997;185(3):393-403. doi: 10.1084/jem.185.3.393 [published Online First: 1997/02/03]

115. Kane LP, Andres PG, Howland KC, et al. Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not TH2 cytokines. *Nat Immunol* 2001;2(1):37-44. doi: 10.1038/83144 [published Online First: 2001/03/29]

116. Pages F, Ragueneau M, Rottapel R, et al. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature* 1994;369(6478):327-9. doi: 10.1038/369327a0 [published Online First: 1994/05/26]

117. Downey SG, Klapper JA, Smith FO, et al. Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. *Clin Cancer Res* 2007;13(22 Pt 1):6681-8. doi: 10.1158/1078-0432.CCR-07-0187 [published Online First: 2007/11/06]

118. Coutzac C, Adam J, Soularue E, et al. Colon Immune-Related Adverse Events: Anti-CTLA-4 and Anti-PD-1 Blockade Induce Distinct Immunopathological Entities. *J Crohns Colitis* 2017;11(10):1238-46. doi: 10.1093/ecco-jcc/jjx081 [published Online First: 2017/10/03]

119. Keir ME, Liang SC, Guleria I, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 2006;203(4):883-95. doi: 10.1084/jem.20051776 [published Online First: 2006/04/12]

120. Garcia-Diaz A, Shin DS, Moreno BH, et al. Interferon Receptor Signaling Pathways Regulating PD-L1 and PD-L2 Expression. *Cell Rep* 2017;19(6):1189-201. doi: 10.1016/j.celrep.2017.04.031 [published Online First: 2017/05/13]

121. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, et al. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* 2012;209(6):1201-17. doi: 10.1084/jem.20112741 [published Online First: 2012/05/30]

122. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192(7):1027-34. doi: 10.1084/jem.192.7.1027 [published Online First: 2000/10/04]

123. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2(3):261-8. doi: 10.1038/85330 [published Online First: 2001/02/27]

124. Nishimura H, Nose M, Hiai H, et al. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999;11(2):141-51. doi: 10.1016/s1074-7613(00)80089-8 [published Online First: 1999/09/15]

125. Nishimura H, Okazaki T, Tanaka Y, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 2001;291(5502):319-22. doi: 10.1126/science.291.5502.319 [published Online First: 2001/02/24]

126. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity* 2016;44(5):989-1004. doi: 10.1016/j.immuni.2016.05.001 [published Online First: 2016/05/19]

127. Patsoukis N, Bardhan K, Chatterjee P, et al. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nat Commun* 2015;6:6692. doi: 10.1038/ncomms7692 [published Online First: 2015/03/27]

128. Wu X, Zhang H, Xing Q, et al. PD-1(+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. *Br J Cancer* 2014;111(7):1391-9. doi: 10.1038/bjc.2014.416 [published Online First: 2014/08/06]

129. Balbaa SI, Hilal SH, Haggag MY. A study of the yield and glycosidal content of the leaves of Digitalis lanata at different stages of plant growth. *Planta Med* 1970;18(3):254-9. doi: 10.1055/s-0028-1099773 [published Online First: 1970/05/01]

130. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014;515(7528):568-71. doi: 10.1038/nature13954 [published Online First: 2014/11/28]

131. Forde PM, Chaft JE, Smith KN, et al. Neoadjuvant PD-1 Blockade in Resectable Lung Cancer. *N Engl J Med* 2018;378(21):1976-86. doi: 10.1056/NEJMoa1716078 [published Online First: 2018/04/17]

132. Ilyas S, Yang JC. Landscape of Tumor Antigens in T Cell Immunotherapy. *J Immunol* 2015;195(11):5117-22. doi: 10.4049/jimmunol.1501657 [published Online First: 2015/11/22]

133. Bonaventura P, Shekarian T, Alcazer V, et al. Cold Tumors: A Therapeutic Challenge for Immunotherapy. *Front Immunol* 2019;10:168. doi: 10.3389/fimmu.2019.00168 [published Online First: 2019/02/26]

134. Topfer K, Kempe S, Muller N, et al. Tumor evasion from T cell surveillance. *J Biomed Biotechnol* 2011;2011:918471. doi: 10.1155/2011/918471 [published Online First: 2011/12/23]

135. Slaney CY, Kershaw MH, Darcy PK. Trafficking of T cells into tumors. *Cancer Res* 2014;74(24):7168-74. doi: 10.1158/0008-5472.CAN-14-2458 [published Online First: 2014/12/06]

136. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991;254(5038):1643-7. doi: 10.1126/science.1840703 [published Online First: 1991/12/13]

137. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J Immunol* 2005;175(9):6169-76. doi: 10.4049/jimmunol.175.9.6169 [published Online First: 2005/10/21]

138. Galon J, Pages F, Marincola FM, et al. Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med* 2012;10:205. doi: 10.1186/1479-5876-10-205 [published Online First: 2012/10/05]

139. Celebi JT, Shendrik I, Silvers DN, et al. Identification of PTEN mutations in metastatic melanoma specimens. *J Med Genet* 2000;37(9):653-7. doi: 10.1136/jmg.37.9.653 [published Online First: 2000/09/09]

140. Frederick DT, Piris A, Cogdill AP, et al. BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. *Clin Cancer Res* 2013;19(5):1225-31. doi: 10.1158/1078-0432.CCR-12-1630 [published Online First: 2013/01/12]

141. Boni A, Cogdill AP, Dang P, et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. *Cancer Res* 2010;70(13):5213-9. doi: 10.1158/0008-5472.CAN-10-0118 [published Online First: 2010/06/17]

142. Cooper ZA, Frederick DT, Juneja VR, et al. BRAF inhibition is associated with increased clonality in tumor-infiltrating lymphocytes. *Oncoimmunology* 2013;2(10):e26615. doi: 10.4161/onci.26615 [published Online First: 2013/11/20]

143. Jo YS, Li S, Song JH, et al. Influence of the BRAF V600E mutation on expression of vascular endothelial growth factor in papillary thyroid cancer. *J Clin Endocrinol Metab* 2006;91(9):3667-70. doi: 10.1210/jc.2005-2836 [published Online First: 2006/06/15]

144. Kuczek DE, Larsen AMH, Thorseth ML, et al. Collagen density regulates the activity of tumor-infiltrating T cells. *J Immunother Cancer* 2019;7(1):68. doi: 10.1186/s40425-019-0556-6 [published Online First: 2019/03/15]

145. Salmon H, Donnadieu E. Within tumors, interactions between T cells and tumor cells are impeded by the extracellular matrix. *Oncoimmunology* 2012;1(6):992-94. doi: 10.4161/onci.20239 [published Online First: 2012/11/20]

146. Kono M, Dunn IS, Durda PJ, et al. Role of the mitogen-activated protein kinase signaling pathway in the regulation of human melanocytic antigen expression. *Mol Cancer Res* 2006;4(10):779-92. doi: 10.1158/1541-7786.MCR-06-0077 [published Online First: 2006/10/20]

147. Sapkota B, Hill CE, Pollack BP. Vemurafenib enhances MHC induction in BRAF(V600E) homozygous melanoma cells. *Oncoimmunology* 2013;2(1):e22890. doi: 10.4161/onci.22890 [published Online First: 2013/03/14]

148. Kwong ML, Neyns B, Yang JC. Adoptive T-cell transfer therapy and oncogene-targeted therapy for melanoma: the search for synergy. *Clin Cancer Res* 2013;19(19):5292-9. doi: 10.1158/1078-0432.CCR-13-0261 [published Online First: 2013/10/04]

149. Liu C, Peng W, Xu C, et al. BRAF inhibition increases tumor infiltration by T cells and enhances the antitumor activity of adoptive immunotherapy in mice. *Clin Cancer Res* 2013;19(2):393-403. doi: 10.1158/1078-0432.CCR-12-1626 [published Online First: 2012/12/04]

150. Wilmott JS, Long GV, Howle JR, et al. Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. *Clin Cancer Res* 2012;18(5):1386-94. doi: 10.1158/1078-0432.CCR-11-2479 [published Online First: 2011/12/14]

151. Peng W, Chen JQ, Liu C, et al. Loss of PTEN Promotes Resistance to T Cell-Mediated Immunotherapy. *Cancer Discov* 2016;6(2):202-16. doi: 10.1158/2159-8290.CD-15-0283 [published Online First: 2015/12/10]

152. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* 2012;13(5):283-96. doi: 10.1038/nrm3330 [published Online First: 2012/04/05]

153. Wang J, Yao Z, Jonsson P, et al. A Secondary Mutation in BRAF Confers Resistance to RAF Inhibition in a BRAF(V600E)-Mutant Brain Tumor. *Cancer Discov* 2018;8(9):1130-41. doi: 10.1158/2159-8290.CD-17-1263 [published Online First: 2018/06/09]

154. Manzano JL, Layos L, Buges C, et al. Resistant mechanisms to BRAF inhibitors in melanoma. *Ann Transl Med* 2016;4(12):237. doi: 10.21037/atm.2016.06.07 [published Online First: 2016/07/19]

155. Luebker SA, Koepsell SA. Diverse Mechanisms of BRAF Inhibitor Resistance in Melanoma Identified in Clinical and Preclinical Studies. *Front Oncol* 2019;9:268. doi: 10.3389/fonc.2019.00268 [published Online First: 2019/05/07]

156. Johansson A, Hamzah J, Payne CJ, et al. Tumor-targeted TNFalpha stabilizes tumor vessels and enhances active immunotherapy. *Proc Natl Acad Sci U S A* 2012;109(20):7841-6. doi: 10.1073/pnas.1118296109 [published Online First: 2012/05/02]

157. Shrimali RK, Yu Z, Theoret MR, et al. Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer.

Cancer Res 2010;70(15):6171-80. doi: 10.1158/0008-5472.CAN-10-0153 [published Online First: 2010/07/16]

