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CHEMOATTRACTANT RECEPTORS BLT1 AND CXCR3 REGULATE ANTI-TUMOR IMMUNITY BY FACILITATING $CD8^+$ T CELL MIGRATION TO TUMORS

By

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A Dissertation
Submitted to the Faculty of the School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

in Microbiology and Immunology

Department of Microbiology and Immunology
University of Louisville
Louisville, Kentucky

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ABSTRACT

CHEMOATTRACTANT RECEPTORS BLT1 AND CXCR3
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Presence of increased numbers of $CD8^+$ T cells in the tumors correspond to better overall survival in the patients. Variety of immuno-therapies have shown considerable efficacy in the clinic, however, a multitude of patients remain unresponsive. Most of these immunotherapies rely on effector T cell responses in the tumor. A major obstacle in the success of these immunotherapies is poor recruitment of $CD8^+$ T cells into tumors despite intact effector responses in the periphery. Therefore understanding the mechanisms that regulate CTL infiltration into tumors becomes essential. Previous studies in our laboratory suggested an important role for BLT1 in immune surveillance against tumors by regulating CTL migration in a syngeneic cervical cancer tumor model. In this thesis, we

investigated the roles of leukotriene B_4 (LTB_4) receptor - BLT1; and CXCR3, the receptor for CXCL9, CXCL10 and CXCL11 in anti-tumor immunity using a syngeneic B16 melanoma tumor model. $BLT1^{-/-}$ mice and $CXCR3^{-/-}$ mice on a C57BL/6 background were used to examine the function of these receptors in tumor progression. Significant acceleration in tumor growth and reduced survival was observed in both $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice as compared to the WT mice. Analysis of tumor infiltrating leukocytes revealed significant reduction of $CD8^+$ T cells in the tumors of $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice as compared to WT tumors; their frequencies being similar in the periphery (spleen and TdLN). Significant reduction of Granzyme-B and IFNγ transcripts were observed in tumors of knockout mice compared to WT mice.

Adoptive transfer of tumor experienced WT but not $BLT1^{-/-}$ or $CXCR3^{-/-}$ $CD8^+$ T cells reduced tumor growth significantly in $Rag2^{-/-}$ mice, which correlated with reduced infiltration of knockout $CD8^+$ T cells into tumors. Co-transfer of WT $CD8^+$ T cells with either of the knockout $CD8^+$ T cells in tumor bearing $Rag2^{-/-}$ mice showed that WT $CD8^+$ T cells did not facilitate additional knockout $CD8^+$ T cell infiltration to tumors. BLT1/CXCR3 double deficient mice displayed similar tumor kinetics as single knockout mice and showed lack of synergism.

The requirement for BLT1 and CXCR3 in inducing checkpoint blockade mediated anti-tumor response was tested. While anti-PD-1 based vaccine significantly attenuated tumor growth in WT mice, the vaccine completely lost its efficacy in $BLT1^{-/-}$, $CXCR3^{-/-}$ or $BLT1^{-/-}CXCR3^{-/-}$ mice that correlated with failure of knockout $CD8^+$ T cell infiltration into tumors. These results demonstrate a critical role for

BLT1 and CXCR3 in CTL migration to tumors and thus can be targeted to enhance effective anti-tumor responses.

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CHAPTER I

GENERAL OVERVIEW

Emerging data clearly demonstrates that the immune system can curtail cancer progression, a concept termed “Immune-surveillance against tumors”. $CD8^+$ T cells act as sentinels against tumors by directly eliminating the tumor cells. The prime focus of this research is to understand the mechanisms regulating $CD8^+$ T cell migration to tumors with a particular emphasis on chemo-attractant receptors. The chapter begins with a brief description of the dual role of immune system in regulating as well as sculpting the tumor, a phenomenon known as immunoediting. The cellular and molecular mechanisms involved in killing tumor cells and how this knowledge is translated into various successful immunotherapies that have revolutionized the field of cancer therapies is described in greater detail with a particular focus on PD-1 blockade based therapies, Adoptive T cell therapies (ACT) and Chimeric Antigen Receptor (CAR) based therapies. Reasons behind failure of immunotherapies in cancer patients is discussed, defective $CD8^+$ T cell infiltration to tumors being a major one as well as hurdles in achieving optimum $CD8^+$ T cell infiltration to tumors.

The importance of chemokines and their cognate receptors in regulating cancer development and $CD8^+$ T cell infiltration to tumors is discussed. Furthermore, we

outlined the background on Leukotriene B_4 receptor BLT1; and CXCR3, receptor for CXCL9 and CXCL10, that are studied in detail in this thesis.

BACKGROUND

Transformed cells arise due to genetic mutations resulting in uncontrolled cellular divisions. Tumor growth is a cumulative effect of three steps viz. *initiation* of a cancerous event in a cell, *promotion* of the cancerous event by proliferation and finally *progression* involving tumor growth and metastases [1]. Cancer development requires attainment of six hallmark capabilities: self-sufficiency in proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replicative potential, the maintenance of vascularization and tissue invasion and metastasis [2].

Multiple intrinsic and extrinsic mechanisms exist to prevent such aberrant cell divisions. These "**intrinsic**" cellular mechanisms protect the cells against various perturbed mutagenic insults or cell stresses including hypoxia, nutrient deprivation, DNA damage, tissue injury etc. Should the cell proliferation go beyond control, the cell activates apoptosis or cell death pathways through activation of pro-apoptotic machinery, engagement of death receptors, activation of tumor suppressor genes like p53, etc. If the transformed cells escape the intrinsic control, they are then subjected to the **"extrinsic"** mechanisms wherein neighboring cells sense the presence of a transformed cell and try to eliminate it. Dependency of cells on trophic environmental cell-cell contact signals or extra-cellular matrix (ECM) dependence; genes controlling cellular junctions and polarity and finally detection and elimination of transformed cells by the immune system; constitute the three extrinsic mechanisms to prevent the maintenance of an oncogenic event [3].

Dual role of immune system in tumor development

The role of immune system in controlling cancer has long been a subject of debate. The initial clues for the involvement of immune system in cancer growth stemmed through the observation made by Virchow in 1863 that tumor biopsy samples contain leukocytes termed as “lymphoreticular infiltrate”. On the other hand, as far back as 1700s the observations that feverish infections in cancer patients occasionally led to cancer remissions suggested that immune activation and cancer remission might be associated. The very first attempt at using immunotherapies as cancer therapies was made by W. Coley in 1893 wherein he injected killed bacterial mixture of *Streptococcus pyogenes* and *Serratia marcescens* to cancer patients and observed tumor regression in some cases [4]. Since then several studies have later proven that immune system can curtail tumor growth as well as promote it.

Inflammation promotion of cancers

Recent studies have expanded the concept that inflammation plays a crucial role in tumor promotion. Only 10-15% of the cancers are hereditary and the rest are caused by somatic mutations at sites of infection, exposure to chemical irritant, cigarette smoke, environmental and dietary factors. The fact that patients suffering from inflammatory bowel disease or Crohn’s disease often develop colon cancers strongly correlates chronic inflammation with cancer development [5]. Patients positive for Hepatitis C infection and Helicobacter pylori infections are predisposed to liver cancer and stomach cancer development respectively,

associating infections to cancers [6, 7]. The use of Non-steroidal anti-inflammatory drugs (NSAIDs) in the prevention of spontaneous tumors in Familial Adenomatous Polyposis (FAP) patients also establishes a strong connection between inflammation and cancer [8].

Oncogenic event also occurs due to mutations caused by reactive oxygen species and nitrogen intermediates (ROS and RNI) as well as superoxide and hydroxyl radicals produced as a result of inflammation [9]. Studies on the role of NF-kB have provided further insights in the participation of inflammation in tumor growth. Murine model of colitis-associated cancer involves azoxymethane (AOM-pre-carcinogen) mediated induction of an oncogenic event that by itself gives rise to fewer numbers of adenomas along the intestines. The adenomas can be augmented by simultaneous induction of colonic inflammation through repeated exposure to intestinal irritant dextran sulfate sodium (DSS). Inactivation of NF-kB pathway in colonic epithelial cells in an AOM-DSS model of intestinal tumorigenesis resulted in a significant reduction in the tumor incidence [10]. NF-kB transcription factor was not only shown to be a key component of inflammation but also known to facilitate the survival of the *initiated* colonic epithelial cells. It was also involved in activating the myeloid immune cells to produce inflammatory cytokines like IL-1β, IL-6, TNFα, IL-12/IL-23; chemokines like KC, MIP-2, and inflammatory mediators like COX-2, STAT-3, MMP-9, etc. that promote the growth of cancerous cells. Ablation of NF-kB activity in pro-tumorigenic tumor associated macrophages (TAMs) re-educated them to gain cytotoxic, anti-tumorigenic potential [11]. Tumor cells also feed into the

inflammation by secreting factors that allow the migration of various other inflammatory myeloid cells that promote the tumor growth. For example, COX-2 is frequently expressed in tumor cells and is involved in the synthesis of prostaglandins and chemokines like IL-8, CCL2, CCL20, up-regulating the chemokine receptor CXCR4 that binds to the chemokine CXCL12 or SDF-1 (stromal cell derived factor-1), activating matrix degrading enzymes, etc. [9]. Tumor suppressor genes are also involved in activating inflammatory pathways in tumor cells. For example, von Hippel-Lindau tumor suppressor protein (VHL) targets the transcription factor Hif-1α or hypoxia-inducible factor 1α for degradation. The role of HIF-1α in cellular response to hypoxia is very well established. Some of the functions of Hif-1α include initiating angiogenesis, interaction with NF-κB to promote TNFα production and is also known to help express CXCR4 chemokine receptor on tumor cells involved in metastasis [12].

Immunesurveillance and Immunoediting of cancer

Despite the semantics of correlation between inflammation and cancer, the immune system is still the third extrinsic mechanism to prevent tumorigenesis. In fact the pro-tumorigenic effects of immune system emerge if the cytotoxic immune cells do not eliminate the cancer cells. The notion that cancerous cells would emerge at an “incredible frequency” if the host defenses would not prevent the growth of continuously arising cancer cells was conceived in 1909 by Paul Erlich. Fifty years later Burnett and Thomas predicted that lymphocytes were responsible for eliminating the nascent tumor cells and hence introduced the concept of “immune-surveillance” against tumors [3]. Since then there has been

unequivocal evidence reinforcing the beneficial role of the immune system in eliminating the tumor cells. By 1990s, the availability of better immunodeficient murine models on pure genetic background led to the importance of interferon gamma (IFNγ) and STAT1 (transcription factor required for IFNγ signaling) in rejection of transplanted tumors. This rekindled the role of lymphocytes in anti-tumor immunity as demonstrated in spontaneously arising tumors as well as chemically induced tumors [13, 14]. The results obtained in immune-compromised mice led to the paradox of tumor formation in immune-competent mice/individuals which led to the concept of “Cancer immunoediting” pioneered by Schreiber and colleagues that describes the dual roles of the immune system in three sequential steps of tumor progression namely Elimination, Equilibrium and Escape phases [13, 15].

In the *Elimination* phase the innate (dendritic cells, macrophages, NK cells) and the adaptive immune cells ($CD8^+$ and $CD4^+$ T cells, γδ T cells) work in concert towards eliminating any nascent tumor cells. Release of effector cytokines like Type I (IFNα and IFNβ) and II (IFNγ) interferons, TNFα, IL-12 by the innate cells upon recognition of tumor antigens recruit the effector cells including effector $CD8^+$ and $CD4^+$ T cells as well as NK cells to the tumors. The CTLs themselves further express granzyme-B, perforin, TRAIL, Fas/FasL, IL-2, IFNγ, TNFα, NKG2D, IL-17 etc. that directly kill the tumor cells and enhance the antigenicity of tumor cells by enhancing the MHC-I expression on tumor cells [3, 16].

In the *Equilibrium* phase, the tumor cells and immune cells exist in a state of equilibrium. At this stage, tumor growth can proceed in either direction -

elimination or escape. Effector cells (T cells) and cytokines (IFNγ, IL-12) are indispensable in this phase and there is a balance established between anti-tumor and pro-tumor immune mechanisms [16, 17]. The immune cells can keep the tumors in a functionally dormant state, wherein tumor cells acquire genetic mutation because of the immune selective pressure leading to the generation of “tumor variants”. The tumor cell variants that emerge in the previous phase grow to become resistant to immune detection or elimination and become tolerized by establishing various immune-suppression mechanisms that allow the tumors to grow and become clinically detectable.

The tumor now progresses to the next phase, *Escape*. The tumor-induced mechanisms include tumor cells acquiring additional somatic mutation, loss of response to IFNγ, loss of antigen presentation due to downregulation of MHC-I molecule on tumor cells, enabling infiltration of immune-suppressive cells as well as expression of immune-suppressive markers like PD-L1 on tumor cells. The immune induced mechanisms include subversion of T cell responses, T cell anergy, immune suppression via myeloid cells like TAMs, MDSCs (myeloid derived suppressor cells) as well as regulatory T cells (Treg) that express various immune suppressive factors like TGFβ, IL-10, IDO (indoleamine 2,3-dioxygenase), FasL, galectin, VEGF, PD-1/PDL1, CTLA4 etc. [3, 16]. The amount of literature in understanding these three phases of immunoediting has been burgeoning in the past decade. The final outcome of tumor growth depends on whether tumor promoting or tumor suppressing inflammation dominates.

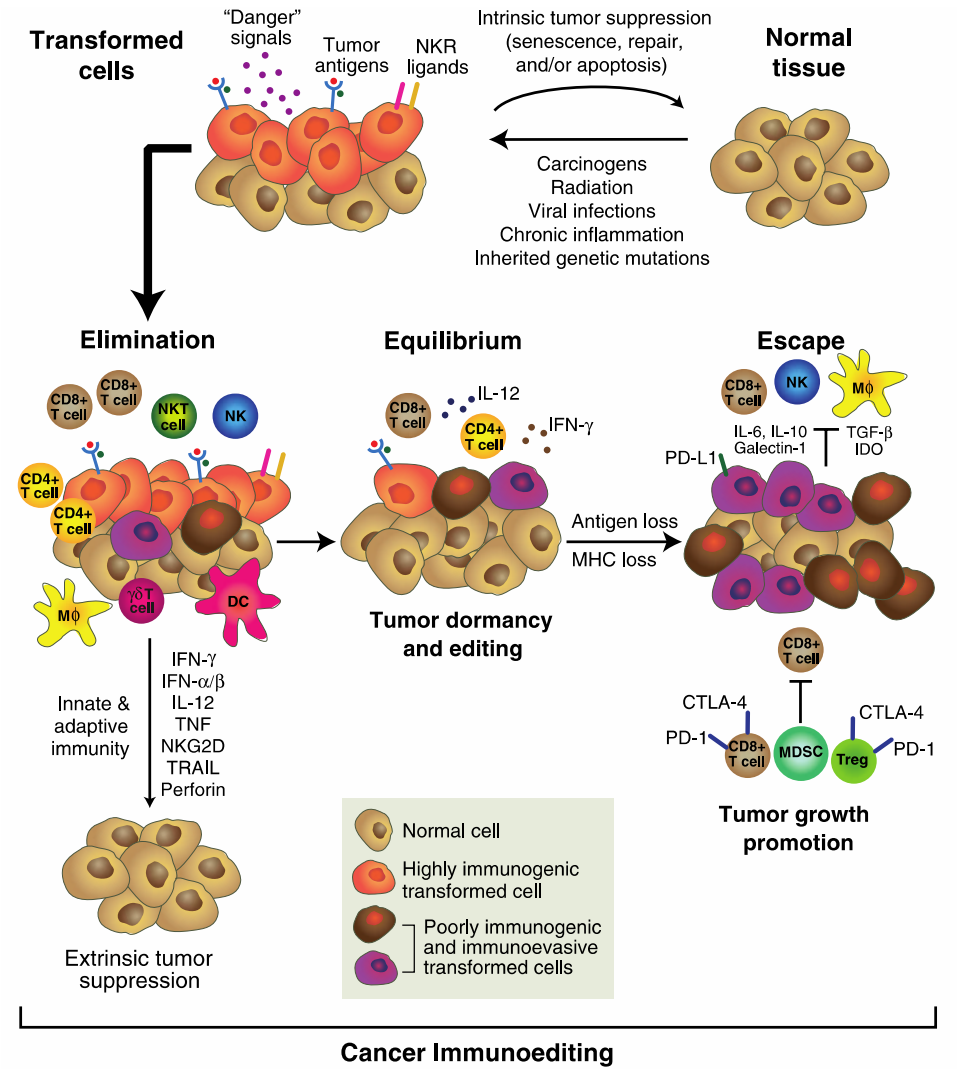

Figure 1: The cancer immunoediting concept. Cancer immunoediting is an extrinsic tumor suppressor mechanism that engages only after cellular transformation has occurred and intrinsic tumor suppressor mechanisms have failed. In its most complex form, cancer immunoediting consists of three sequential phases: elimination, equilibrium, and escape.

Figure adapted from [18].

Patients with established cancers indicate the failure of immune surveillance mechanisms. The immune cells that are present in the tumors of these patients now actually promote the tumor growth by releasing pro-tumorigenic cytokines and chemokines. The conventional tumor therapies like radiotherapy and chemotherapies were originally aimed at killing the cancer cells directly; however, recent studies have demonstrated that these therapies are also immuno-modulatory in nature. Given the beneficial role of immune surveillance in cancer, attempts are being made to jumpstart the anti-tumor responses. Therefore a major focus of the field of tumor immunology is to modulate the immune system such that the anti-tumor responses get activated and pro-tumorigenic responses get subverted.

Cancer Treatment Approaches

Surgery, radiotherapy and chemotherapy are the three most common standard of care treatment approached to most of the cancers. Most of the chemotherapy drugs as well as radiotherapy induce cancer killing by inducing apoptosis in cancer cells. While offering short term benefit, the standard of care treatments can lead to more aggressive cancer cells that become resistant to both chemotherapy and radiotherapy possibly due to disruption of intrinsic apoptosis machinery. One of the reasons behind the aggressiveness of cancer cells post therapy can be due to the negative effect of the radiation and chemotherapy on the immune system. It is now known that certain chemotherapy drugs that targeted cancer cells based on the rapid cell division criterion, also targeted the rapidly dividing hematopoietic cells including immune cells, thus leading to

immunosuppression. Total body irradiation leads to ablation of patient's immune system. Radiation was also shown to promote immunosuppressive mechanisms like activation of transforming growth factor-β and enhancing pro-tumorigenic function of macrophages. Also Treg cells are more resistant to radiation induced death compared to other T cells which leads to presence of more Treg compared to CTLs in the tumor.

Recent studies have demonstrated that the standard of care therapies can have positive impact on immune system. Certain chemotherapy drugs and localized radiotherapy can induce immunogenic cell death, thus enhancing anti-tumor immunity [19, 20]. Local tumor irradiation instead of total body irradiation has recently shown to benefit the anti-tumor effect of the immune system. Even chemotherapies like cyclophosphamide and 5-fluorouracil are considered to be immune-modulatory in nature by eliminating suppressive Treg and MDSC immune cells thereby enhancing anti-tumor immunity. Hence radiotherapy and certain chemotherapy drugs can induce immunogenic cell death mechanisms that involve the activation of type-I IFNs that lead to activation of innate and adaptive system against tumors. Also, immunogenic cell death induces release of various tumor antigens/neo-antigens that act as endogenous vaccines [21-26]. This provides mechanistic rationale behind combining specific chemotherapies and/or radiation therapy with existing immunotherapies, thus opening up new horizons for the treatment of cancers.

Immunotherapy as cancer treatment strategy

Immunotherapies have only recently emerged as a successful treatment modality for cancer [27-29]. Various studies indicated that adjuvants, lectins, IL-2 and interferons could target the tumor cells by activating the lymphocytes [30-32]. A series of landmark approvals by the FDA including a) IFNα2 for the adjuvant treatment of stage IIB/III melanoma in 1995, b) IL-2 for the treatment of metastatic melanoma and renal cell carcinoma in 1998, c) first ever cancer vaccine - "Oncophage/vitespen" for treatment of renal cell carcinoma in Russia in 2008, d) approval of autologous Dendritic cell cancer vaccine (Provenge) for Stage IV hormone refractory prostate cancer and e) immune checkpoint blockade strategies such as anti-CTLA4 (Ipilimumab) in 2011 for melanoma, and anti-PD-1 (Pembrolizumab) in 2014 for advanced metastatic melanoma and non small cell lung carcinoma; have all been significant in reinforcing that immunotherapies hold tremendous potential to combat tumors thus starting a new wave of revolution in cancer therapeutics [33-39]. Three major lines of immunotherapy approaches emerged in recent years against tumors viz. a) employing Adoptive cell transfer therapy including chimeric antigen receptor therapy, b) blocking immune checkpoint inhibitory pathways like PD-1, CTLA, TIM3 and LAG3, and c) activating T cell co-stimulatory pathways by using agonistic antibodies to OX-40, 4-1BB, ICOS. These immune-therapies have higher success rates due to increased specificity to cancer antigens as well as inducing long lasting T cell responses and immunological memory. T cell based therapies including anti-PD-L1 and anti-PD-L2 antagonistic antibodies, adoptive

cell therapies including chimeric antigen receptor therapy, vaccine candidates like Prostvac targeting the prostate specific antigen (PSA) and various agonistic antibodies for co-stimulatory receptor candidates are currently in advanced clinical trials.

An effective T cell dependent anti-tumor response involves five major steps: a) efficient antigen sampling by dendritic cells (DCs) and their maturation; b) migration of these DCs to tumor draining lymph nodes (TdLNs); c) recirculation/migration of naïve T cells to TdLNs; d) antigen presentation by the DCs to the T cells, clonal expansion and acquisition of effector functions in T cells and finally e) migration of these activated CTLs to tumors to execute their anti-tumor functions [40, 41]. Although all these steps are crucial and interconnected, the final outcome must be the presence of $CD8^+$ T cells in tumors and with intact effector functions. Manipulating these steps of anti-tumor immunity has led to various successful immune-therapies against cancer. DC based vaccines, for example, accomplishes step 1 by enhancing the antigen loading and maturation ability of the DCs [34]. Immune checkpoint inhibitors i.e. anti-PD-1 and anti-CTLA4 antagonistic antibodies [39] and co-stimulatory agents (4-1BB, ICOS, GITR agonists, etc. [42]) as well as adjuvants (lectins, IL-2) [43] when used accomplishes step 4 by enhancing the effector functions of the $CD8^+$ T cells against tumor cells. Alternatively, immunotherapies like Adoptive T cell therapies (ACT) including Chimeric Antigen Receptor (CAR) therapy bypass all the steps except the last step of infiltration of CTLs to tumors and anti-tumor

function [44]. The most successful immunotherapies relevant to the current project specifically in melanoma are detailed below.

Immune checkpoint blockade: anti-PD-1 and anti-CTLA4 antibodies

Inhibitory receptors present on the immune cells attenuate the immune responses to prevent excessive inflammation that can be detrimental to the tissues. T cell activation is a complex process and involves antigen specific stimulation via the TCR and a co-stimulatory signal. It is now increasingly clear that both co-stimulatory and co-inhibitory signals are required to maintain an effective T cell response. Enhancing signaling through co-stimulatory molecules (4-1BB, GITR, OX-40) and blocking the co-inhibitory molecules (CTLA4, PD-1, LAG3) can amplify T cell responses to tumors [45-47].

CTLA4: CTLA4 is a type 1 transmembrane glycoprotein inhibitory receptor that is present on T cells and belongs to of the CD28/Immunoglobulin superfamily. By binding to B7-1 (CD80) and B7-2 (CD86) on APCs, CTLA4 competes with CD28 costimulatory molecule to dampen T cell responses. Optimal crosslinking of CTLA4 with TCR-CD28 stimulation led to IL-2 suppression, proliferative arrest without induction of apoptosis. A monoclonal Ab was made by Allison and colleagues to specifically block CTLA4 in a preclinical model wherein significant anti-tumor immunity without any overt immune toxicity was achieved upon CTLA4 blockade [46]. Subsequently two fully humanized CTLA4 antagonistic antibody Ipilimumab and Tremilimumab were introduced in clinical trials in 2000 for metastatic melanoma. Ipilimumab was a better antibody and gave significant

overall response rates of 17% in patients with advanced melanoma with three year survival rates being 20% [48]. Subsequently, in 2010 Ipilimumab was the first immune-checkpoint blockade therapy that was FDA approved for metastatic melanoma [49].

PD-1: PD-1 was originally identified in 1992 as a gene induced upon apoptotic cell death in a T cell hybridoma [50]. Later studies demonstrated that PD-1 deficient mice showed signs of autoimmunity that suggested a role of PD-1 as an inhibitor of lymphocyte responses at peripheral tissues. Further insight on the role of PD-1 as an inhibitory receptor was gained from a chronic viral infection model [51, 52]. While expressed minimally on resting immune cells, PD-1 is broadly expressed on T cells, B cells, NK cells, DCs and macrophages upon activation. PD-1 binds to its partners PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is broadly expressed on cells of both hematopoietic and non-hematopoietic lineage including tumor cells. PD-L1 expression is induced upon inflammatory cytokines like TNFα and interferons. PD-L2 expression is restricted to immune cells like DCs, macrophages and mast cells. CTLA4 and PD-1 control T cell responses by different mechanisms. They both block activation of Akt thereby blocking glucose uptake by CD28. However, PD-1 blocks PI-3K activation while CTLA4 blocks Akt downstream of PI3K. Adoptively transferred T cells express PD-1 and numerous tumor cells express PD-L1 suggesting the role of this signaling pathway in tumor immune evasion [53]. In 2014, Pembrolizumab was the first anti-PD-1 drug approved by the FDA against relapsed or refractory melanoma. At six months, it was shown to enhance progression free survival by

30% as compared to 16% by chemotherapy. Nivolumab was the second anti-PD-1 drug to be FDA approved for metastatic melanoma and metastatic squamous non-small cell lung carcinoma (NSCLC). It showed 72% overall survival rate at one year for metastatic melanoma patients without BRAF mutation [48]. PD-1 and CTLA4 combination blockade was shown to be more effective compared to either treatments alone [54]. Whole exome sequencing of non-small cell lung cancers treated with Pembrolizumab suggested that higher non-synonymous mutational landscape in lung tumors was associated with better objective response and clinical benefit [55]. The higher mutational burden also correlated with molecular smoking signature and increased neo-antigen burden [55]. This also explained why some patients were unresponsive to PD-1 blockade.

Similarly, studies suggest that only a subset of CRC, may be a good candidate to PD-1 blockade therapy [56]. Microsatellite instable (MSI) subset of colorectal cancer comprises 15% of sporadic CRC and most familial CRC [57]. MSI, typically diagnosed by variable length DNA microsatellites, are mutations arising due to epigenetic silencing of DNA mismatch repair genes [58]. The high mutational burden in MSI tumors creates many tumor-specific neo-antigens compared to microsatellite stable tumors [59]. MSI positive tumors correspond to higher level of tumor-infiltrating leukocytes, T cell responses, PD-1 and PD-L1 expression and hence better response to PD-1 blockade therapies [56, 57, 59].

Adoptive T cell therapy (ACT): This technology was pioneered by Dr. Steven Rosenberg and is now widely used in patients. ACT involves the transfer of ex-

vivo activated, stimulated and expanded autologous T cells with high affinity to tumor antigens back into the patients. Ex-vivo activation and culture of tumor specific T cells is possible outside of the endogenous host that has immune-suppressive factors. Anti-tumor T cells with a $CCR7^{+}CD27^{+}$ $CD28^{+}$ $CD62L^{+}$ phenotype characteristic of central memory cells were more effective in ACT protocol than more differentiated effector cells [60]. Antigen specific cells are isolated from the freshly excised tumors and as soon as the anti-tumor activity is detected against specific antigens, cells were expanded in the presence of T cell stimulating antibody OKT3 and IL-2 (6000 IU/ml). Approximately 5×10^{10} cells were infused systemically following a non-myeloablative preparative regimen consisting of 60mg/kg cyclophosphamide for 2 days followed by 5 days of fludarabine at 25 mg/m^2. IL2 was administered for 2–3 days at 7.2×10^{5} IU/kg every 8 hr [61]. ACT today represents one of the most promising T cell based immunotherapy for melanoma, lymphoma and childhood leukemias. The source of the T cells could be PBMCs or tumor itself. The ACT procedure involves pre-conditioning the patient by temporary ablation of immune system or lymphodepletion through total body irradiation or chemotherapy for removal of regulatory T cells, competition for homeostatic cytokines like IL-7 and IL-15 involved in T cell survival and proliferation thereby enhancing the persistence of the transferred T cells [62]. The lymphodepletion regimen before ACT when combined with administering T cell growth factor IL-2 led to tumor eradication for prolonged durations [62-64]. Objective response rates for this therapy against Stage IV melanoma were between 49-72%. Importantly, 22% of the patients had

complete tumor regression and disease free survival for more than 8 yrs [62]. Data from tumor exomic sequencing have enabled the identification and targeting of numerous non-synonymous mutations that result in new epitope generation [65]. ACT also includes TCR gene modified T cells against shared tumor differentiation antigens like MART-1, Carcinoembryonic antigen (CEA), cancer germ line antigens like NY-ESO-1, MAGE-A3 that have shown considerable success in various clinical trials [44]. Despite the success of ACT, a multitude of patients remain unresponsive. Upregulation of immune-suppressive molecules on the transferred CTLs (CTLA4 and PD-1), immune-suppressive tumor microenvironment (IDO, NOS-1), and the nature of tumor vasculature are some of the reasons behind differential response rates of ACT. Attempts are being made to utilize T cells with TCR against a specific antigen or patient specific tumor antigen called neo-antigens based on mutations in tumor DNA, identify T cell subsets to be used for ACT (Naïve, Tcm, Tscm, Tem), identify T cell growth factors used (IL-2, IL-7, IL-15) to enhance persistence, proliferation and survival of T cells, and understand better host preconditioning protocols for lymphodepletion and T cell engraftment [66-69]. Studies are now undertaken to understand specific markers to identify and sort out from the tumor immune infiltrate that would give better anti-tumor responses in an adoptive cell transfer setting. Recent studies showed that $PD1^{+}CD8^{+}$ T cells and not $PD1^{-}CD8^{+}$ T cells represent the cohort of clonally expanded tumor reactive TILs and included T cells that target mutated tumor antigens [70].

Chimeric Antigen Receptor therapy: In line with ACT, Chimeric Antigen Receptor therapy (CAR) is another immunotherapy that has shown considerable success with hematological cancers. It involves genetic modification of T cells such that they stably express chimeric antibodies conferred with antigen specificity. Briefly, chimeric antigen receptor is a fusion of antigen recognition domain of an antibody with the intracellular domain of CD3zeta chain or FcγRI. These first generation CARs effectively demonstrated cytotoxicity and T cell activation but failed to show proliferation and survival of T cells upon continuous antigen exposure. Studies suggested that the first generation CARs become anergic in the absence of co-stimulation in the tumor mileu. Second and third generation CARs encompassed the combination of CD3zeta chains with co-stimulatory molecules like CD28, CD27, 4-1BB and OX-40 [71, 72]. The antigen specificity and HLA independent recognition are some of the benefits of using CARs. Autologous T cells transduced to express CD19 CARs alongwith 4-1BB co-stimulatory molecule have shown considerable success with 50% of them achieving partial or complete remissions and T cells persisting beyond two years in patients with refractory and relapsed B cell chronic lymphocytic leukemia [73]. However, there can be various side effects like cytokine release syndrome and tumor lysis syndrome and B cell aplasia. Nonetheless, there is an increasing excitement with gene modified T cells entering cancer therapeutics.

Reasons behind partial efficacy of immunotherapies

The past decade has witnessed a major revolution in immunotherapy as a promising treatment modality for cancer. Despite the tremendous success of immunotherapies, large numbers of cancer patients still remain unresponsive. For example, PD-1 blockade therapy leads to tumor progression free survival in only 30% of the patients while ACT treatments lead to complete tumor regression in only 22% of the patients [48, 62]. The reason behind this is probably multifold. As indicated before the process of anti-tumor immunity requires proper execution of various steps and involvement of various cells. Any immunotherapy approach would have to overcome various hurdles. For example, *"antigenicity"* of the tumor is an important measure for potential susceptibility to immune therapies. Tumor can express variety of non-mutated or mutated antigens or even lose the antigenicity by reducing the antigen presentation via MHC-I downregulation or dysregulation in antigen presentation machinery. Although recent studies have demonstrated that immune response can be mounted against the neo-antigens or the antigens derived from somatic mutations that are specific to cancer cells (and not normal cells); the mutational landscape could be quite different across every individual that complicates the use of immunotherapies to the generalized population. Also the efficacy of immunotherapies to neo-antigens suggest the possibility that higher number of mutations correlate with enhanced response to immunotherapy [74]. Importantly, the failure of immunotherapies in some patients stems through the off-target toxic side-effects from the use of immunotherapies like CTLA4 blockade, CAR therapies etc. Liver toxicities, respiratory distress

syndrome, cytokine storm syndrome are the various side-effects that limit the efficacy of the T cell based immunotherapies.

Studies have revealed that some immunotherapies including cancer vaccines are able to mount anti-tumor responses in the periphery but not in the tumor. Tumor mileu being inherently tumor suppressive is a major obstacle in success of immunotherapies. Immunotherapies that boost T cell responses fail to maintain the anti-tumor responses in the tumor due to immune-suppressive cells, cytokines, growth factors, hypoxic environment, etc. that inactivate or subvert the T cell based responses [41].

Another major impediment acknowledged only recently is defective $CD8^+$ T cell infiltration to tumors despite effective responses in the periphery [40, 75]. Studies have demonstrated that less than 2% of adoptively transferred T cells actually reach the tumor [76, 77]. Majority of the transferred cells remain in the periphery or are found in lung and liver, which may also be a reason behind off-target responses and toxicities seen in T cell based therapies. Enhancing effector T cell recruitment to tumors by employing chemokine/chemokine receptor pathways is a crucial strategy to circumvent these issues. For an effective immune response to occur it is of importance that specific immune cells (anti-tumorigenic and not pro-tumorigenic) be recruited at specific locations (tumors versus the periphery). A major focus of this thesis is to understand how chemoattractant receptors regulate the efficacy of immunotherapies. Indeed studies conducted herein suggest that absence of BLT1 and CXCR3 chemoattractant receptors completely abrogates anti-PD-1 based vaccine efficacy and therefore suggests an

indispensable role for both BLT1 and CXCR3 in achieving optimum efficacy of PD-1 blockade based immunotherapy.