158. Papadopoulos EB, Ladanyi M, Emanuel D, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med* 1994;330(17):1185-91. doi: 10.1056/NEJM199404283301703 [published Online First: 1994/04/28]

159. Riddell SR, Watanabe KS, Goodrich JM, et al. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science* 1992;257(5067):238-41. doi: 10.1126/science.1352912 [published Online First: 1992/07/10]

160. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014;515(7528):568-71. doi: 10.1038/nature13954

161. Hosoi A, Takeda K, Nagaoka K, et al. Increased diversity with reduced "diversity evenness" of tumor infiltrating T-cells for the successful cancer immunotherapy. *Scientific reports* 2018;8(1):1058. doi: 10.1038/s41598-018-19548-y [published Online First: 2018/01/20]

162. Dammeijer F, Lau SP, van Eijck CHJ, et al. Rationally combining immunotherapies to improve efficacy of immune checkpoint blockade in solid tumors. *Cytokine Growth Factor Rev* 2017;36:5-15. doi: 10.1016/j.cytogfr.2017.06.011 [published Online First: 2017/07/12]

163. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144(5):646-74. doi: 10.1016/j.cell.2011.02.013 [published Online First: 2011/03/08]

164. Kimura H, Braun RD, Ong ET, et al. Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. *Cancer Res* 1996;56(23):5522-8. [published Online First: 1996/12/01]

165. Mole DR, Blancher C, Copley RR, et al. Genome-wide association of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha DNA binding with expression profiling of hypoxia-inducible transcripts. *J Biol Chem* 2009;284(25):16767-75. doi: 10.1074/jbc.M901790200 [published Online First: 2009/04/24]

166. Hu CJ, Wang LY, Chodosh LA, et al. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* 2003;23(24):9361-74. doi: 10.1128/mcb.23.24.9361-9374.2003 [published Online First: 2003/12/04]

167. Lee JW, Ko J, Ju C, et al. Hypoxia signaling in human diseases and therapeutic targets. *Exp Mol Med* 2019;51(6):1-13. doi: 10.1038/s12276-019-0235-1 [published Online First: 2019/06/22]

168. Claesson-Welsh L, Welsh M. VEGFA and tumour angiogenesis. *J Intern Med* 2013;273(2):114-27. doi: 10.1111/joim.12019 [published Online First: 2012/12/12]

169. Lugano R, Ramachandran M, Dimberg A. Tumor angiogenesis: causes, consequences, challenges and opportunities. *Cell Mol Life Sci* 2020;77(9):1745-70. doi: 10.1007/s00018-019-03351-7 [published Online First: 2019/11/07]

170. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol* 2005;7(4):452-64. doi: 10.1215/S1152851705000232 [published Online First: 2005/10/11]

171. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. *Circ Res* 2005;97(6):512-23. doi: 10.1161/01.RES.0000182903.16652.d7 [published Online First: 2005/09/17]

172. Foo SS, Turner CJ, Adams S, et al. Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. *Cell* 2006;124(1):161-73. doi: 10.1016/j.cell.2005.10.034 [published Online First: 2006/01/18]

173. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 2007;8(6):464-78. doi: 10.1038/nrm2183 [published Online First: 2007/05/25]

174. Schaaf MB, Garg AD, Agostinis P. Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis* 2018;9(2):115. doi: 10.1038/s41419-017-0061-0 [published Online First: 2018/01/27]

175. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407(6801):249-57. doi: 10.1038/35025220 [published Online First: 2000/09/23]

176. Jain RK. Barriers to drug delivery in solid tumors. *Sci Am* 1994;271(1):58-65. doi: 10.1038/scientificamerican0794-58 [published Online First: 1994/07/01]

177. Heldin CH, Rubin K, Pietras K, et al. High interstitial fluid pressure - an obstacle in cancer therapy. *Nat Rev Cancer* 2004;4(10):806-13. doi: 10.1038/nrc1456 [published Online First: 2004/10/29]

178. Motz GT, Santoro SP, Wang LP, et al. Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. *Nat Med* 2014;20(6):607-15. doi: 10.1038/nm.3541 [published Online First: 2014/05/06]

179. Yu JS, Lee PK, Ehtesham M, et al. Intratumoral T cell subset ratios and Fas ligand expression on brain tumor endothelium. *J Neurooncol* 2003;64(1-2):55-61. doi: 10.1007/BF02700020 [published Online First: 2003/09/04]

180. Bajou K, Peng H, Laug WE, et al. Plasminogen activator inhibitor-1 protects endothelial cells from FasL-mediated apoptosis. *Cancer Cell* 2008;14(4):324-34. doi: 10.1016/j.ccr.2008.08.012 [published Online First: 2008/10/07]

181. Safa AR. c-FLIP, a master anti-apoptotic regulator. *Exp Oncol* 2012;34(3):176-84. [published Online First: 2012/10/17]

182. Shetty S, Weston CJ, Oo YH, et al. Common lymphatic endothelial and vascular endothelial receptor-1 mediates the transmigration of regulatory T cells across human hepatic sinusoidal endothelium. *J Immunol* 2011;186(7):4147-55. doi: 10.4049/jimmunol.1002961 [published Online First: 2011/03/04]

183. Buckanovich RJ, Facciabene A, Kim S, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. *Nat Med* 2008;14(1):28-36. doi: 10.1038/nm1699 [published Online First: 2007/12/25]

184. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* 2005;69 Suppl 3:4-10. doi: 10.1159/000088478 [published Online First: 2005/11/23]

185. Hardwick JS, Yang Y, Zhang C, et al. Identification of biomarkers for tumor endothelial cell proliferation through gene expression profiling. *Mol Cancer Ther* 2005;4(3):413-25. doi: 10.1158/1535-7163.MCT-04-0209 [published Online First: 2005/03/16]

186. Sun Z, Wang CY, Lawson DA, et al. Single-cell RNA sequencing reveals gene expression signatures of breast cancer-associated endothelial cells. *Oncotarget* 2018;9(13):10945-61. doi: 10.18632/oncotarget.23760 [published Online First: 2018/03/16]

187. Lechleitner S, Gille J, Johnson DR, et al. Interferon enhances tumor necrosis factor-induced vascular cell adhesion molecule 1 (CD106) expression in human endothelial cells by an interferon-related factor 1-dependent pathway. *J Exp Med* 1998;187(12):2023-30. doi: 10.1084/jem.187.12.2023 [published Online First: 1998/06/24]

188. Lin CC, Pan CS, Wang CY, et al. Tumor necrosis factor-alpha induces VCAM-1-mediated inflammation via c-Src-dependent transactivation of EGF receptors in human cardiac

fibroblasts. *J Biomed Sci* 2015;22:53. doi: 10.1186/s12929-015-0165-8 [published Online First: 2015/07/16]

189. Millauer B, Wizigmann-Voos S, Schnurch H, et al. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993;72(6):835-46. doi: 10.1016/0092-8674(93)90573-9 [published Online First: 1993/03/26]

190. Leung DW, Cachianes G, Kuang WJ, et al. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;246(4935):1306-9. doi: 10.1126/science.2479986 [published Online First: 1989/12/08]

191. Senger DR, Galli SJ, Dvorak AM, et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219(4587):983-5. doi: 10.1126/science.6823562 [published Online First: 1983/02/25]

192. Goel S, Duda DG, Xu L, et al. Normalization of the vasculature for treatment of cancer and other diseases. *Physiol Rev* 2011;91(3):1071-121. doi: 10.1152/physrev.00038.2010 [published Online First: 2011/07/12]

193. Yuan F, Chen Y, Dellian M, et al. Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. *Proc Natl Acad Sci U S A* 1996;93(25):14765-70. doi: 10.1073/pnas.93.25.14765 [published Online First: 1996/12/10]

194. Yu L, Wu X, Cheng Z, et al. Interaction between bevacizumab and murine VEGF-A: a reassessment. *Invest Ophthalmol Vis Sci* 2008;49(2):522-7. doi: 10.1167/iovs.07-1175 [published Online First: 2008/02/01]

195. Wildiers H, Guetens G, De Boeck G, et al. Effect of antivascular endothelial growth factor treatment on the intratumoral uptake of CPT-11. *Br J Cancer* 2003;88(12):1979-86. doi: 10.1038/sj.bjc.6601005 [published Online First: 2003/06/12]

196. Lee CG, Heijn M, di Tomaso E, et al. Anti-Vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. *Cancer Res* 2000;60(19):5565-70. [published Online First: 2000/10/18]

197. Dickson PV, Hamner JB, Sims TL, et al. Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. *Clin Cancer Res* 2007;13(13):3942-50. doi: 10.1158/1078-0432.CCR-07-0278 [published Online First: 2007/07/04]

198. Dings RP, Loren M, Heun H, et al. Scheduling of radiation with angiogenesis inhibitors anginex and Avastin improves therapeutic outcome via vessel normalization. *Clin Cancer Res* 2007;13(11):3395-402. doi: 10.1158/1078-0432.CCR-06-2441 [published Online First: 2007/06/05]

199. Hodi FS, Lawrence D, Lezcano C, et al. Bevacizumab plus ipilimumab in patients with metastatic melanoma. *Cancer Immunol Res* 2014;2(7):632-42. doi: 10.1158/2326-6066.CIR-14-0053 [published Online First: 2014/05/20]

200. Nishino M, Giobbie-Hurder A, Ramaiya NH, et al. Response assessment in metastatic melanoma treated with ipilimumab and bevacizumab: CT tumor size and density as markers for response and outcome. *J Immunother Cancer* 2014;2(1):40. doi: 10.1186/s40425-014-0040-2 [published Online First: 2014/11/21]

201. Zhang Z, Li LY. TNFSF15 Modulates Neovascularization and Inflammation. *Cancer Microenviron* 2012;5(3):237-47. doi: 10.1007/s12307-012-0117-8 [published Online First: 2012/07/27]