Chemoattractants and leukocyte migration

Leukocyte migration involving both homeostatic recirculation among lymphoid organs and migration to inflamed sites including tumors is orchestrated and tightly regulated by various chemo-attractants. Chemoattractants include lipids, peptides as well as proteins. These include classic neutrophil chemoattractant peptides such as fMLP that is a tripeptide of N-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) [78]; complement cleavage products C5a and C3a that are involved in migration of various leukocytes [79] and lipid chemoattractants like leukotriene B_4 (LTB_4) and platelet activating factor (PAF) identified as strong chemoattractants for neutrophils. In addition a large family of protein chemoattractants or chemokines broadly divided into four groups i.e. CC, CXC, CX3C and the XC families based on the number and position of vicinal cysteine residues are potent chemoattractants for various leukocytes [40, 80]. Chemokines bind to G-protein coupled receptors with seven transmembrane domains. Differential receptor expression on immune cells is a crucial factor in determining responsiveness to chemokines. Around 50 chemokines and 20 chemokine receptors have been identified to date [81]. This suggests that various chemokines and chemokine receptors bind to multiple counterparts suggesting functional redundancy and complex regulation of cell migration [82]. No chemokine is uniquely active on one particular leukocyte subset [83]. Chemokines can be produced by a variety of immune cells/endothelial

cells/epithelial cells but specific cell types are involved in chemokine production under specific inflammatory conditions. Usually, multiple chemokines are produced concomitantly in a redundant manner in response to the same stimulus by a cell, this phenomenon referred to as “polyspeirism” [83]. Two general modes of chemokine production; constitutive production (eg. SDF-1, CCL19, CCL13, CCL17, CCL25, etc.) or inducible production upon activation (eg. CXCL10, CCL5, CCL3, CCL4, CCL2, IL-8, etc.) are defined [83]. Leukocyte migration is tightly regulated by spatial and temporal expression of chemokines [84].

Chemokines regulate cancer development

It is increasingly evident that a tumor is a complex microenvironment that constitutes various cell types including immune cells, stromal cells like fibroblasts, tumor cells and endothelial cells that communicate with each other. The composition of the tumor microenvironment in terms of the types of immune cells recruited decides whether anti-tumor or pro-tumor immune responses predominate and ultimately the fate of tumor development. Chemokine/cytokine mediated inflammation plays a crucial role in initiation and progression of various cancers [85]. Tumor cells also produce inflammatory chemokines and express chemokine receptors. Melanoma cells have shown to express various chemokines like CCL2, CCL5, CXCL1, CXCL2, CXCL3 and CXCL8 that are pro-tumorigenic [81, 86]. It is well known that various cancer cells express CXCR4, chemokine receptor to SDF-1 and regulates the growth, migration and invasion of tumor cells by activating AKT, MAPK, and JAK-STAT pathways [87, 88]. Apart from CXCR4-SDF-1 axis, various other chemokines and their receptors have

been involved in angiogenesis and metastasis of cancer as well. Chemokines like CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 activating CXCR1 and CXCR2 receptors have been shown to be promoters of angiogenesis [81].

With respect to immune cells in the tumor mileu, CXCR3 receptor and its ligands CXCL9, and CXCL10 are strongly related to Th1 biased responses that is a crucial part of effector anti-tumor responses [89-91]. CXCR3-CXCL9/10 pathway is an important axis involved in $CD4^+$, $CD8^+$ T cell and NK cell infiltration to tumors for anti-tumor immunity. CXCR3 expression was also shown to be crucial to generate cytotoxic anti-tumorigenic M1 polarized macrophages [92]. Another chemokine receptor involved in $CD4^+$ T cell mediated antitumor immunity is CCR5. CCR5 on CD4 and $CD8^+$ T cells was shown to be crucial in anti-tumor responses as deficiency in CCR5 led to enhanced lung adenocarcinoma progression [81, 93].

Tumor promoting cells are also governed through chemoattractant pathways. It is known that tumors shape the microenvironment to its advantage by recruiting immune suppressive cells like Treg cells, TAMs, MDSCs etc. Treg cells constitute a considerable portion of the tumor mileu and through the production of immune suppressive molecules like IL-10 and TGF-β are extremely potent in dampening the anti-tumor response [94]. CCR4-CCL22 axis is shown to be crucial in Treg recruitment to tumors, enhancing VEGF levels followed by angiogenesis and also cancer metastasis [95, 96]. CCR5 [97], CCR10 [98] and CXCR3 [99] are some of the other chemokine receptors shown to be involved in

Treg recruitment to tumors. MDSC myeloid cell subset was shown to secrete chemokines like CCL3, CCL4, and CCL5 that lead to CCR5 mediated recruitment of Treg cells to tumors [97]. Apart from CCR5 chemokines, CCR2-CCL2, CXCR2-CXCL5 and CXCR4-CXCL12 axis have been shown to promote tumor progression by enhancing the suppressive activity of TAMs and MDSCs [81]

Chemoattractants recruit activated $CD8^+$ T cells to target tissues

The mechanisms of chemoattractant-regulated CTL migration to target tissues have been extensively investigated in the context of allergy, inflammation, autoimmune and infectious diseases, as well as in transplantation. However, limited information is available on CTL migration into tumors. Perhaps the knowledge on CTL migration in other diseases might guide further studies in the context of CTL migration to tumors.

In preclinical models of allergic inflammation and airway hyper-responsiveness, the LTB_4–BLT1 axis plays a critical role in controlling the migration of activated CTLs into the airway [100]. The induction of BLT1 in CTLs and BLT1 dependent chemotaxis in effector but not in naive or central memory cells was demonstrated. In addition to BLT1, chemokine receptors CCR5, CXCR3, CXCR6 have all been implicated in CTL migration to lungs under pathophysiological conditions [101-103]. The role of CCR2 on CTLs in autoimmune systemic lupus erythematosus is well recognized; CCR2-deficient CTLs are unable to migrate to the kidney, thus reducing the score of autoimmune disease [104]. CCR9 and its

ligand CCL25 control CTL migration, including to the small intestine, during inflammatory bowel disease [101]. In patients with rheumatoid arthritis, the CX3CR1: CX3CL1 axis is involved in the recruitment of autoimmune CTLs to the synovium [105]. Migration of CTLs in CXCR6, CCR4 and CCR10 dependent fashion to inflamed skin in psoriasis is also well established [106]. Myelin specific CTLs migrate to multi pre-sclerotic lesions in a CXCR3 and CCR5 dependent manner [107]. Thus, a wide range of chemokines and their receptors appear to control CTL migration in a target tissue-dependent manner. This could have implications for CTL migration to tumors in different anatomical locations.

An important physiological function of CTLs is to combat infections. The chemokine receptor CXCR3 and its ligands CXCL9/10 have been shown to mediate the recruitment of CTLs in a variety of infectious diseases [108, 109]. CXCL10 was recently shown to enhance CTL-mediated control of the pathogen Toxoplasma gondii in the brains of the chronically infected mice [110]. In a model of HCV and HSV-2 infections, CXCR3 and its cognate ligands mediated CTL infiltration to the inflamed liver [108] and vaginal mucosa [109], respectively. Chemoattractant mediated CTL migration is also an important part of the pathology of post-transplantation complications. It is well known that $CCR5^+$ and $CXCR3^+$ CTLs are involved in cardiac allograft vasculopathy and acute allograft rejection [111, 112]. In chronic rejection of lungs, CCL5, CXCL9, CXCL10, CXCL11 and CCL2 were upregulated, which might contribute to the post-transplantation complications by recruitment of antigen-specific CTLs to the graft [101]. In graft versus host disease-induced hepatitis, CTL infiltration to the liver

was mainly controlled by CXCR6 [113]. Hence, chemokines/ chemokine receptors are an integral element of CTL recruitment and present an attractive pharmacological target for intervention in various disease pathologies.

Recent advances in the imaging techniques, such as intravital microscopy, have allowed in vivo tracking of CTLs in real time to gain some insights on CTL migration to tumors. It has been demonstrated that recognition of cognate antigen by CTLs within tumors is a critical determinant of optimal CTL infiltration and killing of tumor cell [114]. Furthermore, CD44 dependent CTL migration within the tumor microenvironment was found to be an essential immunologic checkpoint that determines the potency of T-cell effector functions [115]. Boissonnas et al. showed that activated CTLs migrate at high instantaneous velocities in the periphery, but get arrested when in close contact with tumor cells expressing their cognate antigen; therefore, antigen expression by tumor cells determines both $CD8^+$ T cell motility within the tumor and extent of tumor infiltration [116]. In this regard, Deguine et al. demonstrated a sharp contrast between the effector function of CTL and NK cells. Although NK cell formed dynamic contacts with tumor cells, CTLs formed stable contacts with tumor cells expressing their cognate antigen for exerting their cytotoxic functions [117]. Dense matrix was shown to resist the CTL migration because aligned fibers in perivascular regions and around tumor epithelial cell regions dictated the migratory trajectory of T cells and restricted them from entering tumor islets [118].

Chemokine receptors controlling activated CTL migration to tumors

Several studies with human cancers showed a strong correlation between $CD8^+$ T cell infiltration and long-term survival [119]. The newly activated CTLs must infiltrate the tumor to exert their cytotoxic effects for controlling tumor growth [40]. Among the many factors controlling CTL migration, chemoattractants play a pivotal role in shaping the intratumoral infiltration of activated CTLs. The release of individual chemokines, such as CCL3, CCL5, CCL20, CXCL10, CXCL16 and CX3CL1 at the tumor site was shown to enhance CTL recruitment and antitumor immunity, confirming their important role in this process [40]. Likewise, the chemokine receptors known to be crucial in $CD8^+$ T cell recruitment to tumors till date are CX3CR1, CXCR6, CCR5 and CXCR3. Because of it's relevance in this thesis, CXCR3 will be described in detail. We have reviewed all these chemokine receptors in detail here [40].

CX3CR1:

The CX3CL1/CX3CR1 axis has been associated with high numbers of infiltrating CTLs and better prognosis in colorectal cancers [120]. CX3CL1 (fractalkine) gene transfer in tumor cells also showed marked antitumor activity primarily by enhancing the infiltration of T and NK cells in various cancers, [40, 121-123], as also DC maturation and activation in the tumors [124].

CXCR6:

Another relatively new player in this context is CXCL16, which was produced by tumor cells in response to ionizing radiation and the intratumoral CXCL16

enhanced the recruitment of CXCR6- positive CTLs and thus anti-tumor immunity [40, 125, 126].

CCR5

CCR5 was the first chemokine receptor that was shown to be an important regulator of CTL trafficking to tumors because its cognate ligand facilitated the infiltration of CTL into tumors [127]. Intratumoral injection of a chimeric CCL5-Ig encoding DNA plasmid was associated with the infiltration of increased numbers of NK, $CD8^+$ and $CD4^+$ T cells and generation of effective antitumor immunity. This effect was lost in RAG-2 or CCR5-deficient mice, indicating that CCR5 dependent CTL migration was required for this antitumor effect [128]. Adenoviral gene transfer to induce CCL3 expression in B16-ova tumors in vivo increased the efficacy of adoptively transferred tumor-specific effector OT1 T cells expressing its cognate receptor CCR5 [93, 129]. However, in human cancers, the role of CCR5 and its cognate ligands has been elusive because of the diverse observations in various types of cancers. For example, in lung cancer, CCL5 is associated with favorable disease prognosis [130] but in breast, cervical and colon cancers, CCL5 has been associated with adverse prognosis [131, 132]. In case of colon cancer, tumor-derived CCL5 has been demonstrated to recruit regulatory cells and enhance the ability of these cells to induce apoptosis in CTLs within the tumor microenvironment

CXCR3

It has long been recognized that CXCR3 is an important chemokine receptor for controlling $CD8^+$ T cell migration under diverse pathophysiological conditions, including tumors. Expressed on antigen-activated CTLs, this receptor recognizes three cognate ligands, which are interferon-inducible proteins; CXCL9, CXCL10 and CXCL11, produced by stromal cells including endothelial, epithelial and tumor cells in response to IFNγ. CXCR3 has been extensively studied in autoimmune and viral diseases and recently in cancer. CXCR3 gene Cxcr3 is located on the sex-chromosome and is an X-linked gene both in mice and humans [133]. Since certain immune disorders like systemic lupus erythematosus (SLE) have increased incidence in females compared to males, there may be a possible CXCR3 dependent gender predisposition to certain immune disorders. Hyun et.al. have reported a possible correlation of c.12+234G>A polymorphism in CXCR3 with asthma development especially in males [134]. Another study reported an association of the CXCR3 polymorphism rs34334103 with male patients with SLE and pleuritis development in those patients [135].

There may however In SP2/0 myeloma tumor model, CXCL10 expression via adenoviral gene transfer in combination with adoptive T-cell therapy completely eradicated the tumors, whereas both the adoptive T-cell therapy and CXCL10 adenoviral gene transfer treatments had minimal to no beneficial efficacy [136]. These results highlight the importance of the ligand (CXCL10) in tumors and the receptor (CXCR3) on the adoptively transferred CTLs in generating effective

antitumor immunity. In the case of human renal cell carcinoma, intratumoral expression of chemokines CXCL9 and CXCL10 showed positive correlation with CTL infiltration and inverse correlation with tumor size. Moreover, it was also observed that tumors that expressed CXCL9/10 rarely recur after surgery, reinforcing the role of these pathways in antitumor immunity [129]. In a retrospective evaluation of melanoma tumors isolates, expression of CXCR3 by human CTLs was significantly associated with enhanced survival in stage III patients [137]. Human melanoma cell lines have been shown to secrete CXCR3 ligands in response to IFNγ [138]. Analysis of the chemokines and their receptor expression using immunohistochemistry and flow cytometry in situ in colorectal patient's samples revealed pre- dominant IFNγ positive CTLs co-expressing CCR5 and CXCR3 receptors [139]. In human hepatocellular carcinoma (HCC), it was shown that CXCR3 expression correlated with activation markers, such as CD69, and it was suggested to be an important receptor of CTL migration [140]. Conversely, another study on HCC showed functional desensitization of CXCR3 in lymphocytes, including CTLs from HCC patients, by CXCL10 secreted by tumor cells suggesting a new mechanism in HCC to induce dysfunction of active CTL migration and subsequently impaired immune defense against the tumor [141]. These studies emphasize the importance of the CXCR3-CXCL9/10 axis in generating antitumor immunity in mouse models and in multiple human tumors. *Mikuchi et.al.* recently elegantly demonstrated CXCR3 mediated signalling to be a critical and an indispensable checkpoint for tumor antigen specific $CD8^+$ T cells to traffic across the tumor vasculature for carrying out effective tumoricidal

activity in mice and human melanoma [142]. CTL chemokine receptors CCR5 and CCR2 were not essential for CXCR3 mediated CTL extravasation across tumor vessels despite the presence of CCL2 and CCL5 chemokines in the tumor mileu. A recent study demonstrated that adenosine in the tumor milieu suppressed the production of CXCL10 followed by suppression in T cell infiltration; and partial reversion was seen upon adenosine receptor blockade [143].

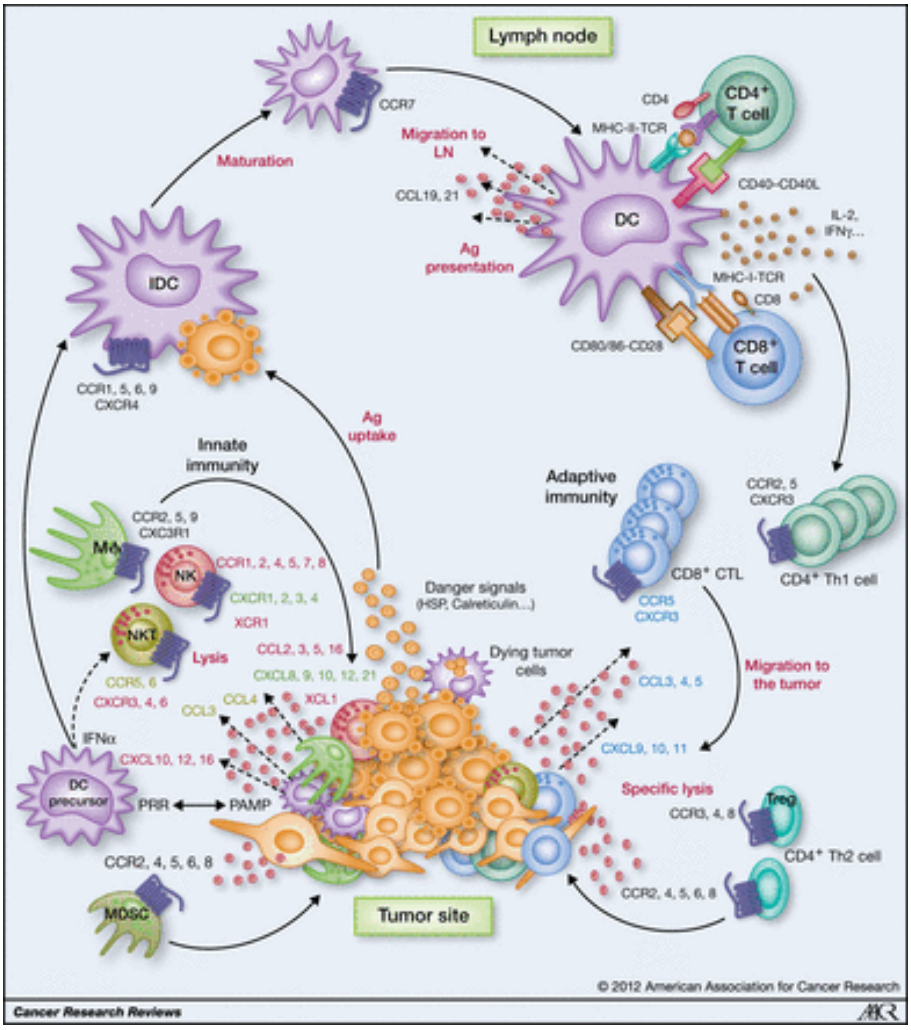

Figure 2: Chemokine network in the anti-tumor immune response. Malignant cells express pathogen-associated molecular patterns (PAMP) that can be recognized by pattern recognition receptors (PRR) on dendritic cells (DC) and macrophages (M), triggering release of chemokines.This results in recruitment and activation of M, NK, and NKT cells, which are able to lyse tumor cells. DC phagocytoses apoptotic tumor cells and HSP-complexed tumor-derived peptides. Upon maturation, DC change their homing proprieties by downregulating tissue-specific chemokine receptors and upregulating CCR7 that guides them to

CCL19/CCL21-rich lymph nodes (LN), where they present processed tumor peptides to $CD4^+$ and $CD8^+$ T cells. Activated T cells upregulate expression of chemokine receptors including CCR5 and CXCR3, and in response to intratumoral chemokines, circulating CTL infiltrate the tumor to destroy malignant cells

Figure adapted from [144].

Chemoattractants & their receptors as targets for enhancing CTL recruitment

Poor CTL trafficking to tumors has become the rate-limiting step in the success of immunotherapies as suggested by evidence from clinical trials [40, 75, 76, 145, 146]. Recently, there have been studies showing modulation of chemokine or chemokine receptor expression to enhance CTL migration to tumors [77, 122, 147-149]. Activation of Toll-like receptors 3 on mast cells through Poly-IC-induced CXCL10 and RANTES production leads to an increase in CTL recruitment [150]. IL-12 treatment of a fibrosarcoma and ovarian cell-derived tumor resulted in tumor regression via CCR5 dependent CTL migration [151]. Ionizing radiation enhanced the recruitment of $CXCR6^{+}$ CTLs and antitumor response in 4T1 breast cancer mouse model [125]. In a recent study, combining radiotherapy and ipilimumab resulted in abscopal effects in a treatment-refractory lung cancer patient that correlated with enhanced CTL infiltration [152]. Chemotherapy, such as low-dose cyclophosphamide and gemcitabine, can eliminate the T regulatory cells [153] and MDSCs [154] respectively, allows for the production of immunostimulatory antitumor cytokines, such as IFNγ and TNF-α, which can induce the CXCR3 and CCR5 mediated feed-forward recovery of CTL recruitment. Radiation therapy, along with its direct cytotoxic effect, also has an immunomodulatory effect through interferon-induced chemokine CXCL10 production by the myeloid cells in tumors. CXCL10 increases CXCR3 mediated recruitment of CTLs into the tumors for this immunomodulatory effect [155].

Direct modulation of CCL5 in the tumor environment was found to enhance CTL infiltration and tumor rejection in an immunogenic fibro- sarcoma genetically modified to express CCL5 in murine model [148].

Various CAR-based therapies using chemokine receptors have been used to enhance the recruitment of tumor specific CTLs into tumors. For instance, in a CAR-based ACT for mesothelioma, the infiltration of human T cells in tumors injected with untransduced T cells or with the mesoCAR T cells was very low (~0.3–0.4%) and not significantly different. In contrast, the infiltration of transferred mesoCAR + CCR2b gene modified T cell group was dramatically improved to 5.2% (>12.5-fold) when compared with the mesoCAR or the untransduced T cell groups [77].

The Reed–Sternberg cells of Hodgkin lymphoma produce CCL17 and CCL22, recruiting $CCR4^{+}$ T regulatory cells to tumor-causing immune suppression [156]. Modification of CTLs instead, to express both CAR directed to the Hodgkins lymphoma associated antigen CD30 and CCR4 resulted in >10-fold increase in tumor infiltration of CTLs by day 12. Infusion of these cells intravenously caused tumor regression in 57% of the mice [156]. These directed therapies enhanced the antitumor responses of the CAR CTLs and also increased the effective migration of these cells into the tumors.

CTL migration to tumors was enhanced by transfecting the CTLs with photoactivable chemokine receptor (PA-CXCR4) that induced their homing to tumors when tumors were exposed to light at 505nm [157]. This study proposes

the use of photoactivatable chemokine receptors as an important tool to enhance CTL recruitment, particularly in CTL based therapies, such as adoptive T cell transfer therapies [157]. All of these studies demonstrate the potential for targeting the chemokine/chemokine receptor directly or indirectly to enhance CTL cell migration into tumors and thus T cell mediated immunotherapies.

Barriers to CTL infiltration to tumors

The tumor evades the immune control by manipulating the immune system into supporting its growth, including restricting the $CD8^+$ T cell infiltration into the tumor microenvironment [40, 158]. Anti-tumor cytokines like IFNγ have shown to be crucial for T cell homing to tumors [159]. However, the tumor mileu is largely immune-suppressive instead due to expression of molecules like TGF-β, IL-10, IDO, VEGF-A, adenosine, etc. that compromise T cell recruitment process [75]. Mechanisms by which T cell homing to tumors gets abrogated is discussed below.

The immune-suppressive environment not only hinders T cell homing to tumors, but those that enter are restricted at the tumor margins or periphery. Studies in breast cancer model showed that tumor dendritic cells (Tu-DCs) and tumor associated macrophages (TAMs) constitutively cross present the tumor antigens to the infiltrating tumor specific T cells engaging them with long-unproductive interactions at the tumor margin [160]. Another study demonstrated that adoptively transferred T cells were trapped in TuDC-mesh like structures that prevented the homing of T cells deeply in the tumor beds [161].

Cytotoxic cell to tumor cell ratio is critical for anti-tumor immunity. T cells are present in higher numbers in tumors that express chemokines like CXCL9, CXCL10, CCL2, CCL3, CCL4 and CCL5 [162]. Tumors usually alter the chemokine profile thus disturbing the balance between T cells and tumors. Tumor mileu can potentially induce chemical modification of chemokines thereby rendering them non-functional for CTL recruitment. For example, nitrosylation of CCL2 preferentially recruits MDSCs and not tumor specific T cells [163]. Likewise tumors under hypoxia also produce CCL28 that preferentially recruit the Tregs [98]. Aberrant EGFR-Ras signaling in skin tumors was shown to suppress the production of CCL27 chemokine by keratinocytes and absence of CCL27 was shown to prevent T cell infiltration to tumors and anti-tumor immunity [164]. Another mechanism for preventing CTL infiltration was postulated to be due to altered proteolytic processing of the CXCR3 chemokine CXCL11 [165]. Thus tumor strategizes immune evasion in various ways including alteration of chemokine production to prevent $CD8^+$ T cell infiltration to tumors.

Yet another mechanism that prevents T cell recruitment to tumors is the disorganized and aberrant nature of the tumor associated endothelial vessels. Growth factors in the tumor mileu like endothelin 1, basic fibroblast growth factor (bFGF) and VEGF-A blocks/downregulates the expression of ICAM1 and VCAM1; a phenomenon called "*Endothelial cell anergy*". This leads to attenuation of T cell adhesion to tumor endothelium and thereby prevents their infiltration into tumors [75, 166]. Although the tumor endothelium is considered as leaky, the tumors with enhanced angiogenesis typically lack T cells. In an ovarian

cancer study, it was reported that VEGF expression was three folds more in tumors without T cells than with T cells [167]. Angiogenesis in the context of tumor leads to the generation of new tumor vessels that are quite irregular compared to the normal vessels. The tumor vessels that originate from the existing ones in the tumors are leaky, disorganized, dilated with abnormal structural morphology, absent or loosely attached pericytes and unusually thick basement membrane [146, 168]. This abnormal nature of the tumor vasculature may increase the interstitial pressure and promote aberrant blood flow making it difficult for selective leukocytes to adhere and traffic into the tumor mass even after being activated in the periphery.

Absence of certain chemokine receptors can seriously compromise T cell homing to tumors. Importance of chemokine-chemokine receptor systems in regulating $CD8^+$ T cell homing to tumors is only recently being acknowledged. Apart from the chemokine receptors discussed in the previous section there are no other known chemokine-chemokine receptor systems that are known to be crucial for $CD8^+$ T cell recruitment into tumors and hence necessitates research in this area. Targeting chemokine-chemokine receptor pathways can enhance T cell homing to tumors. Recent studies have indicated the presence of tumor specific T cells in the tumors for the immunotherapies to be successful. In fact the data presented in this thesis indicate that PD-1 blockade based immunotherapy completely fails in $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice suggesting the importance of chemokine receptors and T cell homing for the success of immune checkpoint blockade based therapies.

Leukotriene B_4 and its high affinity receptor BLT1

Leukotriene B_4 (5(S), 12(R)-dihydroxy-6, 14-cis-8, 10-trans-eicosatetraenoic acid, LTB_4) is a potent lipid inflammatory mediator, a calcium ionophore, that causes adhesion and chemotactic movement in leukocytes and stimulates aggregation, enzyme release and superoxide generation in neutrophils [169, 170]. LTB_4 is derived from arachidonic acid released from membrane phospholipids via the actions of phospholipase A2. Enzymes 5-lipoxygenase (5-LO) [171] and LTA_4 hydrolase [172] catalyze the production of LTB_4 from arachidonic acid in a sequential manner. While LTA_4 hydrolase is universally present in all cells, 5-lipoxygenase is only expressed in hematopoietic cells mostly myeloid cells. LTB_4 is produced by myeloid cells mostly, however, under inflammatory condition, LTB_4 production in other cells (non-immune cells) have also been reported via transcellular transport of LTA_4 from immune cells at the site of inflammation to other cells that have LTA_4 hydrolase activity [173]. LTB_4 signals through two G protein coupled seven-transmembrane domain receptors, BLT1 and BLT2, the high and low affinity receptors, respectively [174-176]. BLT1 receptor activation upon ligand binding leads to IP3 mediated calcium release from intracellular portions and later calcium influx through the cell membrane. In general, BLT1 receptor enhances the production of inositol phosphates, mobilize intracellular calcium, and activate several kinases, including PI3K, MAPK and AKT [177]. BLT1 is expressed on a variety of immune cell subsets including neutrophils, eosinophils, monocytes, dendritic cells, and activated T cells [178]. The role of

BLT1 as a pro-inflammatory mediator was first known when BLT1 deficient mice showed significantly reduced arachidonic-acid induced ear inflammation [179].

The protective phenotype observed in BLT1 knockout mice in various inflammatory disease models like asthma, atherosclerosis, arthritis, autoimmune uveitis, and diet-induced obesity later on indicated that BLT1 mostly acts as a pro-inflammatory mediator [180-184]. However, BLT1 mediated migration of different cell types were the causative reasons behind each of these diseases. For example, BLT1 mediated recruitment of neutrophils was crucial for arthritis to develop; macrophage recruitment via BLT1 was crucial in atherosclerosis and diet induced obesity manifestation; and BLT1 mediated recruitment of activated T cells in the development of autoimmune uveitis and airway-hyperresponsiveness.

The biological significance of this receptor in antitumor immunity has not been explored. The majority of work carried out in the context of inflammatory, autoimmune diseases demonstrated that $CD8^+$ T cells inducibly express BLT1 upon activation and the receptor expression is essential for their recruitment to target organs and disease development [100]. In a model of autoreactive T cell–induced uveitis, BLT1 expression on both T cells and innate immune cells was found critical for full disease development, and absence of BLT1 is highly protective in ocular inflammation [183]. However, in the context of cancer, the lack or delay in recruitment of effector immune cells such as T cells may delay generation of immune response to tumor Ag that can lead to breach of immune surveillance and poor antitumor immunity.

To determine the role of BLT1 in anti-tumor immunity, implantable model of TC-1 cervical cancer was employed and the data from these experiments is briefly described and formed the basis for the experiments conducted in the current thesis. The results obtained after challenging WT and BLT1 deficient mice with TC-1 cervical cancer is discussed in the next chapter.

CHAPTER II

LEUKOTRIENE B_4 RECEPTOR, BLT1 REGULATES ANTI-TUMOR IMMUNITY BY MEDIATING $CD8^+$ T CELL INFILTRATION TO TUMORS

BLT1 the high affinity receptor for Leukotriene B_4 has long been identified as a pro-inflammatory mediator in various disease models of infection, auto-immunity, inflammation and tumors. BLT1 is expressed on a variety of immune cells as discussed before. Migration of specific cell types is crucial in determining the type of inflammation (pro or anti-tumor growth) that ensues. Infiltration of $CD8^+$ T cells into the tumors is a critical event for effective antitumor immunity.

The results presented in this section details the role of BLT1 in regulating $CD8^+$ T cell infiltration to tumors and anti-tumor immunity using a viral antigen based subcutaneous implantable TC-1 cervical cancer model [176]. Implantation of $2x10^4$ TC-1 cells resulted in development of tumors only in 50% of WT mice (sub-lethal dose). However, under these conditions, 100% of $BLT1^{-/-}$ mice developed tumors. At this dose, while 100% of $BLT1^{-/-}$ mice succumbed to disease by day 50, 60% of WT mice still survived at day 80-post tumor challenge **(Figure 3A)**. Subcutaneous implantation of 10^5 TC-1 cells (lethal dose) also resulted in significantly enhanced tumor growth and reduced survival in $BLT1^{-/-}$ mice compared to WT mice **(Figure 3B and C)**. These results indicated that BLT1 deficient mice have poor immune surveillance against TC-1 tumors.

Development of cancer is proposed to be the end result of a malignant transformation that has passed through all three phases viz. elimination, equilibrium, and escape phases [18]. The data obtained with lethal and sub-lethal doses of tumor cells suggest a crucial function for BLT1 in controlling both elimination and equilibrium phases of tumor development. The sub-lethal dose of tumor led to slow growing tumor formation in only 50% of WT mice but showed tumor development in 100% of the BLT1 deficient mice suggesting that BLT1 is a crucial component of immune surveillance to tumors. The lethal dose of tumor cells led to rapidly growing tumors in $BLT1^{-/-}$ mice, indicating its function in antitumor immunity [176].

To determine the cellular mechanisms behind this phenotype, tumor immune infiltration studies were carried out. A marked decrease in infiltration of overall $CD45.2^{+}$ immune cells with most striking decline in $CD8^{+}$ T and NK cell population in tumors of $BLT1^{-/-}$ mice compared with WT **(Figure 4A – 4C)** was observed. To ensure that this difference was not a function of enhanced tumor size in $BLT1^{-/-}$ mice; $CD8^{+}$ T cells at similar tumor sizes were stained that also showed significant reduction of $CD8^{+}$ T cells in tumors of $BLT1^{-/-}$ mice compared to WT mice **(Figure 4D)**. Also no significant difference in $CD8^{+}$ T cell numbers was observed in spleen and tumor draining lymph nodes of WT and $BLT1^{-/-}$ mice **(Figure 4E and 2F)** suggesting that BLT1 deficient $CD8^{+}$ T cells showed defective tumor infiltration ability. Gene expression analysis by RT-PCR showed a significant reduction in effector T cell transcript levels such as IFN-γ, granzyme B, and IL-2 in tumors of $BLT1^{-/-}$ mice relative to WT mice **(Figure 4G).**

To understand whether BLT1 expression on $CD8^+$ T cells or NK cells is crucial in the observed phenotype; $BLT1^{-/-}RAG2^{-/-}$ mice were generated by crossing $BLT1^{-/-}$ mice with $Rag2^{-/-}$ mice. Comparison of tumor growth kinetics in $Rag2^{-/-}$ and $BLT1^{-/-}Rag2^{-/-}$ mice revealed no significant differences in tumor growth indicating that BLT1 expression on innate cells including NK cells does not play a dominant role in this model. Also, similar levels of NK cells were seen in tumors of $Rag2^{-/-}$ and $BLT1^{-/-}Rag2^{-/-}$ mice **(Figure 5A and 5B)** [176]. Although NK cells by themselves may not hinder tumor growth in this model, it is still possible through CD8–NK cross talk that they contribute to effective antitumor immunity [185].

To further understand the importance of BLT1 on $CD8^+$ T cells, $CD8^+$ T cells were depleted in WT and $BLT1^{-/-}$ mice following TC-1 tumor challenge using CD8 depleting antibody (Bioxcell). Depletion of $CD8^+$ T cells resulted in a significant acceleration of tumor growth only in WT and not in $BLT1^{-/-}$ mice. Moreover, the tumor growth in the CD8-depleted WT mice nearly overlapped with the tumor growth in $BLT1^{-/-}$ control mice and/or $CD8^+$ T cell–depleted $BLT1^{-/-}$ mice **(Figure 6)**. Therefore, elimination of $CD8^+$ T cells alone was sufficient for complete loss of the observed phenotype, implicating a central role for BLT1 expression on $CD8^+$ T cells.

Adoptive transfer of tumor experienced WT but not $BLT1^{-/-}$ $CD8^+$ T cells intravenously could significantly reduce tumor growth in $Rag2^{-/-}$ mice challenged with TC-1 tumors **(Figure 7)**. This experiment served as a simplified replica of the adoptive T cell therapy used in the clinic following immune ablation. CTL infiltration studies suggested that the transferred WT $CD8^+$ T cells entered

tumors in greater numbers compared to BLT1$^{-/-}$ CD8^{+} T cells; their numbers being similar in the tumor draining lymph nodes **(Figure 7B and 7C).** Analysis of the major CD8^{+} T cell chemokine receptors, CCR5, CCR9, CXCR3 and BLT1 on the transferred WT and BLT1$^{-/-}$ CTLs in TdLN and tumor revealed no striking differences besides the significantly enhanced expression of BLT1 on WT CTLs in TDLNs compared to the absence of its expression in naive WT and BLT1$^{-/-}$ CTLs **(Figure 8)**. A significant increase in CXCR3 expression was observed in both adoptively transferred WT or BLT1$^{-/-}$ CD8^{+} T cells relative to the CD8^{+} T cells from the naïve mice. Analysis of expression of all these receptors on CD8^{+} T cells showed complete downregulation in tumors which is consistent with our previous study and many others suggesting receptor internalization upon ligand binding upon target sites.