202. Rettig WJ, Garin-Chesa P, Healey JH, et al. Identification of endosialin, a cell surface glycoprotein of vascular endothelial cells in human cancer. *Proc Natl Acad Sci U S A* 1992;89(22):10832-6. doi: 10.1073/pnas.89.22.10832 [published Online First: 1992/11/15]

203. Christian S, Ahorn H, Koehler A, et al. Molecular cloning and characterization of endosialin, a C-type lectin-like cell surface receptor of tumor endothelium. *J Biol Chem* 2001;276(10):7408-14. doi: 10.1074/jbc.M009604200 [published Online First: 2000/11/21]

204. Chi Sabins N, Taylor JL, Fabian KP, et al. DLK1: a novel target for immunotherapeutic remodeling of the tumor blood vasculature. *Mol Ther* 2013;21(10):1958-68. doi: 10.1038/mt.2013.133 [published Online First: 2013/07/31]

205. Fabian KP, Chi-Sabins N, Taylor JL, et al. Therapeutic efficacy of combined vaccination against tumor pericyte-associated antigens DLK1 and DLK2 in mice. *Oncoimmunology* 2017;6(3):e1290035. doi: 10.1080/2162402X.2017.1290035 [published Online First: 2017/04/14]

206. Zhao X, Bose A, Komita H, et al. Intratumoral IL-12 gene therapy results in the crosspriming of Tc1 cells reactive against tumor-associated stromal antigens. *Mol Ther* 2011;19(4):805-14. doi: 10.1038/mt.2010.295 [published Online First: 2010/12/30]

207. Hatano M, Kuwashima N, Tatsumi T, et al. Vaccination with EphA2-derived T cell-epitopes promotes immunity against both EphA2-expressing and EphA2-negative tumors. *J Transl Med* 2004;2(1):40. doi: 10.1186/1479-5876-2-40 [published Online First: 2004/11/26]

208. Bondjers C, Kalen M, Hellstrom M, et al. Transcription profiling of platelet-derived growth factor-B-deficient mouse embryos identifies RGS5 as a novel marker for pericytes and vascular smooth muscle cells. *Am J Pathol* 2003;162(3):721-9. doi: 10.1016/S0002-9440(10)63868-0 [published Online First: 2003/02/25]

209. Liu W, Parikh AA, Stoeltzing O, et al. Upregulation of neuropilin-1 by basic fibroblast growth factor enhances vascular smooth muscle cell migration in response to VEGF. *Cytokine* 2005;32(5):206-12. doi: 10.1016/j.cyto.2005.09.009 [published Online First: 2005/11/18]

210. Niethammer AG, Xiang R, Becker JC, et al. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat Med* 2002;8(12):1369-75. doi: 10.1038/nm1202-794 [published Online First: 2002/11/05]

211. Luo Y, Markowitz D, Xiang R, et al. FLK-1-based minigene vaccines induce T cell-mediated suppression of angiogenesis and tumor protective immunity in syngeneic BALB/c mice. *Vaccine* 2007;25(8):1409-15. doi: 10.1016/j.vaccine.2006.10.043 [published Online First: 2006/11/23]

212. Facciponte JG, Ugel S, De Sanctis F, et al. Tumor endothelial marker 1-specific DNA vaccination targets tumor vasculature. *J Clin Invest* 2014;124(4):1497-511. doi: 10.1172/JCI67382 [published Online First: 2014/03/20]

213. Browder T, Butterfield CE, Kraling BM, et al. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 2000;60(7):1878-86. [published Online First: 2000/04/15]

214. Luqmani YA. Mechanisms of drug resistance in cancer chemotherapy. *Med Princ Pract* 2005;14 Suppl 1:35-48. doi: 10.1159/000086183 [published Online First: 2005/08/17]

215. Hida K, Ohga N, Akiyama K, et al. Heterogeneity of tumor endothelial cells. *Cancer Sci* 2013;104(11):1391-5. doi: 10.1111/cas.12251 [published Online First: 2013/08/13]

216. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 2000;105(8):R15-24. doi: 10.1172/JCI8829 [published Online First: 2000/04/20]

217. Luan X, Guan YY, Lovell JF, et al. Tumor priming using metronomic chemotherapy with neovasculature-targeted, nanoparticulate paclitaxel. *Biomaterials* 2016;95:60-73. doi: 10.1016/j.biomaterials.2016.04.008 [published Online First: 2016/05/01]

218. Bocci G, Nicolaou KC, Kerbel RS. Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various

chemotherapeutic drugs. *Cancer Res* 2002;62(23):6938-43. [published Online First: 2002/12/04]

219. Steinbild S, Arends J, Medinger M, et al. Metronomic antiangiogenic therapy with capecitabine and celecoxib in advanced tumor patients--results of a phase II study. *Onkologie* 2007;30(12):629-35. doi: 10.1159/000110580 [published Online First: 2007/12/08]

220. Slaton JW, Perrotte P, Inoue K, et al. Interferon-alpha-mediated down-regulation of angiogenesis-related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule. *Clin Cancer Res* 1999;5(10):2726-34. [published Online First: 1999/10/28]

221. Garcia-Barros M, Paris F, Cordon-Cardo C, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 2003;300(5622):1155-9. doi: 10.1126/science.1082504 [published Online First: 2003/05/17]

222. Girard JP, Moussion C, Forster R. HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat Rev Immunol* 2012;12(11):762-73. doi: 10.1038/nri3298 [published Online First: 2012/09/29]

223. Browning JL, Allaire N, Ngam-Ek A, et al. Lymphotoxin-beta receptor signaling is required for the homeostatic control of HEV differentiation and function. *Immunity* 2005;23(5):539-50. doi: 10.1016/j.immuni.2005.10.002 [published Online First: 2005/11/16]

224. Ager A, May MJ. Understanding high endothelial venules: Lessons for cancer immunology. *Oncoimmunology* 2015;4(6):e1008791. doi: 10.1080/2162402X.2015.1008791 [published Online First: 2015/07/15]

225. Lee M, Kiefel H, LaJevic MD, et al. Transcriptional programs of lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing. *Nat Immunol* 2014;15(10):982-95. doi: 10.1038/ni.2983 [published Online First: 2014/09/01]

226. Liao S, Ruddle NH. Synchrony of high endothelial venules and lymphatic vessels revealed by immunization. *J Immunol* 2006;177(5):3369-79. doi: 10.4049/jimmunol.177.5.3369 [published Online First: 2006/08/22]

227. Liu K, Victora GD, Schwickert TA, et al. In vivo analysis of dendritic cell development and homeostasis. *Science* 2009;324(5925):392-7. doi: 10.1126/science.1170540 [published Online First: 2009/03/17]

228. Chen S, Kawashima H, Lowe JB, et al. Suppression of tumor formation in lymph nodes by L-selectin-mediated natural killer cell recruitment. *J Exp Med* 2005;202(12):1679-89. doi: 10.1084/jem.20051473 [published Online First: 2005/12/15]

229. Hong SA, Hwang HW, Kim MK, et al. High Endothelial Venule with Concomitant High CD8+ Tumor-Infiltrating Lymphocytes Is Associated with a Favorable Prognosis in Resected Gastric Cancer. *J Clin Med* 2020;9(8) doi: 10.3390/jcm9082628 [published Online First: 2020/08/23]

230. Dieu-Nosjean MC, Goc J, Giraldo NA, et al. Tertiary lymphoid structures in cancer and beyond. *Trends Immunol* 2014;35(11):571-80. doi: 10.1016/j.it.2014.09.006

231. Martinet L, Garrido I, Filleron T, et al. Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer. *Cancer Res* 2011;71(17):5678-87. doi: 10.1158/0008-5472.CAN-11-0431 [published Online First: 2011/08/19]

232. Helmink BA, Reddy SM, Gao J, et al. B cells and tertiary lymphoid structures promote immunotherapy response. *Nature* 2020;577(7791):549-55. doi: 10.1038/s41586-019-1922-8 [published Online First: 2020/01/17]

233. He B, Jabouille A, Steri V, et al. Vascular targeting of LIGHT normalizes blood vessels in primary brain cancer and induces intratumoural high endothelial venules. *J Pathol* 2018;245(2):209-21. doi: 10.1002/path.5080 [published Online First: 2018/04/01]

234. Wieland A, Patel MR, Cardenas MA, et al. Defining HPV-specific B cell responses in patients with head and neck cancer. *Nature* 2020 doi: 10.1038/s41586-020-2931-3 [published Online First: 2020/11/20]

235. Petitprez F, de Reynies A, Keung EZ, et al. B cells are associated with survival and immunotherapy response in sarcoma. *Nature* 2020;577(7791):556-60. doi: 10.1038/s41586-019-1906-8 [published Online First: 2020/01/17]

236. Colbeck EJ, Jones E, Hindley JP, et al. Treg Depletion Licenses T Cell-Driven HEV Neogenesis and Promotes Tumor Destruction. *Cancer Immunol Res* 2017;5(11):1005-15. doi: 10.1158/2326-6066.CIR-17-0131 [published Online First: 2017/09/28]

237. Weinstein AM, Chen L, Brzana EA, et al. Tbet and IL-36gamma cooperate in therapeutic DC-mediated promotion of ectopic lymphoid organogenesis in the tumor microenvironment. *Oncoimmunology* 2017;6(6):e1322238. doi: 10.1080/2162402X.2017.1322238 [published Online First: 2017/07/07]

238. Hindley JP, Jones E, Smart K, et al. T-cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T-cell depletion. *Cancer Res* 2012;72(21):5473-82. doi: 10.1158/0008-5472.CAN-12-1912 [published Online First: 2012/09/11]

239. Messina JL, Fenstermacher DA, Eschrich S, et al. 12-Chemokine gene signature identifies lymph node-like structures in melanoma: potential for patient selection for immunotherapy? *Scientific reports* 2012;2:765. doi: 10.1038/srep00765 [published Online First: 2012/10/26]