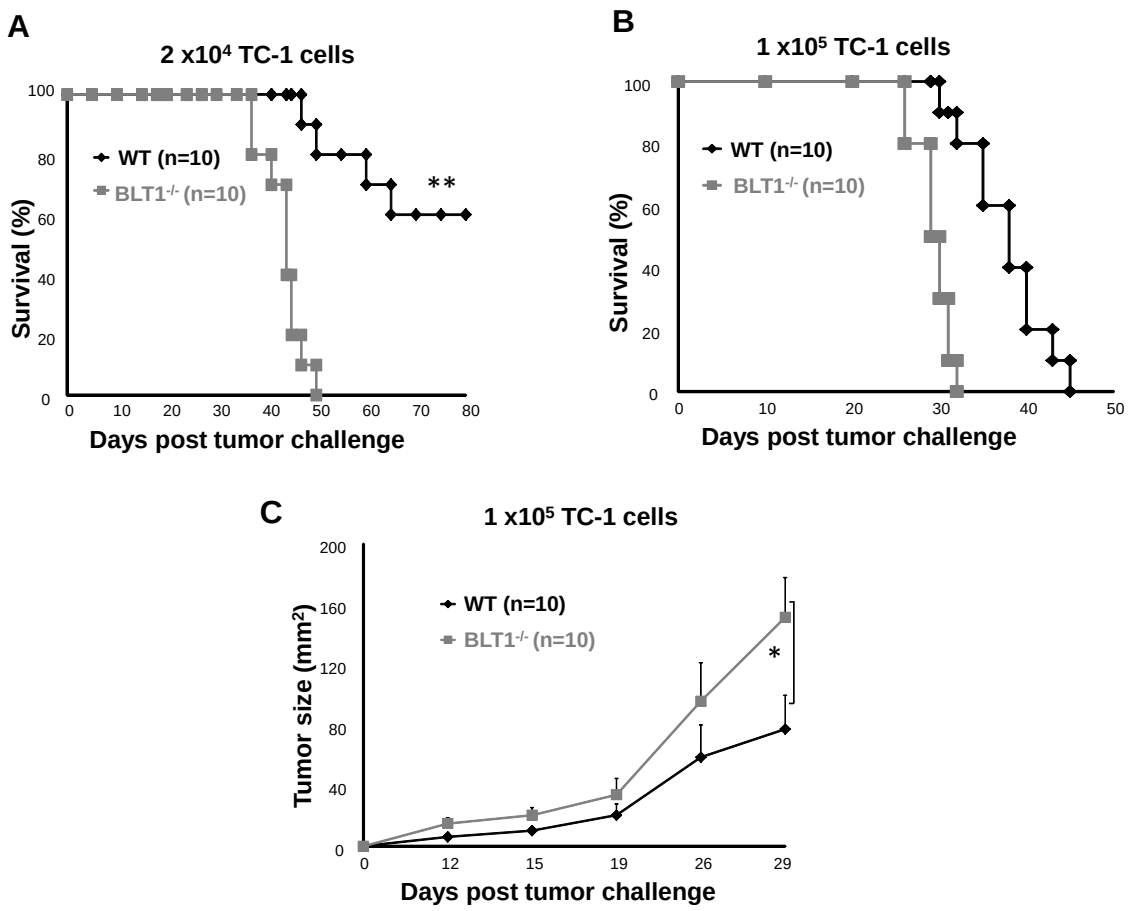

Figure 3: Decreased survival and increased tumor growth in BLT1$^{-/-}$ mice. (A and B) Significantly reduced survival of BLT1$^{-/-}$ mice using sub-lethal and lethal TC-1 dose. Kaplan–Meier survival plots of BLT1$^{+/+}$ and BLT1$^{-/-}$ mice. **(A)** The mice were injected s.c. with a sublethal dose (2.0 x 10^4) of TC-1 cells and their survival was monitored up to 80d**. (B)** BLT1$^{+/+}$ and BLT1$^{-/-}$ mice were injected s.c. with a lethal dose (1.0 x 10^5) of TC-1 cells and survival was followed. (C) Significantly enhanced tumor growth in BLT1$^{-/-}$ mice. Tumor size in lethal dose challenged group was measured and calculated by multiplication of two perpendicular diameters (length x width). Log rank tests were performed for statistical analysis of survival, and student t test was used for tumor sizes.

Experiment shown is representative of three independent experiments. *p< 0.05 (significant), **p < 0.001 (very significant).

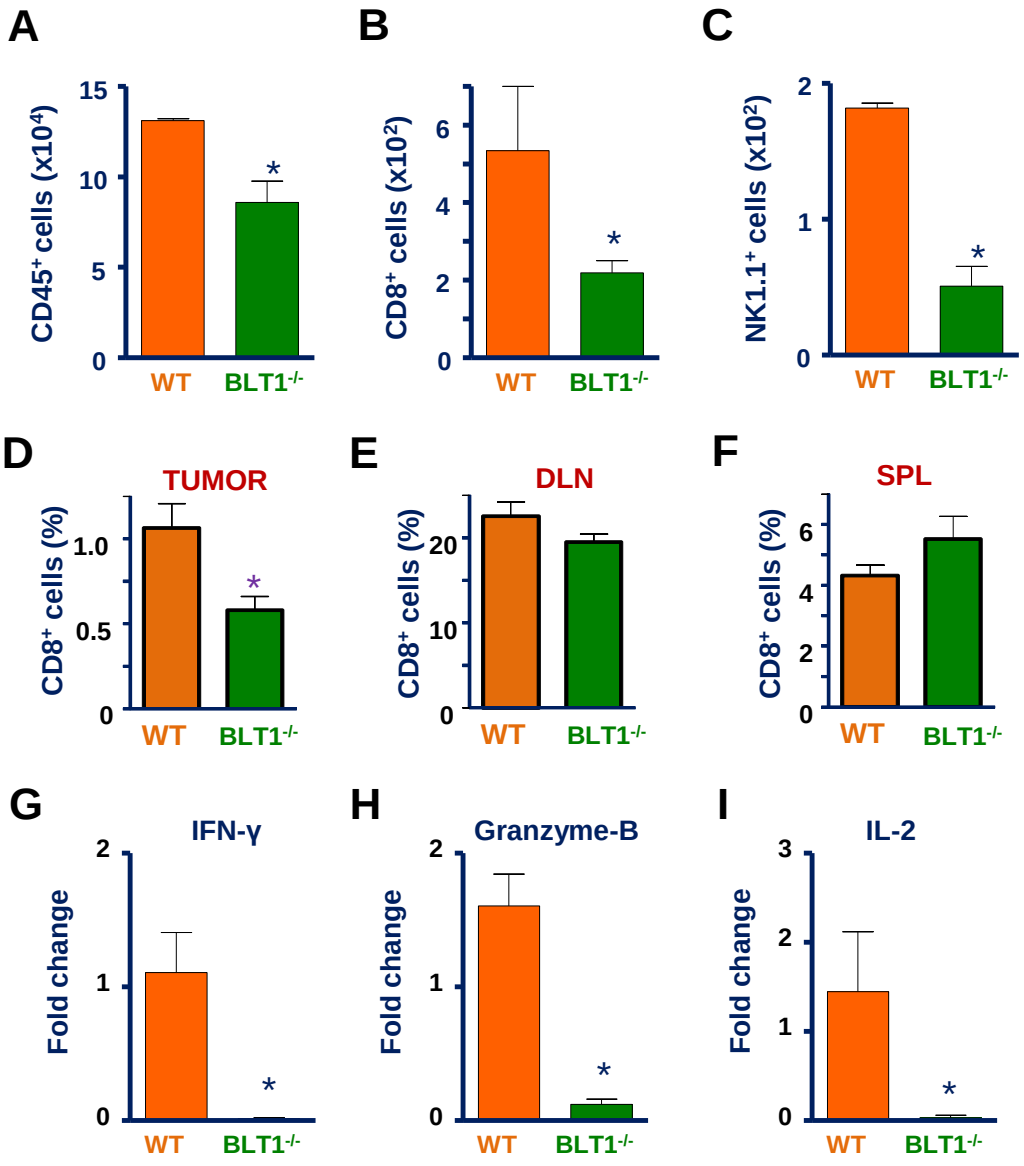

Figure 4: Reduced infiltration of effector antitumor immune cells into TC-1 tumors growing in BLT1$^{-/-}$ mice. (A–C) BLT1$^{+/+}$ and BLT1$^{-/-}$ mice were injected s.c. with 1.0 x 10^5 TC-1 cells, and tumors were harvested at 29d of post tumor challenge. Numbers of total CD45$^+$ immune cells **(A)**, CD8$^+$ T cells **(B),** and NK

cells **(C)** per million of total tumor cells were analyzed from WT and BLT1$^{-/-}$ mice using standard flow cytometry methods. **(D–F)** CD8^{+} T cell staining in size matched tumors showing %CD8^{+} T cells (frequency of total) in tumor **(D),** % CD8^{+} T cells (frequency of CD45^{+}) in TdLN **(E),** and spleen **(F)** from WT and BLT1$^{-/-}$ mice. **(G-I)** Quantitative real-time PCR analysis: The levels of IFNγ **(G),** granzyme B **(H),** and IL-2 **(I)** mRNA expression in BLT1$^{-/-}$ tumors as compared with WT tumors by qRT-PCR were determined. Data are representative of two to three independent experiments involving at least n = 4 mice/group in each experiment. *p< 0.05

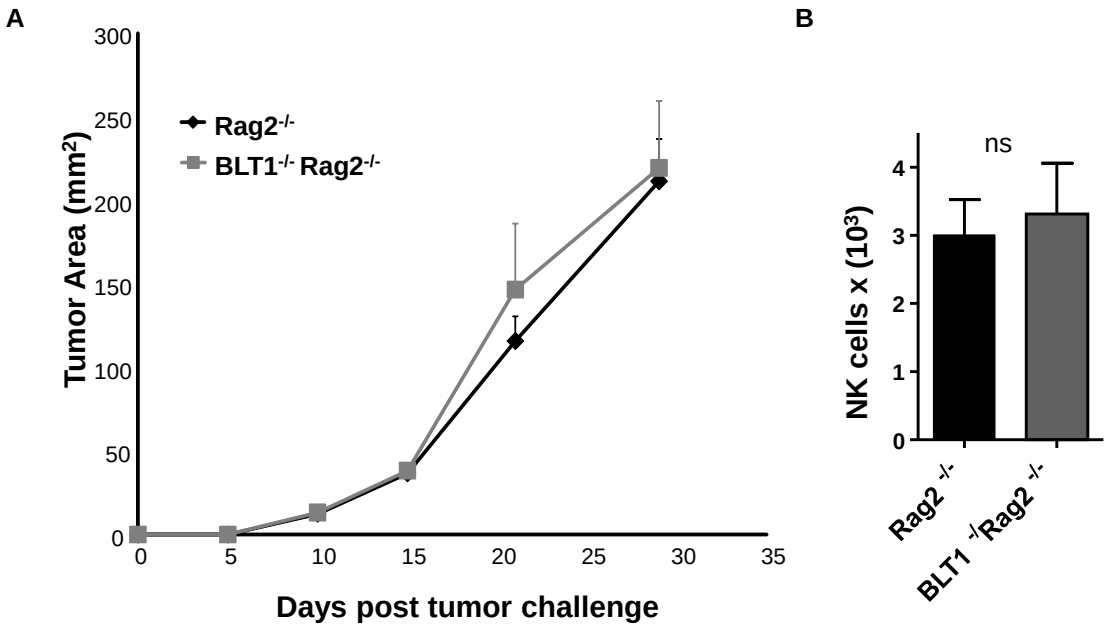

Figure 5: Unaltered tumor growth and intratumoral NK cell numbers in Rag2$^{-/-}$ and Rag2$^{-/-}$BLT1$^{-/-}$ mice. (A) Rag2$^{-/-}$ and Rag2$^{-/-}$BLT1$^{-/-}$ mice were challenged s.c. with 5 x 10^4 TC-1 cells on the right flank and observed for the rate of tumor growth. **(B)** Absolute numbers of tumor-infiltrating NK cells per million of total tumor cells were analyzed from Rag2$^{-/-}$ and Rag2$^{-/-}$BLT1$^{-/-}$ mice using standard flow cytometry methods. Data shown are representative of three independent experiments.

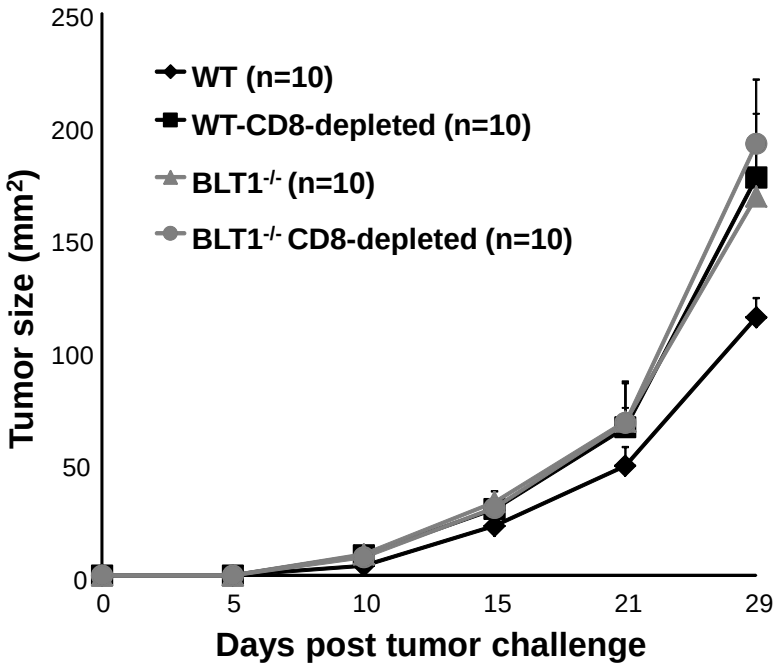

Figure 6: Depletion of $CD8^+$ T cells accelerates TC-1 tumor growth in $BLT1^{+/+}$ but not in $BLT1^{-/-}$ mice. A single dose of 500 µg CD8-depleting Ab was injected i.p. in WT and $BLT1^{-/-}$mice. The next day, 1 x 10^5 TC-1 tumor cells were inoculated s.c in the right flank in WT and $BLT1^{-/-}$ mice, and the tumor growth was monitored. Data shown are representative of three independent experiments. *p< 0.05

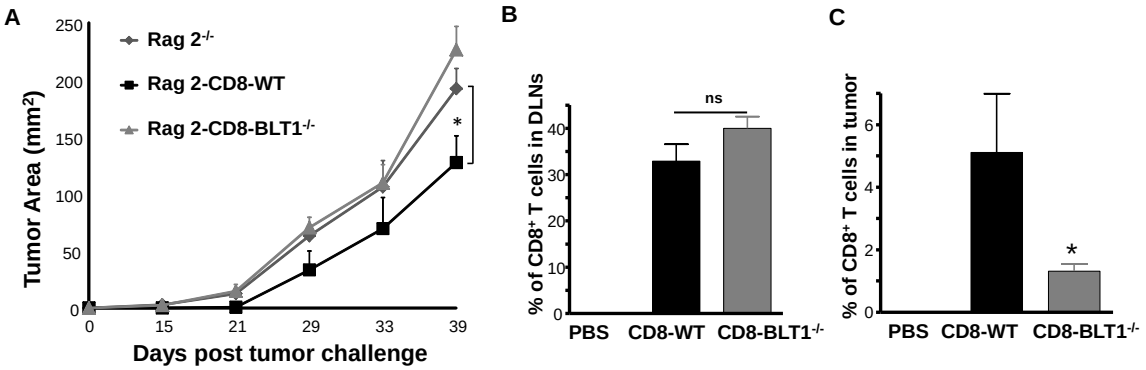

Figure 7: Adoptive transfer of tumor primed CD8$^+$ T cells from BLT1$^{+/+}$ but not from BLT1$^{-/-}$ mice retards tumor growth. Rag2$^{-/-}$ mice were challenged with 5 x 10^4 TC-1 tumor cells in the right flank. Two days later, CD8$^+$ T cells were isolated from the spleens and lymph nodes of tumor bearing (3–5 mm) WT or BLT1$^{-/-}$ mice by magnetic sorting. A total of 8 x 10^5 CD8 T cells (97% purity) or PBS was injected i.v. in the tumor inoculated Rag2$^{-/-}$mice. **(A)** Tumor growth curve of Rag2$^{-/-}$, Rag2$^{-/-}$ transferred either with BLT1$^{+/+}$ or BLT1$^{-/-}$ CD8$^+$ T cells. **(B and C)** The %CD8$^+$ T cells of total cells recovered from TDLNs **(B)** and %CD8$^+$ T cells of total CD45$^+$ cells within tumors **(C)** are shown. Data shown are representative of three independent experiments (n = 5). * p <0.05

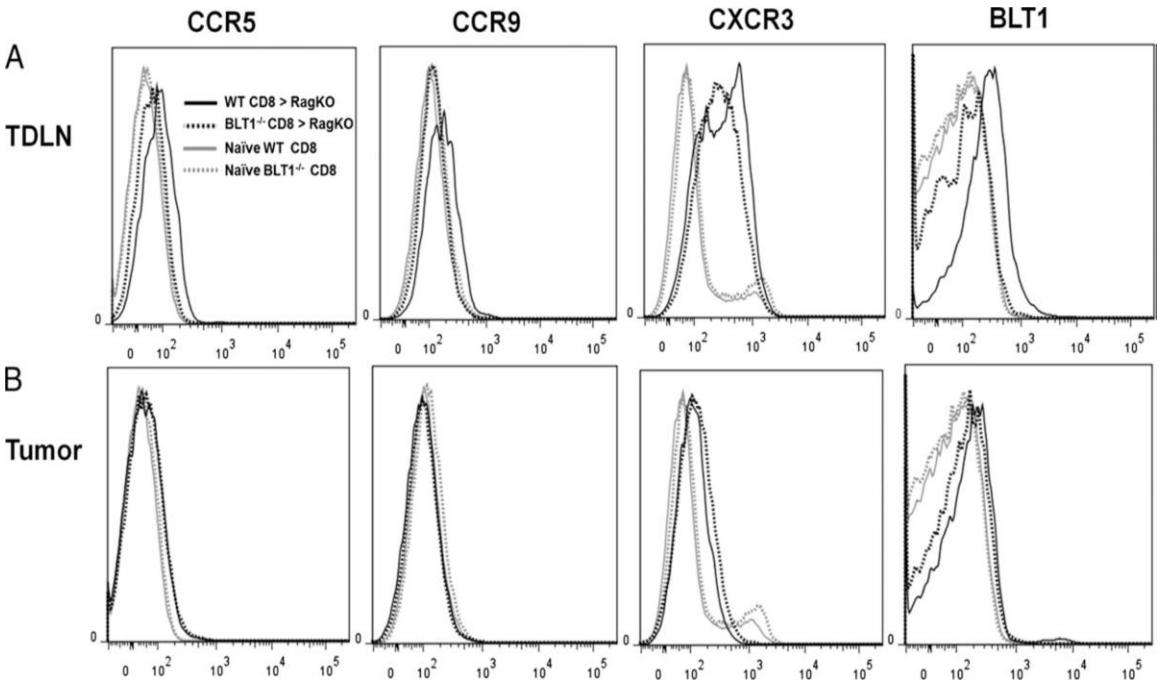

Figure 8: Chemokine receptor expression on the adoptively transferred CD8$^+$ T cells from TDLNs and tumors. Adoptively transferred CD8$^+$ T cells from WT and BLT1$^{-/-}$ mice into Rag2$^{-/-}$ mice were analyzed for the expression levels of chemokine receptors. Naïve WT and BLT1$^{-/-}$ CD8$^+$ T cells were used as controls. Chemokine receptors CCR5, CCR9, CXCR3, and BLT1 were stained and analyzed on the transferred CD8$^+$ WT or BLT1$^{-/-}$ CD8$^+$ T cells from TDLNs (A) and tumor (B). Data shown are representative of two independent experiments.

Taken together, using an implantable cervical cancer model, we demonstrated that expression of BLT1 on $CD8^+$ T cells plays a crucial role in mediating their recruitment to tumors, thereby initiating and sustaining antitumor immunity. In the context of cancer, BLT1 was shown to play a dual role in controlling tumor promoting inflammation; as seen in silica induced lung tumorigenesis [186] and or tumor suppressive inflammation; as shown in the TC-1 cervical cancer model [176]. A recent article by Yokota et al. used GM-CSF–based tumor vaccine setting in BALB/c leukemia model to evaluate the vaccine and secondary/recall immune responses. Their results showed similar or better primary and recall immune responses in the $BLT1^{-/-}$ mice [187]. Several differences that might account for the divergent results include: 1) different mouse strains (BALB/c), 2) different cancer type (leukemia), and 3) GM-CSF transformed cancer cell lines. In addition, Yokota et al.'s study found differences WT and $BLT1^{-/-}$ mice only in the recall responses with $CD4^+$ T cells playing a dominant role. In our studies, the numbers of tumor-infiltrating $CD4^+$ T cells in WT and $BLT1^{-/-}$ mice were similar, indicating a limited, if any, direct role of $CD4^+$ T cells in controlling the tumor growth.

Hence, in context of cancers, BLT1 seems to have dual roles in tumor progression. Given the results on the role of BLT1 in CTL migration to tumors in viral antigen based cervical cancer model, the experiments conducted in this thesis are aimed at developing an understanding about the role of BLT1 in endogenous immune surveillance against other solid tumors, specifically non-viral, self-antigen based B16 melanoma model. We also studied the role of

CXCR3 chemokine receptor in CTL migration to tumors and anti-tumor immunity and the possibility of interdependence of BLT1 and CXCR3 in achieving an anti-tumor immune response through the generation of BLT1/CXCR3 double knockout mice (DKO). The experiments also determine the importance of BLT1 and CXCR3 receptors in regulating PD-1 blockade based therapeutic vaccinate mediated anti-tumor effector responses.

CHAPTER III

MATERIALS AND METHODS

Mice and cell lines:

C57BL/6 mice, CXCR3$^{-/-}$ mice and UBC-GFP mice in C57BL/6 background (6-7wks old) were purchased from The Jackson Laboratory and/or bred in our animal facility at the University of Louisville. Previously described BLT1$^{-/-}$ mice in C57BL/6 background were also bred in our animal facility at University of Louisville [179]. Rag2$^{-/-}$ mice in C57BL/6 background were purchased from Taconic (Germantown, NY). BLT1$^{-/-}$CXCR3$^{-/-}$ double knockout mice were generated by crossing BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice at our animal facility. All animals were cared for in accordance with institutional and National Institute of Health guidelines and under IACUC protocol. B16 melanoma cell line was purchased from American Type Culture Collection (Manassas, VA) and cultured in complete RPMI media supplemented with 10% FBS.

Reagents:

Fluorochrome-conjugated Abs (anti-CD45.2-PE-Cy7, anti-CD3-APC-Cy7, anti-CD4-APC, anti-CD8-PerCP-Cy5.5, NK1.1-PE, CD11b-APC, Ly6G-PE, Ly6C-FITC, CXCR3-PE, IFNγ APC, TNFα APC-Cy7, Streptavidin-APC and 7-AAD)

were purchased from BD PharMingen and eBioscience. Trp-2 peptide (Trp2180: SVYDFFVWL) was purchased from Peptide 2.O Inc. Anti-mouse BLT1 antibody conjugated to biotin was developed in the lab (unpublished data). Anti-m-OX-40 agonistic Ab (Clone OX-86) and Anti-m-PD-1 antagonistic Ab (Clone: RMP1-14) were purchased from BioXcell. RT primers for IFN-γ, granzyme B, and IL-2 genes were obtained from Real Time Primers, LLC.

Tumor model and vaccinations:

Naive WT, BLT1$^{-/-}$, CXCR3$^{-/-}$ and BLT1$^{-/-}$CXCR3$^{-/-}$ mice were challenged with 10^5 live B16 melanoma cells by reconstituting in 200 µl PBS and injecting subcutaneously at the right flank of mice to form tumors. For sub-lethal tumor dose experiments, $4x10^4$ live B16 cells were injected s.c. Tumor diameter was measured every alternate day using a calliper. Average tumor diameter was calculated by measuring two perpendicular diameters and tumor area was calculated by multiplying the two perpendicular diameters. For survival experiments, mice were allowed to reach 15 mm tumor diameter as experimental endpoint. Percentage survival was calculated and plotted using Kaplan Meier survival plots. Tumor bearing animals were euthanized once the tumors reached 15 mm or 7-9 mm diameter or earlier if they showed any signs of discomfort. For in vivo-cytotoxicity studies, animals were immunized s.c. with 50 µg/mice Trp-2 peptide and 100 µg/mice OX-86 Ab (Clone OX-86, BioXcell) in PBS or PBS alone as control. For vaccination studies; WT, BLT1$^{-/-}$, CXCR3$^{-/-}$ and BLT1$^{-/-}$ CXCR3$^{-/-}$ were challenged with 10^5 live B16 cells s.c. on the right flank on day 0. On day +5 and day +15 post tumor challenge, the mice were vaccinated i.v. with

50 µg/mice Trp-2 peptide and 100 µg/mice anti-PD-1 antagonistic Ab (clone RMP1-14, BioXcell). The control mice were administered with PBS. The tumor growth was monitored every alternate day. The mice were euthanized when the knockout animals or the control unvaccinated animals reached 15 mm tumor diameter. Tumors, blood, spleen and tumor draining lymph nodes (TdLN) were then analyzed for $CD8^+$ T cells by flow cytometry.

Flow Cytometry:

Tumors were harvested and cut into small pieces after removal of connective tissue and tissue stroma. To obtain single cell tumor suspension, the small tumor pieces were incubated in an enzyme mixture consisting of Collagenase A (2 mg/ml) and DNase-I (1 mg/ml) in incomplete RPMI medium for 1hr at 37 °C on a rocking platform. After 1hr digestion, single cell suspension was obtained by passing the digested tissue through 40µm nylon mesh and the resultant cells washed twice in PBS before staining for flow cytometry. Cells were stained with fluorochrome labelled anti-mouse Ab like CD45.2, CD3, CD4, CD8, NK1.1, CD11b, Ly6G, Ly6C, etc. Two million total tumor cells were stained and analyzed using multi-parameter flow cytometry. Similarly, spleen and tumor draining lymph nodes (TdLN- inguinal, brachial and axillary) were harvested, processed into single suspension, stained and analyzed via flow cytometry. For intracellular cytokine staining single cell suspensions from tumor, spleen and TdLNs were stimulated with cell stimulation cocktail (eBiosciences, 500X used at 1X) consisting of PMA (40.5 µM), Ionomycin (670 µM) and protein transport inhibitors – Brefeldin A (5.3 mM) and Monensin (1 mM) for 6hrs at 37 °C, 5%CO_2. After 6

hrs the cells were harvested and washed, surface stained with CD45, CD3 and CD8 and fixed and permeabilized (IC fixation and Permeabilisation buffer – eBiosciences) and stained for IFNγ and TNFα using anti-mIFNγ Ab and anti-mTNFα Ab (BD Biosciences). Isotype controls with the same fluorochrome were used as controls. Cells were acquired using FACS Canto II machine and analyzed by FlowJo (TreeStar) software.

Immune-fluorescence microscopy:

Immune-fluorescence staining for $CD8^+$ T cells in the tumors of WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice was analyzed using Nikon-A1R Confocal microscope. Tumors were embedded in OCT medium and snap frozen in liquid nitrogen and later cut into 5 µm sections using a cryostat. Sections were fixed using ice-cold acetone and then blocked using 1X PBS supplemented with 3% BSA and 5% goat serum for 1 hr at RT. To stain for $CD8^+$ T cells, the sections were incubated with rat anti-mouse CD8a Ab (BD Pharmingen) in 1X PBS + 3% BSA for 1hr at RT. After 3 washes with PBS, the sections were then incubated with the secondary Ab goat anti-rat Alexa 594 (2mg/ml, Invitrogen). After washing with PBS, the sections were mounted with Vectashield mounting medium containing DAPI (Vector Labs) and analyzed at 200X magnification. A minimum of 4 fields for each tumor section was analyzed.

Real-time PCR

Total RNA from the excised tumors was isolated using Trizol followed by RNase mini prep kit from Qiagen. The RNA was treated with DNase using Turbo DNAse kit (Ambion). For quantitative real-time PCR, 1µg total RNA was reverse transcribed in 50 µl reaction using TaqMan reverse transcription reagents (Applied Biosystems) using random hexamer primers. A total of 2 µl cDNA and the 1 µM real-time PCR primers were used in a final 20 µl PCR reaction with "power SYBR-green master mix" (Applied Biosystems). The real-time primers were purchased from Real Time Primers, LLC (Elkins Park, PA). The sequence of the primers will be provided upon request. Real-time PCR reaction was performed in Bio-Rad CFX-96 Real Time System. Expression of the target genes was normalized to GAPDH and displayed as fold change relative to the WT sample. Data are representative of tumors isolated from at least five different mice for each genotype.

In vivo cytotoxicity assay:

A standard in vivo-cytotoxicity assay was performed by injecting peptide pulsed target cells into immunized mice as previously described [176]. WT, $BLT1^{-/-}$, $CXCR3^{-/-}$ and $BLT1^{-/-}CXCR3^{-/-}$ recipient mice were immunized s.c. with 50 µg/mice of Trp-2 peptide and an adjuvant i.e. 100 µg/mice anti-OX-40 agonistic Ab (Clone OX-86, BioXcell). 7 days later, C57BL/6 splenocytes were divided into $CFSE_{high}$ and $CFSE_{low}$ populations by staining with 2.5 µM and 0.25 µM CFSE fluorescent dye. $CFSE_{high}$ cells were pulsed with 2 µg/ml Trp-2 peptide for 90 min

at 37°C in a 5% CO_2 incubator. CFSE high and low cells were extensively washed and mixed at 1:1 ratio and injected i.v. into the immunized WT, $BLT1^{-/-}$, $CXCR3^{-/-}$ mice. Their spleens were harvested after two days and analyzed by flow cytometry to determine the ratio of $CFSE_{high}/CFSE_{low}$ target cells and percent killing. The percentage of in vivo killing was calculated by the following formula:

$[1 – ([CFSE^{high}/CFSE^{low}$ for experimental$]/[CFSE^{high}/CFSE^{low}$ for naive$])] \times 100$.

Purification of $CD8^+$ T Cells

CD8+ T cells were isolated from spleen and tumor draining lymph nodes of tumor bearing (3-4mm tumor diameter) WT, $BLT1^{-/-}$, $CXCR3^{-/-}$ or $BLT1^{-/-}CXCR3^{-/-}$ double knockout mice using cell sorting using magnetic beads for CD8 (MACS, Miltenyi Biotec). In co-transfer experiments, tumor bearing UBC-GFP mice were used to obtain $CD8^+$ T cells instead of WT mice. Briefly, spleen or lymph nodes were crushed and passed through 70 µm strainer (Corning). The red blood cells were lysed with RBC lysis buffer (Biolegend). After lysis step, cells were resuspended in Automax buffer (Miltenyi Biotec) containing 0.5% FBS and incubated with $CD8^+a$ (Ly-2) micro beads (Miltenyi Biotec). The $CD8^+$ T cells were isolated as described by the manufacturers protocol and were positively selected on MACS MS columns. The purified $CD8^+$ T cells were > 98% pure as analyzed by Flow Cytometer.

Adoptive transfer studies in Rag2$^{-/-}$ mice:

Rag2$^{-/-}$ immune-deficient mice were challenged s.c. with 10^5 live B16 cells. Two days later, CD8$^+$ T cells were isolated from the spleen and TdLN of tumor bearing WT, BLT1$^{-/-}$, CXCR3$^{-/-}$ or BLT1$^{-/-}$CXCR3$^{-/-}$ mice by magnetic sorting using CD8a-Ly2 microbeads (Miltenyi Biotec) with >98% purity. 1 million purified CD8$^+$ T cells were injected i.v. into the Rag2$^{-/-}$ mice challenged with live B16 tumors and vehicle alone i.e. PBS was used as the control. Tumor growth was monitored every alternate day. Animals were euthanized once they reached 15mm tumor diameter and TdLNs as well as tumors were analyzed for CD8$^+$ T cell numbers. For, co-transfer experiments, UBC-GFP mice were used as WT mice in order to distinguish between WT and knockout (non-GFP) CD8$^+$ T cells. Rag2$^{-/-}$ mice were challenged with 10^5 live B16 cells. Two days later, CD8$^+$ T cells were isolated from tumor bearing WT (UBC-GFP), BLT1$^{-/-}$, CXCR3$^{-/-}$ and BLT1$^{-/-}$ CXCR3$^{-/-}$ mice. 1 million total CD8$^+$ T cells consisting of WT (GFP$^+$) and either BLT1$^{-/-}$, CXCR3$^{-/-}$ or BLT1$^{-/-}$CXCR3$^{-/-}$ CD8$^+$ T cells were injected into Rag2$^{-/-}$ mice in equal proportion and tumor growth was monitored. Animals were euthanized once they reached 7-9mm diameter. Spleen, blood, TdLN and tumors were harvested and CD8$^+$ T cells were analysed for GFP$^+$ (WT) and GFP$^-$ (knockout) populations.

Statistical Analysis:

Statistical analysis was done using the Student t test and Mann Whitney U test. The survival assays were analyzed using long-rank test in Graph Pad Prism software. Student's t-test were used for comparisons between two experimental groups, with a *p* value of <0.05 considered as significant using Graph Pad Prism software (***=$p<0.001$; **=$p<0.01$, *=$p<0.05$). Error bars represent ±SD.

CHAPTER IV

BLT1 AND CXCR3 REGULATE ANTI-TUMOR IMMUNITY BY FACILITATING $CD8^+$ T CELL MIGRATION TO TUMORS

INTRODUCTION

Chemokine-chemokine receptor pathways are one of the major factors governing CTL recruitment to tumors and anti-tumor immunity [40, 75]. Till date only a few chemokine receptor systems are known to regulate T cell homing to tumors and anti-tumor immunity. Herein, we studied the roles of leukotriene B4 receptor BLT1 and CXCR3 in regulating an endogenous anti-tumor immune response using a syngeneic murine model of B16 melanoma. **We hypothesize that $BLT1^{-/-}$ mice would demonstrate defective immune-surveillance and anti-tumor immunity against melanoma tumor, as seen in TC-1 cancer. We also hypothesize that tumors in $CXCR3^{-/-}$ mice would rapidly develop compared to WT mice.** Experiments involved challenging WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice with B16 melanoma cells subcutaneously. Experiments such as assessing tumor infiltrating leukocytes and adoptive transfer of WT or knockout $CD8^+$ T cell in tumor bearing $Rag2^{-/-}$ mice were carried out to further investigate the importance of BLT1 and CXCR3 expression on $CD8^+$ T cells for generating effective anti-

tumor immunity. Adoptive transfer of either WT, BLT1$^{-/-}$ or CXCR3$^{-/-}$ tumor educated CD8^{+} T cells in Rag2$^{-/-}$ mice somewhat recapitulates the ACT procedure employed in clinic post chemotherapy mediated immune ablation. The effector functions of WT and knockout CTLs in periphery as well as in tumors were then assessed to understand if apart from migration, the effector functions of the T cells are regulated by BLT1 and CXCR3. This is of relevance, since previous studies have indicated a co-stimulatory role for chemokine receptors on T cells [188].

RESULTS

Defective immune surveillance and anti-tumor immunity in BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice:

Data presented in the previous chapter demonstrated a crucial role for BLT1 in $CD8^+$ T cell migration to tumors and anti-tumor immunity in a viral antigen derived TC-1 cervical cancer model. To determine the requirement for BLT1 and CXCR3 in mediating anti-tumor immunity in an autologous (non-viral) tumor model, syngeneic spontaneous B16 melanoma murine model was employed. WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice were subcutaneously challenged with either a lethal tumor dose (10^5 cells) or sub-lethal tumor dose (4×10^4 cells) of B16 cells. BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice showed significantly enhanced tumor growth as compared to the WT mice at both doses of tumor challenge **(Figure 9A and 9B)** and significantly reduced survival as compared to the WT mice at the sub-lethal dose **(Figure 9C)**. At the sub-lethal tumor dose both BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice demonstrated 100% mortality by day 28 post tumor challenge, however, 50% of the WT mice still survived post day 40 with all of them developing relatively slow growing tumors (**Figure 9C**). These results suggest that both BLT1 and CXCR3 are crucial for immune surveillance and endogenous anti-tumor response. There was no difference between the tumor kinetics of BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice at both lethal and sub-lethal tumor doses, suggesting that they both are crucial to an equal extent in achieving effective immune surveillance and anti-tumor immunity.