240. Sautes-Fridman C, Petitprez F, Calderaro J, et al. Tertiary lymphoid structures in the era of cancer immunotherapy. *Nat Rev Cancer* 2019;19(6):307-25. doi: 10.1038/s41568-019-0144-6 [published Online First: 2019/05/17]

241. Pimenta EM, Barnes BJ. Role of Tertiary Lymphoid Structures (TLS) in Anti-Tumor Immunity: Potential Tumor-Induced Cytokines/Chemokines that Regulate TLS Formation in Epithelial-Derived Cancers. *Cancers (Basel)* 2014;6(2):969-97. doi: 10.3390/cancers6020969 [published Online First: 2014/04/26]

242. Wirsing AM, Rikardsen OG, Steigen SE, et al. Characterisation and prognostic value of tertiary lymphoid structures in oral squamous cell carcinoma. *BMC Clin Pathol* 2014;14:38. doi: 10.1186/1472-6890-14-38 [published Online First: 2014/09/02]

243. Martinet L, Filleron T, Le Guellec S, et al. High endothelial venule blood vessels for tumor-infiltrating lymphocytes are associated with lymphotoxin beta-producing dendritic cells in human breast cancer. *J Immunol* 2013;191(4):2001-8. doi: 10.4049/jimmunol.1300872 [published Online First: 2013/07/05]

244. Giraldo NA, Becht E, Pages F, et al. Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer. *Clin Cancer Res* 2015;21(13):3031-40. doi: 10.1158/1078-0432.CCR-14-2926 [published Online First: 2015/02/18]

245. Peske JD, Thompson ED, Gemta L, et al. Effector lymphocyte-induced lymph node-like vasculature enables naive T-cell entry into tumours and enhanced anti-tumour immunity. *Nat Commun* 2015;6:7114. doi: 10.1038/ncomms8114 [published Online First: 2015/05/15]

246. Tang H, Zhu M, Qiao J, et al. Lymphotoxin signalling in tertiary lymphoid structures and immunotherapy. *Cell Mol Immunol* 2017;14(10):809-18. doi: 10.1038/cmi.2017.13 [published Online First: 2017/04/18]

247. Kratz A, Campos-Neto A, Hanson MS, et al. Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J Exp Med* 1996;183(4):1461-72. doi: 10.1084/jem.183.4.1461 [published Online First: 1996/04/01]

248. Luther SA, Lopez T, Bai W, et al. BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. *Immunity* 2000;12(5):471-81. doi: 10.1016/s1074-7613(00)80199-5 [published Online First: 2000/06/08]

249. Drayton DL, Ying X, Lee J, et al. Ectopic LT alpha beta directs lymphoid organ neogenesis with concomitant expression of peripheral node addressin and a HEV-restricted sulfotransferase. *J Exp Med* 2003;197(9):1153-63. doi: 10.1084/jem.20021761 [published Online First: 2003/05/07]

250. Schrama D, thor Straten P, Fischer WH, et al. Targeting of lymphotoxin-alpha to the tumor elicits an efficient immune response associated with induction of peripheral lymphoid-like tissue. *Immunity* 2001;14(2):111-21. doi: 10.1016/s1074-7613(01)00094-2 [published Online First: 2001/03/10]

251. Dohi T, Rennert PD, Fujihashi K, et al. Elimination of colonic patches with lymphotoxin beta receptor-Ig prevents Th2 cell-type colitis. *J Immunol* 2001;167(5):2781-90. doi: 10.4049/jimmunol.167.5.2781 [published Online First: 2001/08/18]

252. Ettinger R, Munson SH, Chao CC, et al. A critical role for lymphotoxin-beta receptor in the development of diabetes in nonobese diabetic mice. *J Exp Med* 2001;193(11):1333-40. doi: 10.1084/jem.193.11.1333 [published Online First: 2001/06/08]

253. Gantsev SK, Umezawa K, Islamgulov DV, et al. The role of inflammatory chemokines in lymphoid neoorganogenesis in breast cancer. *Biomed Pharmacother* 2013;67(5):363-6. doi: 10.1016/j.biopha.2013.03.017 [published Online First: 2013/04/23]

254. Fan Z, Yu P, Wang Y, et al. NK-cell activation by LIGHT triggers tumor-specific CD8+ T-cell immunity to reject established tumors. *Blood* 2006;107(4):1342-51. doi: 10.1182/blood-2005-08-3485 [published Online First: 2005/10/15]

255. Yu P, Lee Y, Liu W, et al. Priming of naive T cells inside tumors leads to eradication of established tumors. *Nat Immunol* 2004;5(2):141-9. doi: 10.1038/ni1029 [published Online First: 2004/01/06]

256. Denton AE, Innocentin S, Carr EJ, et al. Type I interferon induces CXCL13 to support ectopic germinal center formation. *J Exp Med* 2019;216(3):621-37. doi: 10.1084/jem.20181216 [published Online First: 2019/02/07]

257. Adachi Y, Onodera T, Yamada Y, et al. Distinct germinal center selection at local sites shapes memory B cell response to viral escape. *J Exp Med* 2015;212(10):1709-23. doi: 10.1084/jem.20142284 [published Online First: 2015/09/02]

258. Nacionales DC, Kelly-Scumpia KM, Lee PY, et al. Deficiency of the type I interferon receptor protects mice from experimental lupus. *Arthritis Rheum* 2007;56(11):3770-83. doi: 10.1002/art.23023 [published Online First: 2007/10/31]

259. Nacionales DC, Kelly KM, Lee PY, et al. Type I interferon production by tertiary lymphoid tissue developing in response to 2,6,10,14-tetramethyl-pentadecane (pristane). *Am J Pathol* 2006;168(4):1227-40. doi: 10.2353/ajpath.2006.050125 [published Online First: 2006/03/28]

260. Ogasawara K, Hida S, Weng Y, et al. Requirement of the IFN-alpha/beta-induced CXCR3 chemokine signalling for CD8+ T cell activation. *Genes Cells* 2002;7(3):309-20. doi: 10.1046/j.1365-2443.2002.00515.x [published Online First: 2002/03/29]

261. Banks TA, Rickert S, Benedict CA, et al. A lymphotoxin-IFN-beta axis essential for lymphocyte survival revealed during cytomegalovirus infection. *J Immunol* 2005;174(11):7217-25. doi: 10.4049/jimmunol.174.11.7217 [published Online First: 2005/05/21]

262. Weinstein AM, Giraldo NA, Petitprez F, et al. Association of IL-36gamma with tertiary lymphoid structures and inflammatory immune infiltrates in human colorectal cancer. *Cancer Immunol Immunother* 2019;68(1):109-20. doi: 10.1007/s00262-018-2259-0 [published Online First: 2018/10/14]

263. Chelvanambi M, Weinstein AM, Storkus WJ. IL-36 Signaling in the Tumor Microenvironment. *Adv Exp Med Biol* 2020;1240:95-110. doi: 10.1007/978-3-030-38315-2_8 [published Online First: 2020/02/16]

264. Gu-Trantien C, Migliori E, Buisseret L, et al. CXCL13-producing TFH cells link immune suppression and adaptive memory in human breast cancer. *JCI Insight* 2017;2(11) doi: 10.1172/jci.insight.91487 [published Online First: 2017/06/02]

265. Luther SA, Bidgol A, Hargreaves DC, et al. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol* 2002;169(1):424-33. doi: 10.4049/jimmunol.169.1.424 [published Online First: 2002/06/22]

266. Furtado GC, Marinkovic T, Martin AP, et al. Lymphotoxin beta receptor signaling is required for inflammatory lymphangiogenesis in the thyroid. *Proc Natl Acad Sci U S A* 2007;104(12):5026-31. doi: 10.1073/pnas.0606697104 [published Online First: 2007/03/16]

267. Hiraoka N, Ino Y, Yamazaki-Itoh R. Tertiary Lymphoid Organs in Cancer Tissues. *Front Immunol* 2016;7:244. doi: 10.3389/fimmu.2016.00244 [published Online First: 2016/07/23]

268. Spiotto MT, Yu P, Rowley DA, et al. Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. *Immunity* 2002;17(6):737-47. doi: 10.1016/s1074-7613(02)00480-6 [published Online First: 2002/12/14]

269. Ching LM, Cao Z, Kieda C, et al. Induction of endothelial cell apoptosis by the antivascular agent 5,6-Dimethylxanthenone-4-acetic acid. *Br J Cancer* 2002;86(12):1937-42. doi: 10.1038/sj.bjc.6600368 [published Online First: 2002/06/27]

270. Gulen MF, Koch U, Haag SM, et al. Signalling strength determines proapoptotic functions of STING. *Nat Commun* 2017;8(1):427. doi: 10.1038/s41467-017-00573-w [published Online First: 2017/09/07]

271. Larkin B, Ilyukha V, Sorokin M, et al. Cutting Edge: Activation of STING in T Cells Induces Type I IFN Responses and Cell Death. *J Immunol* 2017;199(2):397-402. doi: 10.4049/jimmunol.1601999 [published Online First: 2017/06/16]

272. Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 2008;455(7213):674-8. doi: 10.1038/nature07317 [published Online First: 2008/08/30]

273. Sun L, Wu J, Du F, et al. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* 2013;339(6121):786-91. doi: 10.1126/science.1232458 [published Online First: 2012/12/22]

274. Li XD, Wu J, Gao D, et al. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science* 2013;341(6152):1390-4. doi: 10.1126/science.1244040 [published Online First: 2013/08/31]

275. Zhong B, Yang Y, Li S, et al. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 2008;29(4):538-50. doi: 10.1016/j.immuni.2008.09.003 [published Online First: 2008/09/27]

276. Zhang L, Liang Y, Li S, et al. The interplay of circulating tumor DNA and chromatin modification, therapeutic resistance, and metastasis. *Mol Cancer* 2019;18(1):36. doi: 10.1186/s12943-019-0989-z [published Online First: 2019/03/10]