Reduced homing of $CD8^+$ T cells into tumors of $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice

To explore the basis for enhanced tumor growth in the knockout mice, leukocyte sub-populations in tumors, spleen and TdLN of tumor bearing WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice were profiled by flow cytometry. WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice were challenged with 10^5 B16 cells and the tumors were harvested when the knockout tumors reach 7-9 mm (mid-sized) tumor diameter. Single cell suspensions were obtained from the tumor, spleen and TdLN and stained with CD45.2 for all immune cell populations and CD3, CD4 and CD8 for T cells, NK1.1 for NK cells, CD11b, Ly6G and Ly6C for myeloid cell populations. The $BLT1^{-/-}$ and $CXCR3^{-/-}$ tumors showed significant reduction in $CD8^+$ T cell numbers as compared to WT tumors **(Figure 10A)**. Moreover, $CXCR3^{-/-}$ tumors, but not $BLT1^{-/-}$ tumors, had significant reduction in other effector cell populations like $CD4^+$ T cell and NK cells as compared to the WT tumors**.** To ensure that reduced CTL numbers are not a function of differential tumor sizes, TIL infiltration, studies were carried out in size-matched tumors. Similar reduction in $CD8^+$ T cell numbers in tumors of knockout mice as compared to WT mice was observed at size matched (end stage) tumors as well **(Figure 10B)**. The significant reduction in $CD8^+$ T cells in the tumors of $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice was confirmed by immune-fluorescence staining and confocal microscopy **(Figure 10C)**. Immune cell profiling in the spleen **(Figure 11)** and TdLN **(Figure 12)** revealed that knockout mice had similar percentages of $CD8^+$ T cells, $CD4^+$ T cells and NK cells as compared to WT mice. Myeloid cell populations constitute a significant part of the tumor microenvironment. Analysis of $CD11b^+$ myeloid cells and

myeloid derived suppressive cells subsets (MDSC) i.e. $CD11b^{+}Ly6G^{+}$ (granulocytic-MDSC) and $CD11b^{+}Ly6C^{+}$ (monocytic - MDSC) in the tumors of WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice showed no significant differences **(Figure 13)**. These results suggest that enhanced tumor growth in $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice may be related to the reduced numbers of cytotoxic cells as compared to tumors of WT mice.

Effector responses in WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice

To assess the effector responses controlled by BLT1 and CXCR3, transcript expression of various effector molecules and IFNγ regulated genes were analyzed in total tumor RNA of WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice by quantitative real time PCR as described in methods. Transcript expression levels of CTL effector molecules like granzyme-B and IFNγ were significantly reduced in tumors of $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice as compared to WT mice as shown by RT-PCR **(Figure 14 A and 14 B)**. Expression of interferon gamma inducible genes like CXCL9 and CXCL10 were also determined. CXCL9, ligand for CXCR3 induced by IFNγ was significantly reduced in tumors of both $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice while CXCL10, another ligand for CXCR3 was significantly reduced in $CXCR3^{-/-}$ mice **(Figure 14 C and 14D).** Significantly less IFNγ, Granzyme-b and interferon gamma inducible chemokines CXCL9 and CXCL10 in $CXCR3^{-/-}$ mice precludes the infiltration of T cells and NK cells that are major producers of IL-2 and IFNγ. $CXCR3^{-/-}$ tumors showed significantly reduced IL-2 and prevents the feed-forward loop of T cell infiltration>IFNγ>CXCL9/10>T cell infiltration and reduces the overall effector responses in the tumor. Tumors in $CXCR3^{-/-}$ mice

show reduced NK cells and CD4+ T cells that are major These results suggest defective effector responses in tumors of BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice compared to WT mice reflective of reduction in CTL numbers to tumors.

Effector responses of WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ CD8^{+} T cells

In order to understand if BLT1 and CXCR3 receptors contribute to effector functions in the CD8^{+} T cellS; IFNγ and TNFα effector cytokines were analyzed in tumor infiltrated CD8^{+} T cells as well as CD8^{+} T cells from spleen and TdLN of tumor bearing mice. Invivo cytotoxicity assay was also performed to examine the cytotoxic ability of WT and knockout CD8^{+} T cells in the spleens of immunized WT and knockout mice.

To assess whether the function of CD8^{+} T cells in BLT1$^{-/-}$ and CXCR3$^{-/-}$ is intact or defective, we assessed the in vivo killing activity of CD8^{+} T cells in WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice. Briefly, WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ recipient mice were immunized s.c. with 50µg/mice of Trp-2 peptide and an adjuvant i.e. 100µg/mice anti-OX-40 agonistic Ab (Clone OX-86, BioXcell). 7 days later, C57BL/6 splenocytes were divided into CFSE$_{high}$ and CFSE$_{low}$ populations by staining with 2.5µM and 0.25µM CFSE fluorescent dye. CFSE$_{high}$ cells were pulsed with 2µg/ml Trp-2 peptide. CFSE high and low cells were extensively washed and mixed at 1:1 ratio and injected i.v. into the immunized and naïve WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice. Their spleens were harvested after two days and analyzed by flow cytometry to determine the ratio of CFSE$_{high}$/CFSE$_{low}$ target cells and percent killing. No significant difference was observed in the killing abilities of WT

as well as BLT1$^{-/-}$ and CXCR3$^{-/-}$ CD8^{+} T cells **(Figure 15 A-D).** These results suggest that there is no intrinsic defect in the cytotoxic function of the knockout CD8^{+} T cells in the periphery. However, in the tumor microenvironment, the lack of CXCR3 receptor on the CD8^{+} T cells may render them defective in terms of IFNγ production and effector functions.

Given that BLT1 and CXCR3 are essential for homing into tumors, we sought to determine if the effector functions were also controlled by BLT1 and CXCR3. To measure their cytotoxic function within the tumor, levels of IFNγ, an effector cytokine was determined in the CD8^{+} T cells by intracellular cytokine staining. The percent IFNγ^{+} cells of total CD8^{+} T cells in the tumors of CXCR3$^{-/-}$ mice was significantly reduced as compared to WT mice; but remained similar in the tumors of BLT1$^{-/-}$ mice **(Figure 16A).** In contrast, percent IFNγ^{+} cells of total CD8^{+} T cells in spleens and TdLNs of tumor bearing WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice were similar. Interestingly, CXCR3$^{-/-}$ CD8^{+} T cells showed intact TNFα production in the tumor as also in spleen and TdLN **(Figure 16B).**

Adoptively transferred WT but not BLT1$^{-/-}$ or CXCR3$^{-/-}$ CD8^{+} T cells delayed tumor growth in Rag2$^{-/-}$ mice

To further examine the importance of BLT1 and CXCR3 expression on CD8^{+} T cells for effective infiltration to tumors, adoptive transfer model involving Rag2$^{-/-}$ mice was employed. Rag2$^{-/-}$ mice have intact innate systems and hence allow us to understand the role of BLT1 and CXCR3 specifically on CD8^{+} T cells and their

function in anti-tumor immunity. $Rag2^{-/-}$ mice were challenged with 10^5 B16 cells and 2 days later were adoptively transferred with tumor educated sorted (>98% pure) WT or $BLT1^{-/-}$ $CD8^+$ T cells and the tumor growth was recorded. PBS transferred tumor bearing $Rag2^{-/-}$ mice served as controls. WT $CD8^+$ T cells significantly reduced the tumor progression in $Rag2^{-/-}$ animals. However, $BLT1^{-/-}$ $CD8^+$ T cells failed to retard the tumor growth and showed tumor growth kinetics similar to control $Rag2^{-/-}$ mice without any transferred $CD8^+$ T cells; reinforcing the crucial role of BL1 in anti-tumor immunity **(Figure 17A)**. CTL infiltration studies showed that $BLT1^{-/-}$ CTLs were significantly reduced in tumors of $Rag2^{-/-}$ mice as compared to WT CTLs. Analysis of $CD8^+$ T cells in TdLN revealed no difference in homeostatic proliferation and numbers between transferred WT and $BLT1^{-/-}$ $CD8^+$ T cells **(Figure 17B)**. This suggests a defective tumor homing ability of $BLT1^{-/-}$ CTLs.

Similar studies were carried out with WT and $CXCR3^{-/-}$ CTLs. $CXCR3^{-/-}$ CTLs also failed to retard tumor growth in $Rag2^{-/-}$ mice suggesting CXCR3 is crucial in anti-tumor immunity (**Figure 18A)**. This defective anti-tumor response was attributed to significantly reduced levels of $CXCR3^{-/-}$ CTLs in the tumors, their levels remaining similar in the TdLN suggesting no difference between the proliferation of WT and $CXCR3^{-/-}$ CTLs (**Figure 18B**). These studies demonstrate that expression of both BLT1 and CXCR3 on CTLs is necessary for their effective infiltration to tumors and subsequent anti-tumor immunity.

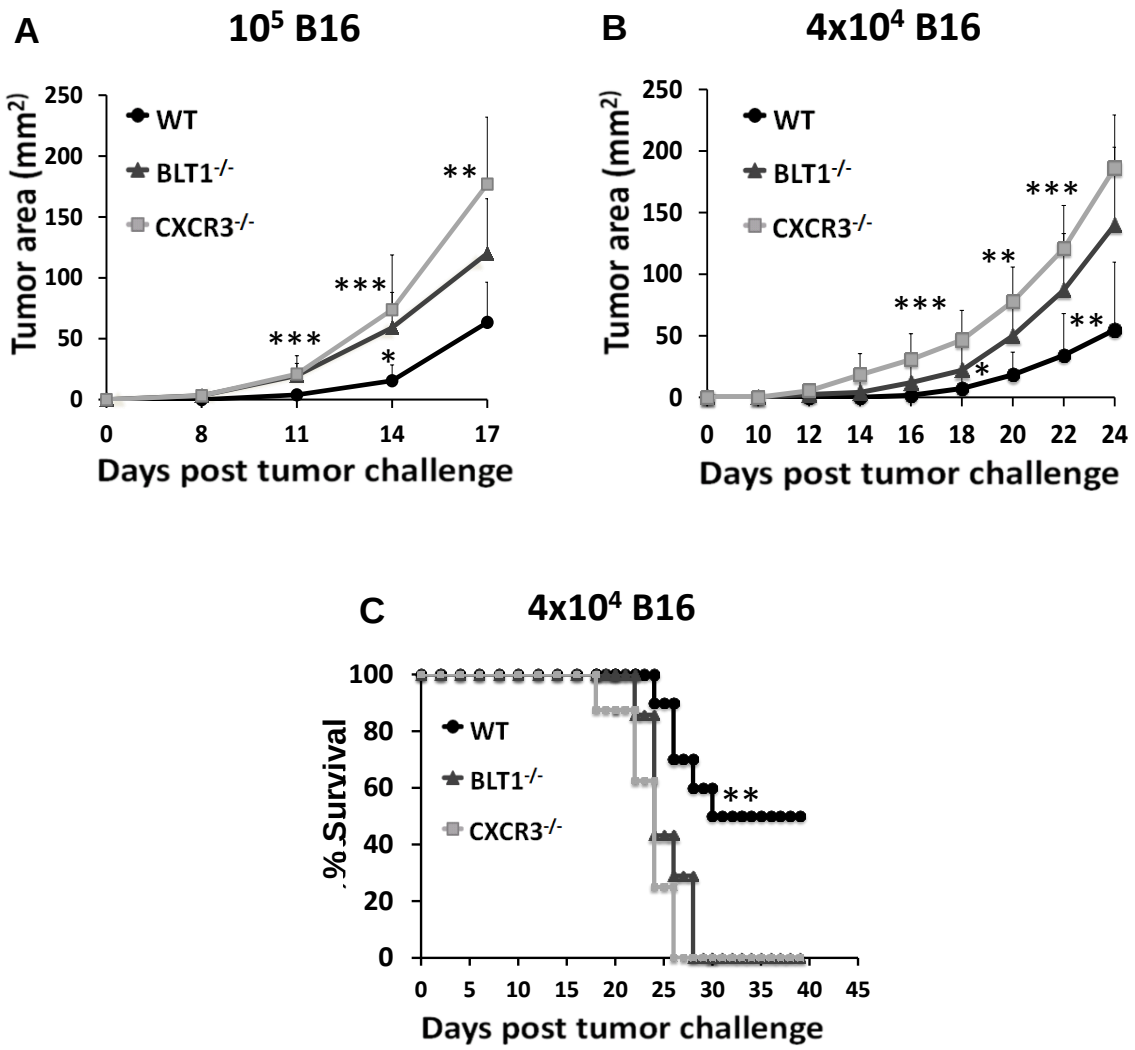

Figure 9: Enhanced tumor growth and reduced survival in BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice. A, WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice were challenged subcutaneously with 10^5 B16 cells (lethal dose). Tumor area was determined by multiplication of two perpendicular diameters (LxW). n=9 for each group. **B,** WT (n=10), BLT1$^{-/-}$ (n=7) and CXCR3$^{-/-}$ (n=8) mice were challenged subcutaneously with 4x10^4 B16 cells (sub-lethal dose) and the tumor area calculated. **C,** Survival in sub-lethal dose challenge group was monitored till day 45 post tumor

challenge. Log-rank test and Kaplan-Meier methods were used for survival analyses and student t tests were used for tumor sizes. **A,** Data is representative of three independent experiments. **B, C,** Data representative of two independent experiments.

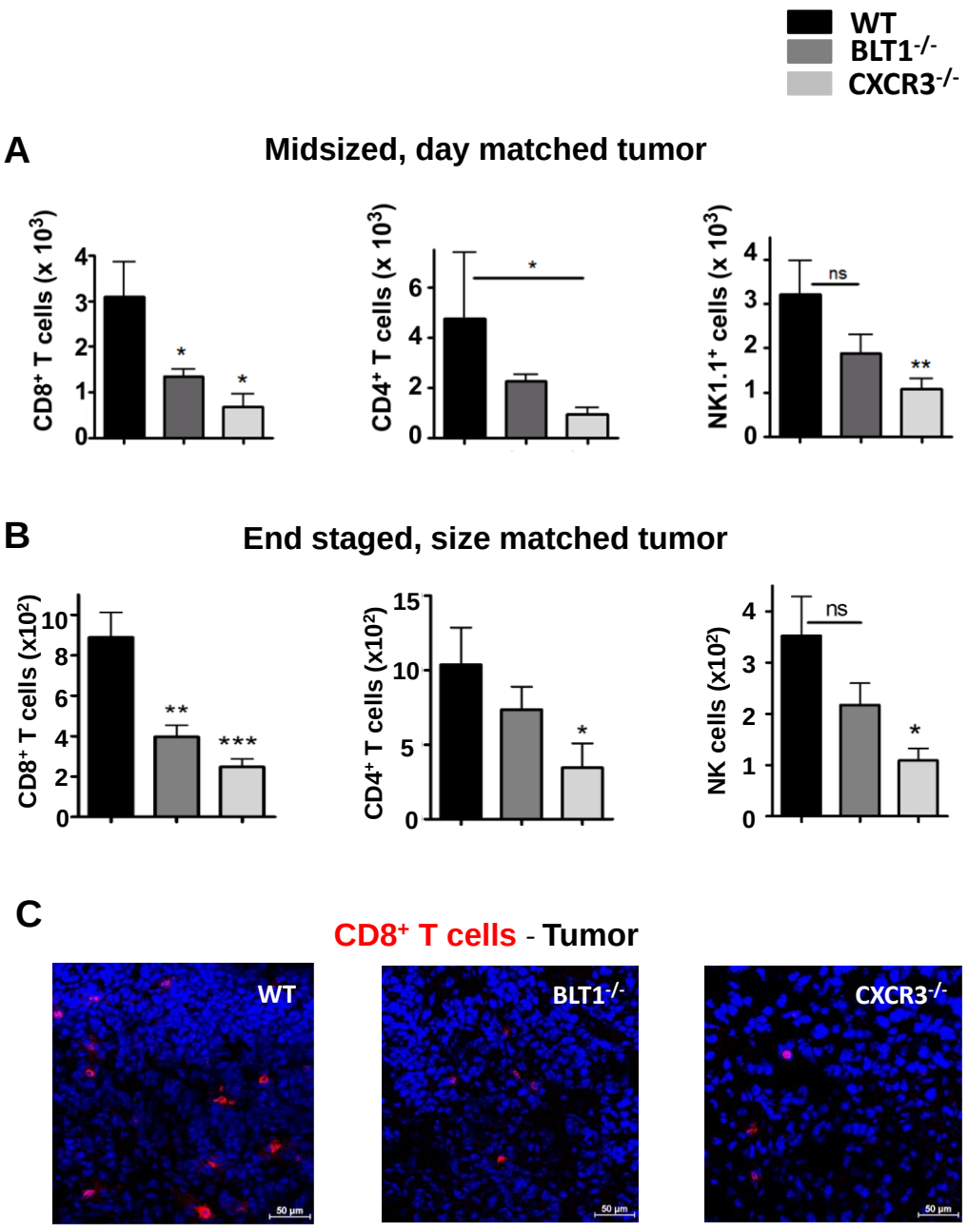

Figure 10: Reduced infiltration of CD8$^+$ T cells in BLT1$^{-/-}$ and CXCR3$^{-/-}$ tumors. A) Reduced CD8$^+$ T cells in tumors of knockout mice at mid-sized tumor, day matched. Numbers of tumor infiltrating CD8$^+$ T cells, CD4$^+$ T cells and NK1.1$^+$ cells per million total tumor cells (frequency of total) were analyzed from WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice using standard flow cytometry protocol as

described in Methods. All the mice were sacrificed and tumors harvested when the knockout tumors reached 7-9mm tumor diameter (mid-sized)**. B) Reduced $CD8^+$ T cells in size-matched end staged tumors of knockout mice.** Numbers of tumor infiltrating $CD8^+$ T cells, $CD4^+$ T cells and $NK1.1^+$ cells per million total tumor cells (frequency of total) were analyzed from WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice using standard flow cytometry protocol as described in Methods. All tumors were analyzed at large end staged (15 mm tumor diameter), size matched tumors. n=4 in each group. **C) Representative immunofluorescence staining images of $CD8^+$ T cells in WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ tumors.** Tumors harvested were frozen, sectioned and stained as described in Methods, CD8 represented in Red, DAPI in blue. The images were captured using Nikon A1R confocal microscope. The scale represents 50µM. Data representative of three independent experiments.