277. Woo SR, Fuertes MB, Corrales L, et al. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* 2014;41(5):830-42. doi: 10.1016/j.immuni.2014.10.017 [published Online First: 2014/12/18]

278. Marcus A, Mao AJ, Lensink-Vasan M, et al. Tumor-Derived cGAMP Triggers a STING-Mediated Interferon Response in Non-tumor Cells to Activate the NK Cell Response. *Immunity* 2018;49(4):754-63 e4. doi: 10.1016/j.immuni.2018.09.016 [published Online First: 2018/10/18]

279. Musella M, Manic G, De Maria R, et al. Type-I-interferons in infection and cancer: Unanticipated dynamics with therapeutic implications. *Oncoimmunology* 2017;6(5):e1314424. doi: 10.1080/2162402X.2017.1314424 [published Online First: 2017/06/24]

280. Ergun SL, Li L. Structural Insights into STING Signaling. *Trends Cell Biol* 2020;30(5):399-407. doi: 10.1016/j.tcb.2020.01.010 [published Online First: 2020/04/18]

281. Kim S, Li L, Maliga Z, et al. Anticancer flavonoids are mouse-selective STING agonists. *ACS Chem Biol* 2013;8(7):1396-401. doi: 10.1021/cb400264n [published Online First: 2013/05/21]

282. Conlon J, Burdette DL, Sharma S, et al. Mouse, but not human STING, binds and signals in response to the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid. *J Immunol* 2013;190(10):5216-25. doi: 10.4049/jimmunol.1300097 [published Online First: 2013/04/16]

283. Chin KH, Tu ZL, Su YC, et al. Novel c-di-GMP recognition modes of the mouse innate immune adaptor protein STING. *Acta Crystallogr D Biol Crystallogr* 2013;69(Pt 3):352-66. doi: 10.1107/S0907444912047269 [published Online First: 2013/03/23]

284. Cong X, Yuan Z, Du Y, et al. Crystal structures of porcine STING(CBD)-CDN complexes reveal the mechanism of ligand recognition and discrimination of STING proteins. *J Biol Chem* 2019;294(30):11420-32. doi: 10.1074/jbc.RA119.007367 [published Online First: 2019/06/07]

285. Shang G, Zhang C, Chen ZJ, et al. Cryo-EM structures of STING reveal its mechanism of activation by cyclic GMP-AMP. *Nature* 2019;567(7748):389-93. doi: 10.1038/s41586-019-0998-5 [published Online First: 2019/03/08]

286. Tsuchiya Y, Jounai N, Takeshita F, et al. Ligand-induced Ordering of the C-terminal Tail Primes STING for Phosphorylation by TBK1. *EBioMedicine* 2016;9:87-96. doi: 10.1016/j.ebiom.2016.05.039 [published Online First: 2016/06/23]

287. Zhang C, Shang G, Gui X, et al. Structural basis of STING binding with and phosphorylation by TBK1. *Nature* 2019;567(7748):394-98. doi: 10.1038/s41586-019-1000-2 [published Online First: 2019/03/08]

288. Tanaka Y, Chen ZJ. STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Sci Signal* 2012;5(214):ra20. doi: 10.1126/scisignal.2002521 [published Online First: 2012/03/08]

289. Liu S, Cai X, Wu J, et al. Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* 2015;347(6227):aaa2630. doi: 10.1126/science.aaa2630 [published Online First: 2015/02/01]

290. Zhao B, Shu C, Gao X, et al. Structural basis for concerted recruitment and activation of IRF-3 by innate immune adaptor proteins. *Proc Natl Acad Sci U S A* 2016;113(24):E3403-12. doi: 10.1073/pnas.1603269113 [published Online First: 2016/06/16]

291. Wang Z, Ji J, Peng D, et al. Complex Regulation Pattern of IRF3 Activation Revealed by a Novel Dimerization Reporter System. *J Immunol* 2016;196(10):4322-30. doi: 10.4049/jimmunol.1502458 [published Online First: 2016/04/06]

292. Takahasi K, Suzuki NN, Horiuchi M, et al. X-ray crystal structure of IRF-3 and its functional implications. *Nat Struct Biol* 2003;10(11):922-7. doi: 10.1038/nsb1001 [published Online First: 2003/10/14]

293. Sato M, Tanaka N, Hata N, et al. Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene. *FEBS Lett* 1998;425(1):112-6. doi: 10.1016/s0014-5793(98)00210-5 [published Online First: 1998/04/16]

294. Yoneyama M, Suhara W, Fukuhara Y, et al. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J* 1998;17(4):1087-95. doi: 10.1093/emboj/17.4.1087 [published Online First: 1998/03/28]

295. Qin BY, Liu C, Lam SS, et al. Crystal structure of IRF-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation. *Nat Struct Biol* 2003;10(11):913-21. doi: 10.1038/nsb1002 [published Online First: 2003/10/14]

296. Sivick KE, Desbien AL, Glickman LH, et al. Magnitude of Therapeutic STING Activation Determines CD8(+) T Cell-Mediated Anti-tumor Immunity. *Cell Rep* 2018;25(11):3074-85 e5. doi: 10.1016/j.celrep.2018.11.047 [published Online First: 2018/12/13]

297. Li W, Quan YY, Li Y, et al. Monitoring of tumor vascular normalization: the key points from basic research to clinical application. *Cancer Manag Res* 2018;10:4163-72. doi: 10.2147/CMAR.S174712 [published Online First: 2018/10/17]

298. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;307(5706):58-62. doi: 10.1126/science.1104819 [published Online First: 2005/01/08]

299. Huang Y, Kim BYS, Chan CK, et al. Improving immune-vascular crosstalk for cancer immunotherapy. *Nat Rev Immunol* 2018;18(3):195-203. doi: 10.1038/nri.2017.145 [published Online First: 2018/01/16]

300. Shekarian T, Valsesia-Wittmann S, Brody J, et al. Pattern recognition receptors: immune targets to enhance cancer immunotherapy. *Ann Oncol* 2017;28(8):1756-66. doi: 10.1093/annonc/mdx179 [published Online First: 2017/04/27]

301. Zhu Y, An X, Zhang X, et al. STING: a master regulator in the cancer-immunity cycle. *Mol Cancer* 2019;18(1):152. doi: 10.1186/s12943-019-1087-y [published Online First: 2019/11/05]

302. Cooper ZA, Juneja VR, Sage PT, et al. Response to BRAF inhibition in melanoma is enhanced when combined with immune checkpoint blockade. *Cancer Immunol Res* 2014;2(7):643-54. doi: 10.1158/2326-6066.CIR-13-0215 [published Online First: 2014/06/07]

303. Corrales L, Glickman LH, McWhirter SM, et al. Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. *Cell Rep* 2015;11(7):1018-30. doi: 10.1016/j.celrep.2015.04.031 [published Online First: 2015/05/12]

304. Baguley BC, Siemann DW. Temporal aspects of the action of ASA404 (vadimezan; DMXAA). *Expert Opin Investig Drugs* 2010;19(11):1413-25. doi: 10.1517/13543784.2010.529128 [published Online First: 2010/10/23]

305. Lemos H, Ou R, McCardle C, et al. Overcoming resistance to STING agonist therapy to incite durable protective antitumor immunity. *J Immunother Cancer* 2020;8(2) doi: 10.1136/jitc-2020-001182 [published Online First: 2020/08/28]

306. Ching LM, Zwain S, Baguley BC. Relationship between tumour endothelial cell apoptosis and tumour blood flow shutdown following treatment with the antivascular agent DMXAA in mice. *Br J Cancer* 2004;90(4):906-10. doi: 10.1038/sj.bjc.6601606 [published Online First: 2004/02/19]

307. Yu J, Tian S, Metheny-Barlow L, et al. Modulation of endothelial cell growth arrest and apoptosis by vascular endothelial growth inhibitor. *Circ Res* 2001;89(12):1161-7. doi: 10.1161/hh2401.101909 [published Online First: 2001/12/12]

308. Romagnani P, Lasagni L, Annunziato F, et al. CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trends Immunol* 2004;25(4):201-9. doi: 10.1016/j.it.2004.02.006 [published Online First: 2004/03/25]

309. Vermeulen PB, Gasparini G, Fox SB, et al. Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. *Eur J Cancer* 2002;38(12):1564-79. doi: 10.1016/s0959-8049(02)00094-1 [published Online First: 2002/07/27]

310. Willam C, Schindler R, Frei U, et al. Increases in oxygen tension stimulate expression of ICAM-1 and VCAM-1 on human endothelial cells. *Am J Physiol* 1999;276(6):H2044-52. doi: 10.1152/ajpheart.1999.276.6.H2044 [published Online First: 1999/06/11]

311. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol* 2005;5(8):617-28. doi: 10.1038/nri1670 [published Online First: 2005/08/02]

312. Stylianopoulos T, Munn LL, Jain RK. Reengineering the Physical Microenvironment of Tumors to Improve Drug Delivery and Efficacy: From Mathematical Modeling to Bench to Bedside. *Trends Cancer* 2018;4(4):292-319. doi: 10.1016/j.trecan.2018.02.005 [published Online First: 2018/04/03]

313. Lund AW, Wagner M, Fankhauser M, et al. Lymphatic vessels regulate immune microenvironments in human and murine melanoma. *J Clin Invest* 2016;126(9):3389-402. doi: 10.1172/JCI79434 [published Online First: 2016/08/16]

314. Zhu G, Falahat R, Wang K, et al. Tumor-Associated Tertiary Lymphoid Structures: Gene-Expression Profiling and Their Bioengineering. *Front Immunol* 2017;8:767. doi: 10.3389/fimmu.2017.00767 [published Online First: 2017/07/18]

315. Webster B, Ekland EH, Agle LM, et al. Regulation of lymph node vascular growth by dendritic cells. *J Exp Med* 2006;203(8):1903-13. doi: 10.1084/jem.20052272 [published Online First: 2006/07/13]

316. Furtado GC, Pacer ME, Bongers G, et al. TNFalpha-dependent development of lymphoid tissue in the absence of RORgammat(+) lymphoid tissue inducer cells. *Mucosal Immunol* 2014;7(3):602-14. doi: 10.1038/mi.2013.79 [published Online First: 2013/10/17]

317. Coppola D, Nebozhyn M, Khalil F, et al. Unique ectopic lymph node-like structures present in human primary colorectal carcinoma are identified by immune gene array profiling. *Am J Pathol* 2011;179(1):37-45. doi: 10.1016/j.ajpath.2011.03.007 [published Online First: 2011/06/28]

318. Goc J, Germain C, Vo-Bourgais TKD, et al. Dendritic Cells in Tumor-Associated Tertiary Lymphoid Structures Signal a Th1 Cytotoxic Immune Contexture and License the Positive Prognostic Value of Infiltrating CD8⁺ T Cells. *Cancer Research* 2014;74(3):705-15. doi: 10.1158/0008-5472.Can-13-1342

319. Johansson-Percival A, He B, Li ZJ, et al. De novo induction of intratumoral lymphoid structures and vessel normalization enhances immunotherapy in resistant tumors. *Nat Immunol* 2017;18(11):1207-17. doi: 10.1038/ni.3836 [published Online First: 2017/09/12]

320. Stowman AM, Hickman AW, Mauldin IS, et al. Lymphoid aggregates in desmoplastic melanoma have features of tertiary lymphoid structures. *Melanoma Res* 2018;28(3):237-45. doi: 10.1097/CMR.0000000000000439 [published Online First: 2018/03/15]

321. Martinet L, Le Guellec S, Filleron T, et al. High endothelial venules (HEVs) in human melanoma lesions: Major gateways for tumor-infiltrating lymphocytes. *Oncoimmunology* 2012;1(6):829-39. doi: 10.4161/onci.20492 [published Online First: 2012/11/20]

322. Konno H, Yamauchi S, Berglund A, et al. Suppression of STING signaling through epigenetic silencing and missense mutation impedes DNA damage mediated cytokine production. *Oncogene* 2018;37(15):2037-51. doi: 10.1038/s41388-017-0120-0 [published Online First: 2018/01/26]

323. Yang H, Lee WS, Kong SJ, et al. STING activation reprograms tumor vasculatures and synergizes with VEGFR2 blockade. *J Clin Invest* 2019;129(10):4350-64. doi: 10.1172/JCI125413 [published Online First: 2019/07/26]

324. Ager CR, Reilley MJ, Nicholas C, et al. Intratumoral STING Activation with T-cell Checkpoint Modulation Generates Systemic Antitumor Immunity. *Cancer Immunol Res* 2017;5(8):676-84. doi: 10.1158/2326-6066.CIR-17-0049 [published Online First: 2017/07/05]

325. Fu J, Kanne DB, Leong M, et al. STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade. *Sci Transl Med* 2015;7(283):283ra52. doi: 10.1126/scitranslmed.aaa4306 [published Online First: 2015/04/17]

326. Sallets A, Robinson S, Kardosh A, et al. Enhancing immunotherapy of STING agonist for lymphoma in preclinical models. *Blood Adv* 2018;2(17):2230-41. doi: 10.1182/bloodadvances.2018020040 [published Online First: 2018/09/09]

327. Peske JD, Thompson ED, Gemta L, et al. Effector lymphocyte-induced lymph node-like vasculature enables naive T-cell entry into tumours and enhanced anti-tumour immunity. *Nature Communications* 2015;6:7114. doi: 10.1038/ncomms8114
https://www.nature.com/articles/ncomms8114#supplementary-information

328. McMahon EJ, Bailey SL, Castenada CV, et al. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nat Med* 2005;11(3):335-9. doi: 10.1038/nm1202 [published Online First: 2005/03/01]

329. Cabrita R, Lauss M, Sanna A, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature* 2020;577(7791):561-65. doi: 10.1038/s41586-019-1914-8

330. Helmink BA, Reddy SM, Gao J, et al. B cells and tertiary lymphoid structures promote immunotherapy response. *Nature* 2020;577(7791):549-55. doi: 10.1038/s41586-019-1922-8

331. Rodriguez-Pinto D. B cells as antigen presenting cells. *Cell Immunol* 2005;238(2):67-75. doi: 10.1016/j.cellimm.2006.02.005 [published Online First: 2006/04/01]

332. Rodriguez-Pinto D, Moreno J. B cells can prime naive CD4+ T cells in vivo in the absence of other professional antigen-presenting cells in a CD154-CD40-dependent manner. *Eur J Immunol* 2005;35(4):1097-105. doi: 10.1002/eji.200425732 [published Online First: 2005/03/10]

333. Rubtsov AV, Rubtsova K, Kappler JW, et al. CD11c-Expressing B Cells Are Located at the T Cell/B Cell Border in Spleen and Are Potent APCs. *J Immunol* 2015;195(1):71-9. doi: 10.4049/jimmunol.1500055 [published Online First: 2015/06/03]

334. Lochner M, Ohnmacht C, Presley L, et al. Microbiota-induced tertiary lymphoid tissues aggravate inflammatory disease in the absence of RORgamma t and LTi cells. *J Exp Med* 2011;208(1):125-34. doi: 10.1084/jem.20100052 [published Online First: 2010/12/22]

335. Cohen KW, Dugast AS, Alter G, et al. HIV-1 single-stranded RNA induces CXCL13 secretion in human monocytes via TLR7 activation and plasmacytoid dendritic cell-derived type I IFN. *J Immunol* 2015;194(6):2769-75. doi: 10.4049/jimmunol.1400952 [published Online First: 2015/02/11]

336. Yim LY, Lau CS, Chan VS. Heightened TLR7/9-Induced IL-10 and CXCL13 Production with Dysregulated NF-B Activation in CD11c(hi)CD11b(+) Dendritic Cells in NZB/W F1 Mice. *Int J Mol Sci* 2019;20(18) doi: 10.3390/ijms20184639 [published Online First: 2019/09/25]

337. Ma D, Fan SB, Hua N, et al. Hypermethylation of single CpG dinucleotides at the promoter of CXCL13 gene promote cell migration in cervical cancer. *Curr Cancer Drug Targets* 2020 doi: 10.2174/1568009620666200102123635 [published Online First: 2020/01/04]

338. Kobayashi T, Hamaguchi Y, Hasegawa M, et al. B cells promote tumor immunity against B16F10 melanoma. *Am J Pathol* 2014;184(11):3120-9. doi: 10.1016/j.ajpath.2014.07.003 [published Online First: 2014/09/01]

339. Wouters MCA, Nelson BH. Prognostic Significance of Tumor-Infiltrating B Cells and Plasma Cells in Human Cancer. *Clin Cancer Res* 2018;24(24):6125-35. doi: 10.1158/1078-0432.CCR-18-1481 [published Online First: 2018/07/28]

340. Largeot A, Pagano G, Gonder S, et al. The B-side of Cancer Immunity: The Underrated Tune. *Cells* 2019;8(5) doi: 10.3390/cells8050449 [published Online First: 2019/05/16]

341. Somasundaram R, Zhang G, Fukunaga-Kalabis M, et al. Tumor-associated B-cells induce tumor heterogeneity and therapy resistance. *Nat Commun* 2017;8(1):607. doi: 10.1038/s41467-017-00452-4 [published Online First: 2017/09/21]

342. Liu B, Qu L, Yan S. Cyclooxygenase-2 promotes tumor growth and suppresses tumor immunity. *Cancer Cell Int* 2015;15:106. doi: 10.1186/s12935-015-0260-7 [published Online First: 2015/11/10]

343. Idos GE, Kwok J, Bonthala N, et al. The Prognostic Implications of Tumor Infiltrating Lymphocytes in Colorectal Cancer: A Systematic Review and Meta-Analysis. *Scientific reports* 2020;10(1):3360. doi: 10.1038/s41598-020-60255-4 [published Online First: 2020/02/27]

344. Teng MW, Ngiow SF, Ribas A, et al. Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Res* 2015;75(11):2139-45. doi: 10.1158/0008-5472.CAN-15-0255 [published Online First: 2015/05/16]

345. Liu Y, Jesus AA, Marrero B, et al. Activated STING in a vascular and pulmonary syndrome. *N Engl J Med* 2014;371(6):507-18. doi: 10.1056/NEJMoa1312625 [published Online First: 2014/07/17]

346. Mattila P, Majuri ML, Mattila PS, et al. TNF alpha-induced expression of endothelial adhesion molecules, ICAM-1 and VCAM-1, is linked to protein kinase C activation. *Scand J Immunol* 1992;36(2):159-65. doi: 10.1111/j.1365-3083.1992.tb03087.x [published Online First: 1992/08/01]

347. Brown MD, Wick TM, Eckman JR. Activation of vascular endothelial cell adhesion molecule expression by sickle blood cells. *Pediatr Pathol Mol Med* 2001;20(1):47-72. [published Online First: 2003/04/04]

348. Kobayashi T, Takaku Y, Yokote A, et al. Interferon-beta augments eosinophil adhesion-inducing activity of endothelial cells. *Eur Respir J* 2008;32(6):1540-7. doi: 10.1183/09031936.00059507 [published Online First: 2008/07/26]

349. Wculek SK, Cueto FJ, Mujal AM, et al. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol* 2020;20(1):7-24. doi: 10.1038/s41577-019-0210-z [published Online First: 2019/08/31]

350. Cillo AR, Kurten CHL, Tabib T, et al. Immune Landscape of Viral- and Carcinogen-Driven Head and Neck Cancer. *Immunity* 2020;52(1):183-99 e9. doi: 10.1016/j.immuni.2019.11.014 [published Online First: 2020/01/12]

351. Sharonov GV, Serebrovskaya EO, Yuzhakova DV, et al. B cells, plasma cells and antibody repertoires in the tumour microenvironment. *Nat Rev Immunol* 2020;20(5):294-307. doi: 10.1038/s41577-019-0257-x [published Online First: 2020/01/29]

352. Nzula S, Going JJ, Stott DI. Antigen-driven clonal proliferation, somatic hypermutation, and selection of B lymphocytes infiltrating human ductal breast carcinomas. *Cancer Res* 2003;63(12):3275-80. [published Online First: 2003/06/18]

353. Overdijk MB, Verploegen S, Bogels M, et al. Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. *MAbs* 2015;7(2):311-21. doi: 10.1080/19420862.2015.1007813 [published Online First: 2015/03/12]

354. Gul N, van Egmond M. Antibody-Dependent Phagocytosis of Tumor Cells by Macrophages: A Potent Effector Mechanism of Monoclonal Antibody Therapy of Cancer. *Cancer Res* 2015;75(23):5008-13. doi: 10.1158/0008-5472.CAN-15-1330 [published Online First: 2015/11/18]

355. Kurai J, Chikumi H, Hashimoto K, et al. Antibody-dependent cellular cytotoxicity mediated by cetuximab against lung cancer cell lines. *Clin Cancer Res* 2007;13(5):1552-61. doi: 10.1158/1078-0432.CCR-06-1726 [published Online First: 2007/03/03]

356. Gilbert AE, Karagiannis P, Dodev T, et al. Monitoring the systemic human memory B cell compartment of melanoma patients for anti-tumor IgG antibodies. *PLoS One* 2011;6(4):e19330. doi: 10.1371/journal.pone.0019330 [published Online First: 2011/05/12]

357. Nishioka K, Kawamura K, Hirayama T, et al. The complement system in tumor immunity: significance of elevated levels of complement in tumor bearing hosts. *Ann N Y Acad Sci* 1976;276:303-15. doi: 10.1111/j.1749-6632.1976.tb41656.x [published Online First: 1976/01/01]

358. Niculescu F, Rus HG, Retegan M, et al. Persistent complement activation on tumor cells in breast cancer. *Am J Pathol* 1992;140(5):1039-43. [published Online First: 1992/05/01]

359. Yamakawa M, Yamada K, Tsuge T, et al. Protection of thyroid cancer cells by complement-regulatory factors. *Cancer* 1994;73(11):2808-17. doi: 10.1002/1097-0142(19940601)73:11<2808::aid-cncr2820731125>3.0.co;2-p [published Online First: 1994/06/01]

360. Pio R, Corrales L, Lambris JD. The role of complement in tumor growth. *Adv Exp Med Biol* 2014;772:229-62. doi: 10.1007/978-1-4614-5915-6_11 [published Online First: 2013/11/26]

361. Schroeder HW, Jr., Cavacini L. Structure and function of immunoglobulins. *J Allergy Clin Immunol* 2010;125(2 Suppl 2):S41-52. doi: 10.1016/j.jaci.2009.09.046 [published Online First: 2010/03/05]

362. Bruhns P, Iannascoli B, England P, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. *Blood* 2009;113(16):3716-25. doi: 10.1182/blood-2008-09-179754 [published Online First: 2008/11/20]

363. Chen X, Song X, Li K, et al. FcgammaR-Binding Is an Important Functional Attribute for Immune Checkpoint Antibodies in Cancer Immunotherapy. *Front Immunol* 2019;10:292. doi: 10.3389/fimmu.2019.00292 [published Online First: 2019/03/14]

364. Deola S, Panelli MC, Maric D, et al. Helper B cells promote cytotoxic T cell survival and proliferation independently of antigen presentation through CD27/CD70 interactions. *J Immunol* 2008;180(3):1362-72. doi: 10.4049/jimmunol.180.3.1362 [published Online First: 2008/01/23]

365. Ma DY, Clark EA. The role of CD40 and CD154/CD40L in dendritic cells. *Semin Immunol* 2009;21(5):265-72. doi: 10.1016/j.smim.2009.05.010 [published Online First: 2009/06/16]

366. Wykes M, Poudrier J, Lindstedt R, et al. Regulation of cytoplasmic, surface and soluble forms of CD40 ligand in mouse B cells. *Eur J Immunol* 1998;28(2):548-59. doi: 10.1002/(SICI)1521-4141(199802)28:02<548::AID-IMMU548>3.0.CO;2-2 [published Online First: 1998/04/01]

367. Grammer AC, McFarland RD, Heaney J, et al. Expression, regulation, and function of B cell-expressed CD154 in germinal centers. *J Immunol* 1999;163(8):4150-9. [published Online First: 1999/10/08]

368. Colbeck EJ, Ager A, Gallimore A, et al. Tertiary Lymphoid Structures in Cancer: Drivers of Antitumor Immunity, Immunosuppression, or Bystander Sentinels in Disease? *Front Immunol* 2017;8:1830. doi: 10.3389/fimmu.2017.01830 [published Online First: 2018/01/10]

369. Avram G, Sanchez-Sendra B, Martin JM, et al. The density and type of MECA-79-positive high endothelial venules correlate with lymphocytic infiltration and tumour regression in primary cutaneous melanoma. *Histopathology* 2013;63(6):852-61. doi: 10.1111/his.12235 [published Online First: 2013/10/10]

370. Ladanyi A, Kiss J, Somlai B, et al. Density of DC-LAMP(+) mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. *Cancer Immunol Immunother* 2007;56(9):1459-69. doi: 10.1007/s00262-007-0286-3 [published Online First: 2007/02/07]

371. Ohandjo AQ, Liu Z, Dammer EB, et al. Transcriptome Network Analysis Identifies CXCL13-CXCR5 Signaling Modules in the Prostate Tumor Immune Microenvironment. *Scientific reports* 2019;9(1):14963. doi: 10.1038/s41598-019-46491-3 [published Online First: 2019/10/20]

372. Prabakaran T, Bodda C, Krapp C, et al. Attenuation of cGAS-STING signaling is mediated by a p62/SQSTM1-dependent autophagy pathway activated by TBK1. *EMBO J* 2018;37(8) doi: 10.15252/embj.201797858 [published Online First: 2018/03/03]

373. Liu D, Sheng C, Gao S, et al. SOCS3 Drives Proteasomal Degradation of TBK1 and Negatively Regulates Antiviral Innate Immunity. *Mol Cell Biol* 2015;35(14):2400-13. doi: 10.1128/MCB.00090-15 [published Online First: 2015/05/06]

374. Pauli EK, Schmolke M, Wolff T, et al. Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression. *PLoS Pathog* 2008;4(11):e1000196. doi: 10.1371/journal.ppat.1000196 [published Online First: 2008/11/08]

375. Pothlichet J, Chignard M, Si-Tahar M. Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-dependent pathway. *J Immunol* 2008;180(4):2034-8. doi: 10.4049/jimmunol.180.4.2034 [published Online First: 2008/02/06]

376. Zhang K, Zhang Y, Xue J, et al. DDX19 Inhibits Type I Interferon Production by Disrupting TBK1-IKKepsilon-IRF3 Interactions and Promoting TBK1 and IKKepsilon Degradation.

Cell Rep 2019;26(5):1258-72 e4. doi: 10.1016/j.celrep.2019.01.029 [published Online First: 2019/01/31]

377. Deng M, Tam JW, Wang L, et al. TRAF3IP3 negatively regulates cytosolic RNA induced anti-viral signaling by promoting TBK1 K48 ubiquitination. *Nat Commun* 2020;11(1):2193. doi: 10.1038/s41467-020-16014-0 [published Online First: 2020/05/06]

378. Lesinski GB, Zimmerer JM, Kreiner M, et al. Modulation of SOCS protein expression influences the interferon responsiveness of human melanoma cells. *BMC Cancer* 2010;10:142. doi: 10.1186/1471-2407-10-142 [published Online First: 2010/04/20]

379. Fojtova M, Boudny V, Kovarik A, et al. Development of IFN-gamma resistance is associated with attenuation of SOCS genes induction and constitutive expression of SOCS 3 in melanoma cells. *Br J Cancer* 2007;97(2):231-7. doi: 10.1038/sj.bjc.6603849 [published Online First: 2007/06/21]

380. Nasarre P, Bonilla IV, Metcalf JS, et al. TRAF3-interacting protein 3, a new oncotarget, promotes tumor growth in melanoma. *Melanoma Res* 2018;28(3):185-94. doi: 10.1097/CMR.0000000000000440 [published Online First: 2018/03/20]

381. Xu H, An H, Hou J, et al. Phosphatase PTP1B negatively regulates MyD88- and TRIF-dependent proinflammatory cytokine and type I interferon production in TLR-triggered macrophages. *Mol Immunol* 2008;45(13):3545-52. doi: 10.1016/j.molimm.2008.05.006 [published Online First: 2008/06/24]

382. Xia T, Yi XM, Wu X, et al. PTPN1/2-mediated dephosphorylation of MITA/STING promotes its 20S proteasomal degradation and attenuates innate antiviral response. *Proc Natl Acad Sci U S A* 2019;116(40):20063-69. doi: 10.1073/pnas.1906431116 [published Online First: 2019/09/19]

383. Qi D, Hu L, Jiao T, et al. Phosphatase Cdc25A Negatively Regulates the Antiviral Immune Response by Inhibiting TBK1 Activity. *J Virol* 2018;92(19) doi: 10.1128/JVI.01118-18 [published Online First: 2018/07/20]

384. Png CW, Zhang Y. MAPK phosphatase 5 inhibits IRF3. *Oncotarget* 2015;6(23):19348-9. doi: 10.18632/oncotarget.5096 [published Online First: 2015/08/26]

385. Ouyang W, Liu C, Pan Y, et al. SHP2 deficiency promotes Staphylococcus aureus pneumonia following influenza infection. *Cell Prolif* 2020;53(1):e12721. doi: 10.1111/cpr.12721 [published Online First: 2019/11/30]

386. Walsh N, Kennedy S, Larkin AM, et al. Membrane transport proteins in human melanoma: associations with tumour aggressiveness and metastasis. *Br J Cancer* 2010;102(7):1157-62. doi: 10.1038/sj.bjc.6605590 [published Online First: 2010/03/18]

387. Luteijn RD, Zaver SA, Gowen BG, et al. SLC19A1 transports immunoreactive cyclic dinucleotides. *Nature* 2019;573(7774):434-38. doi: 10.1038/s41586-019-1553-0 [published Online First: 2019/09/13]

388. Ablasser A, Schmid-Burgk JL, Hemmerling I, et al. Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP. *Nature* 2013;503(7477):530-4. doi: 10.1038/nature12640 [published Online First: 2013/10/01]

389. Mason PA, Cox LS. The role of DNA exonucleases in protecting genome stability and their impact on ageing. *Age (Dordr)* 2012;34(6):1317-40. doi: 10.1007/s11357-011-9306-5 [published Online First: 2011/09/29]

390. Yan N. Immune Diseases Associated with TREX1 and STING Dysfunction. *J Interferon Cytokine Res* 2017;37(5):198-206. doi: 10.1089/jir.2016.0086 [published Online First: 2017/05/06]

391. Xia T, Konno H, Ahn J, et al. Deregulation of STING Signaling in Colorectal Carcinoma Constrains DNA Damage Responses and Correlates With Tumorigenesis. *Cell Rep* 2016;14(2):282-97. doi: 10.1016/j.celrep.2015.12.029 [published Online First: 2016/01/11]

392. Xia T, Konno H, Barber GN. Recurrent Loss of STING Signaling in Melanoma Correlates with Susceptibility to Viral Oncolysis. *Cancer Res* 2016;76(22):6747-59. doi: 10.1158/0008-5472.CAN-16-1404 [published Online First: 2016/11/05]

393. Falahat R, Perez-Villarroel P, Mailloux AW, et al. STING Signaling in Melanoma Cells Shapes Antigenicity and Can Promote Antitumor T-cell Activity. *Cancer Immunol Res* 2019;7(11):1837-48. doi: 10.1158/2326-6066.CIR-19-0229 [published Online First: 2019/08/30]

394. Hu HG, Li YM. Emerging Adjuvants for Cancer Immunotherapy. *Front Chem* 2020;8:601. doi: 10.3389/fchem.2020.00601 [published Online First: 2020/08/28]

395. Lara PN, Jr., Douillard JY, Nakagawa K, et al. Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent vadimezan (ASA404) in advanced non-small-cell lung cancer. *J Clin Oncol* 2011;29(22):2965-71. doi: 10.1200/JCO.2011.35.0660 [published Online First: 2011/06/29]

396. Simm R, Morr M, Kader A, et al. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol* 2004;53(4):1123-34. doi: 10.1111/j.1365-2958.2004.04206.x [published Online First: 2004/08/13]

397. Yadav M, Pal K, Sen U. Structures of c-di-GMP/cGAMP degrading phosphodiesterase VcEAL: identification of a novel conformational switch and its implication. *Biochem J* 2019;476(21):3333-53. doi: 10.1042/BCJ20190399 [published Online First: 2019/10/28]

398. Yan H, Wang X, KuoLee R, et al. Synthesis and immunostimulatory properties of the phosphorothioate analogues of cdiGMP. *Bioorg Med Chem Lett* 2008;18(20):5631-4. doi: 10.1016/j.bmcl.2008.08.088 [published Online First: 2008/09/19]

399. Li A, Yi M, Qin S, et al. Activating cGAS-STING pathway for the optimal effect of cancer immunotherapy. *J Hematol Oncol* 2019;12(1):35. doi: 10.1186/s13045-019-0721-x [published Online First: 2019/04/03]

400. Ghaffari A, Peterson N, Khalaj K, et al. STING agonist therapy in combination with PD-1 immune checkpoint blockade enhances response to carboplatin chemotherapy in high-grade serous ovarian cancer. *Br J Cancer* 2018;119(4):440-49. doi: 10.1038/s41416-018-0188-5 [published Online First: 2018/07/27]

401. Nakanishi M, Rosenberg DW. Multifaceted roles of PGE2 in inflammation and cancer. *Semin Immunopathol* 2013;35(2):123-37. doi: 10.1007/s00281-012-0342-8 [published Online First: 2012/09/22]

402. Lemos H, Mohamed E, Huang L, et al. STING Promotes the Growth of Tumors Characterized by Low Antigenicity via IDO Activation. *Cancer Res* 2016;76(8):2076-81. doi: 10.1158/0008-5472.CAN-15-1456 [published Online First: 2016/03/12]

403. Hornyak L, Dobos N, Koncz G, et al. The Role of Indoleamine-2,3-Dioxygenase in Cancer Development, Diagnostics, and Therapy. *Front Immunol* 2018;9:151. doi: 10.3389/fimmu.2018.00151 [published Online First: 2018/02/16]

404. Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends Immunol* 2013;34(3):137-43. doi: 10.1016/j.it.2012.10.001 [published Online First: 2012/10/30]

405. Maria NI, van Helden-Meeuwsen CG, Brkic Z, et al. Association of Increased Treg Cell Levels With Elevated Indoleamine 2,3-Dioxygenase Activity and an Imbalanced Kynurenine Pathway in Interferon-Positive Primary Sjogren's Syndrome. *Arthritis Rheumatol* 2016;68(7):1688-99. doi: 10.1002/art.39629 [published Online First: 2016/02/13]

406. Nguyen NT, Kimura A, Nakahama T, et al. Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. *Proc Natl Acad Sci U S A* 2010;107(46):19961-6. doi: 10.1073/pnas.1014465107 [published Online First: 2010/11/03]

407. Ohkuri T, Kosaka A, Ishibashi K, et al. Intratumoral administration of cGAMP transiently accumulates potent macrophages for anti-tumor immunity at a mouse tumor site. *Cancer Immunol Immunother* 2017;66(6):705-16. doi: 10.1007/s00262-017-1975-1 [published Online First: 2017/03/01]

408. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* 2014;6:13. doi: 10.12703/P6-13 [published Online First: 2014/03/29]

409. Yin Y, Pham TL, Shin J, et al. Arginase 2 Deficiency Promotes Neuroinflammation and Pain Behaviors Following Nerve Injury in Mice. *J Clin Med* 2020;9(2) doi: 10.3390/jcm9020305 [published Online First: 2020/01/26]

410. Hardbower DM, Asim M, Murray-Stewart T, et al. Arginase 2 deletion leads to enhanced M1 macrophage activation and upregulated polyamine metabolism in response to Helicobacter pylori infection. *Amino Acids* 2016;48(10):2375-88. doi: 10.1007/s00726-016-2231-2 [published Online First: 2016/04/15]

411. Kalinski P. Regulation of immune responses by prostaglandin E2. *J Immunol* 2012;188(1):21-8. doi: 10.4049/jimmunol.1101029 [published Online First: 2011/12/22]

412. Muthuswamy R, Mueller-Berghaus J, Haberkorn U, et al. PGE(2) transiently enhances DC expression of CCR7 but inhibits the ability of DCs to produce CCL19 and attract naive T cells. *Blood* 2010;116(9):1454-9. doi: 10.1182/blood-2009-12-258038 [published Online First: 2010/05/26]

413. Joshi PC, Zhou X, Cuchens M, et al. Prostaglandin E2 suppressed IL-15-mediated human NK cell function through down-regulation of common gamma-chain. *J Immunol* 2001;166(2):885-91. doi: 10.4049/jimmunol.166.2.885 [published Online First: 2001/01/06]

414. Liu L, Ge D, Ma L, et al. Interleukin-17 and prostaglandin E2 are involved in formation of an M2 macrophage-dominant microenvironment in lung cancer. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* 2012;7(7):1091-100. doi: 10.1097/JTO.0b013e3182542752 [published Online First: 2012/04/27]

415. Castellone MD, Teramoto H, Williams BO, et al. Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. *Science* 2005;310(5753):1504-10. doi: 10.1126/science.1116221 [published Online First: 2005/11/19]

416. Buchanan FG, Wang D, Bargiacchi F, et al. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. *J Biol Chem* 2003;278(37):35451-7. doi: 10.1074/jbc.M302474200 [published Online First: 2003/06/26]

417. Zheng M, Huang J, Tong A, et al. Oncolytic Viruses for Cancer Therapy: Barriers and Recent Advances. *Mol Ther Oncolytics* 2019;15:234-47. doi: 10.1016/j.omto.2019.10.007 [published Online First: 2019/12/25]

418. Thorne SH. Adding STING to the Tale of Oncolytic Virotherapy. *Trends Cancer* 2016;2(2):67-68. doi: 10.1016/j.trecan.2016.01.002 [published Online First: 2016/03/24]

419. Heo J, Reid T, Ruo L, et al. Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat Med* 2013;19(3):329-36. doi: 10.1038/nm.3089 [published Online First: 2013/02/12]

420. Kaufman HL, Bines SD. OPTIM trial: a Phase III trial of an oncolytic herpes virus encoding GM-CSF for unresectable stage III or IV melanoma. *Future Oncol* 2010;6(6):941-9. doi: 10.2217/fon.10.66 [published Online First: 2010/06/10]

421. Arwert EN, Milford EL, Rullan A, et al. STING and IRF3 in stromal fibroblasts enable sensing of genomic stress in cancer cells to undermine oncolytic viral therapy. *Nat Cell Biol* 2020;22(7):758-66. doi: 10.1038/s41556-020-0527-7 [published Online First: 2020/06/03]