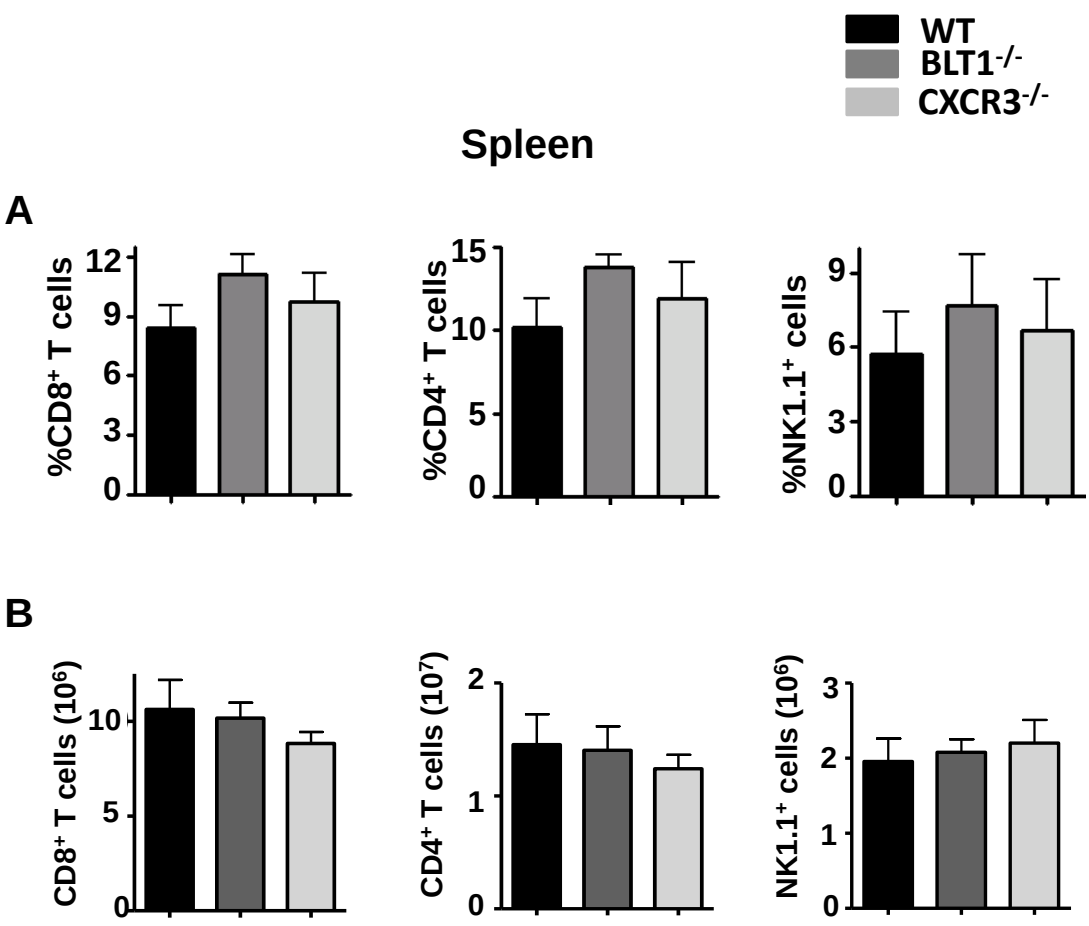

Figure 11: Similar numbers of $CD8^+$ T cells in spleens of WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ tumor bearing mice: Spleens were harvested, processed into single suspension, stained for CD3, CD8, CD4 and NK1.1 markers and analyzed via flow cytometry as mentioned in the methods. $CD8^+$ T cells, $CD4^+$ T cells, $NK1.1^+$ cells in spleen of size-matched, end staged (14-15mm tumor diameter) tumor bearing WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice. Cells represented as percent of total $CD45^+$ cells (A) as well as absolute numbers represented as frequency of total splenocytes (B). n=4 in each group. Data is representative of atleast three independent experiments.

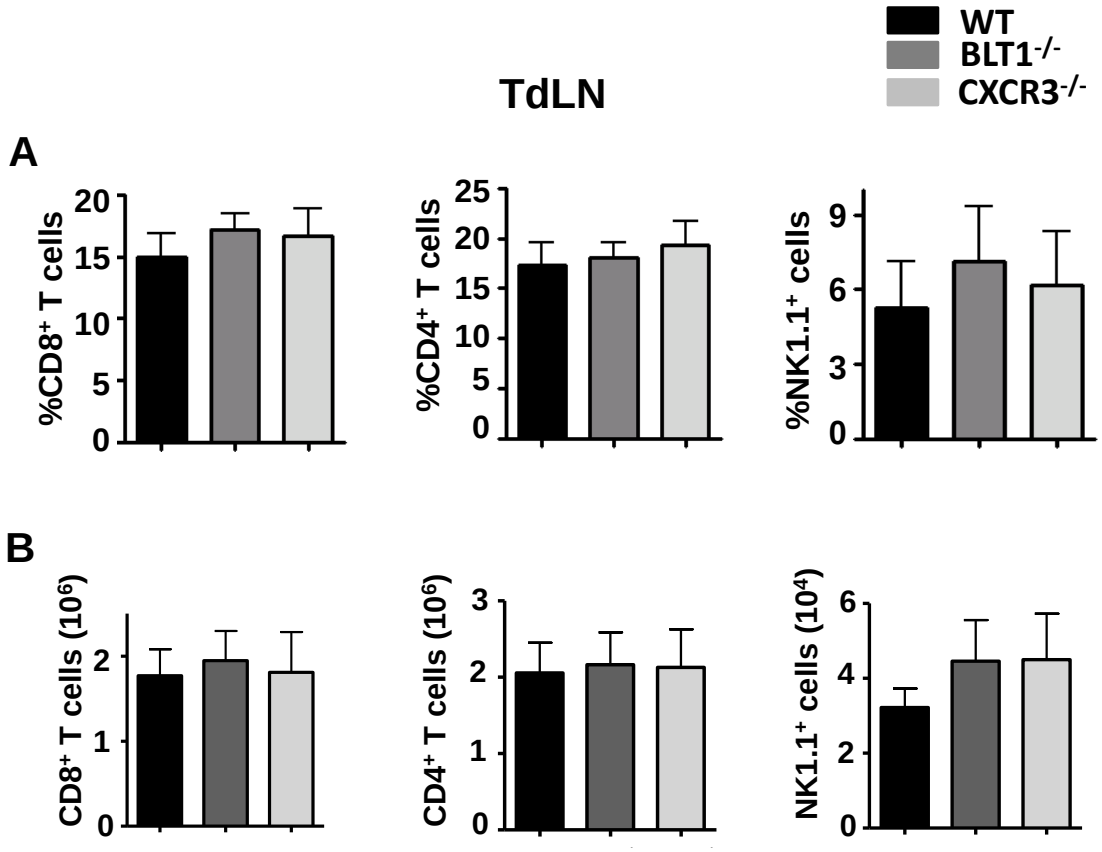

Figure 12: Similar numbers of $CD8^+$ T cells in TdLNs of WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ tumor bearing mice: Tumor draining lymph nodes (TdLN- inguinal, brachial and axillary) were harvested, processed into single suspension, stained for CD3, CD8, CD4 and NK1.1 markers and analyzed via flow cytometry as mentioned in the methods. $CD8^+$ T cells, $CD4^+$ T cells, $NK1.1^+$ cells in TdLN of size-matched, end staged (14-15mm tumor diameter) tumor bearing WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice. Cells represented as percent of total $CD45^+$ cells **(A)** as well as absolute numbers represented as frequency of total lymph node cells **(B)**. n=4 in each group. Data is representative of atleast three independent experiments.

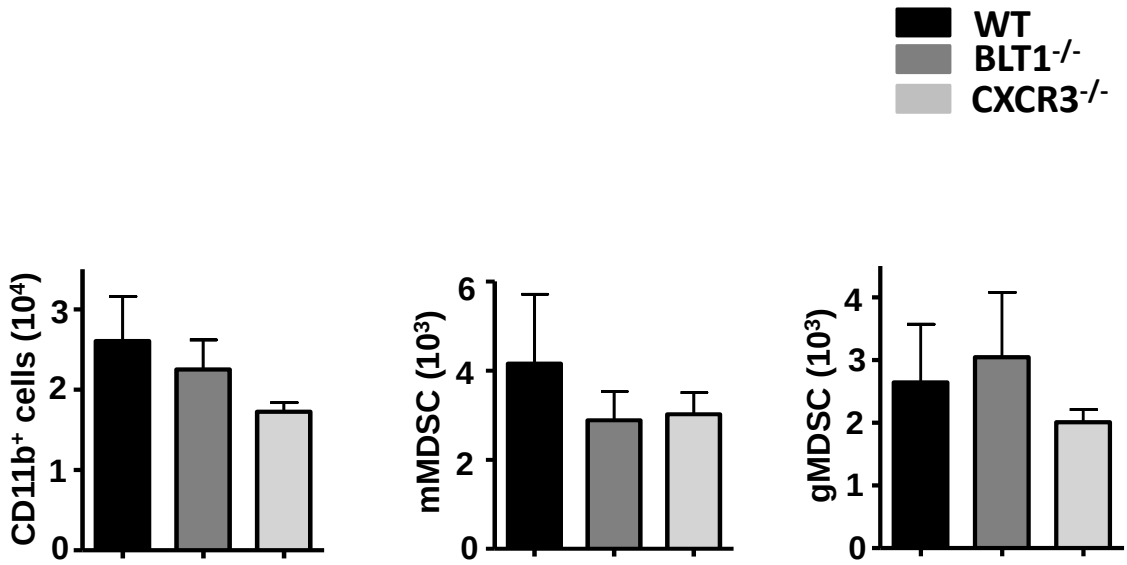

Figure 13: Similar numbers of of myeloid cell subsets in tumors of WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$. Numbers of total CD11b^{+}, mMDSC (CD11b^{+}Ly6C^{hi}) and gMDSC (CD11b^{+}Ly6G^{hi}) subsets per million total tumor cells (frequency of total) were analyzed from size matched, end staged (14-15 mm tumor diameter) WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice using standard flow cytometry protocol as described in Methods. n=4 in each group. Data is representative of at least three independent experiments.

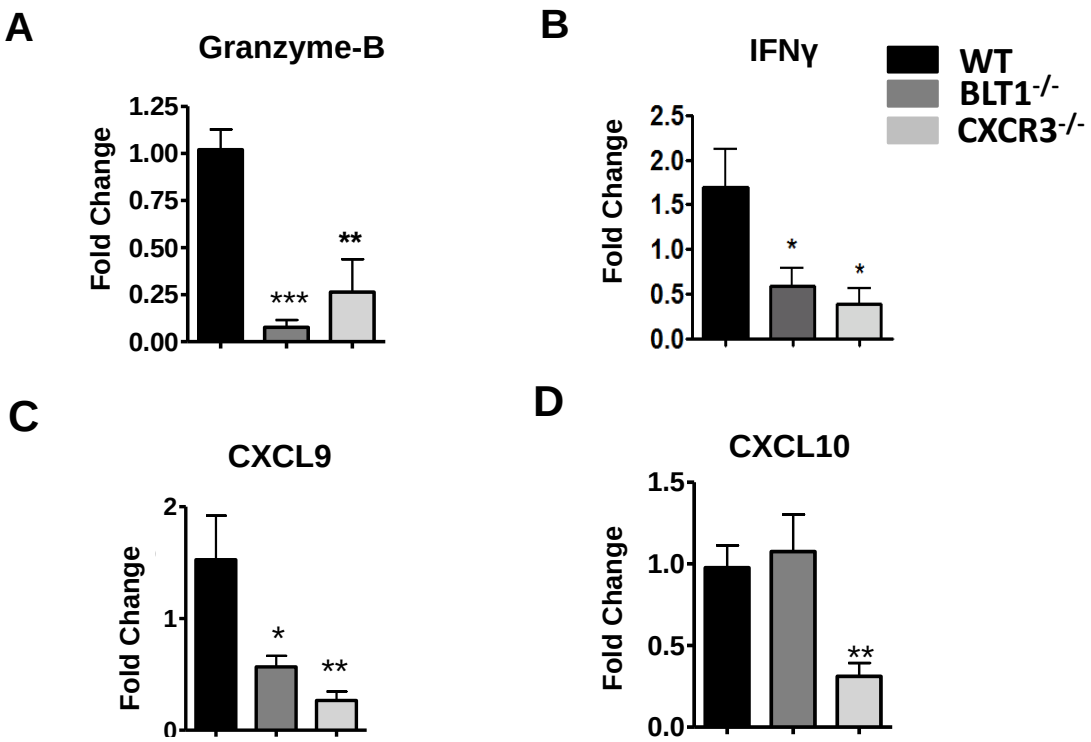

Figure 14: Effector responses in tumors of WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice. Quantitative real time PCR of effector molecules and interferon gamma regulated genes. The levels of Granzyme-B **(A)**, IFNγ **(B),** CXCL9 **(C)** and CXCL10 **(D)** mRNA expression in tumors from WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice were determined by RT-PCR as described in methods. Expression of the target genes was normalized to GAPDH. Data displayed as fold change relative to the WT sample. n=4 in each group.

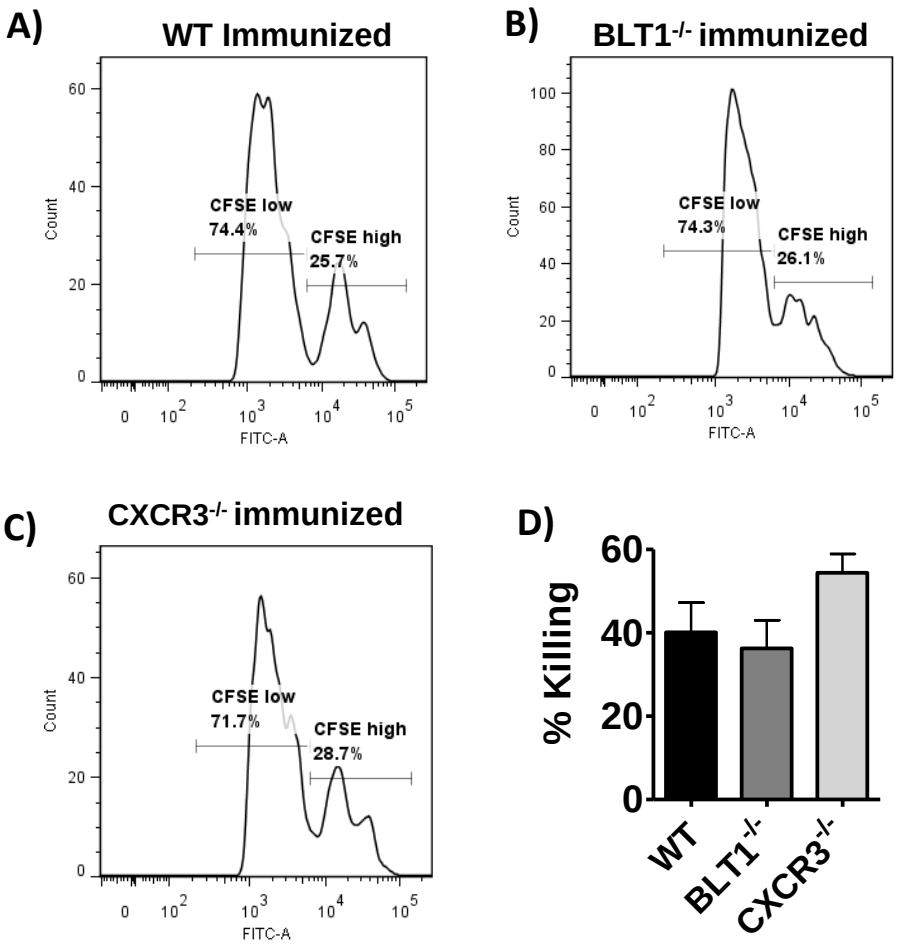

Figure 15: BLT1$^{-/-}$ and CXCR3$^{-/-}$ CD8^{+} T cells display normal cytotoxic function in the spleen. The CD8^{+} T cell based In vivo killing assay was performed as described in the Methods. WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ recipient mice were immunized s.c. with 50 µg/mice of Trp-2 peptide and an adjuvant i.e. 100 µg/mice anti-OX-40 agonistic Ab (Clone OX-86, BioXcell). Naïve WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice were used as controls. Representative histograms of CFSE-labeled targets viz. CFSE hi and CFSE low determining the killing in **A)** Immunized WT mice, **B)** Immunized BLT1$^{-/-}$ mice and **C)** Immunized CXCR3$^{-/-}$ mice. **D)** Cumulative levels of percent killing activity by respective CTLs are shown as a bar graph. n=4 in each group.

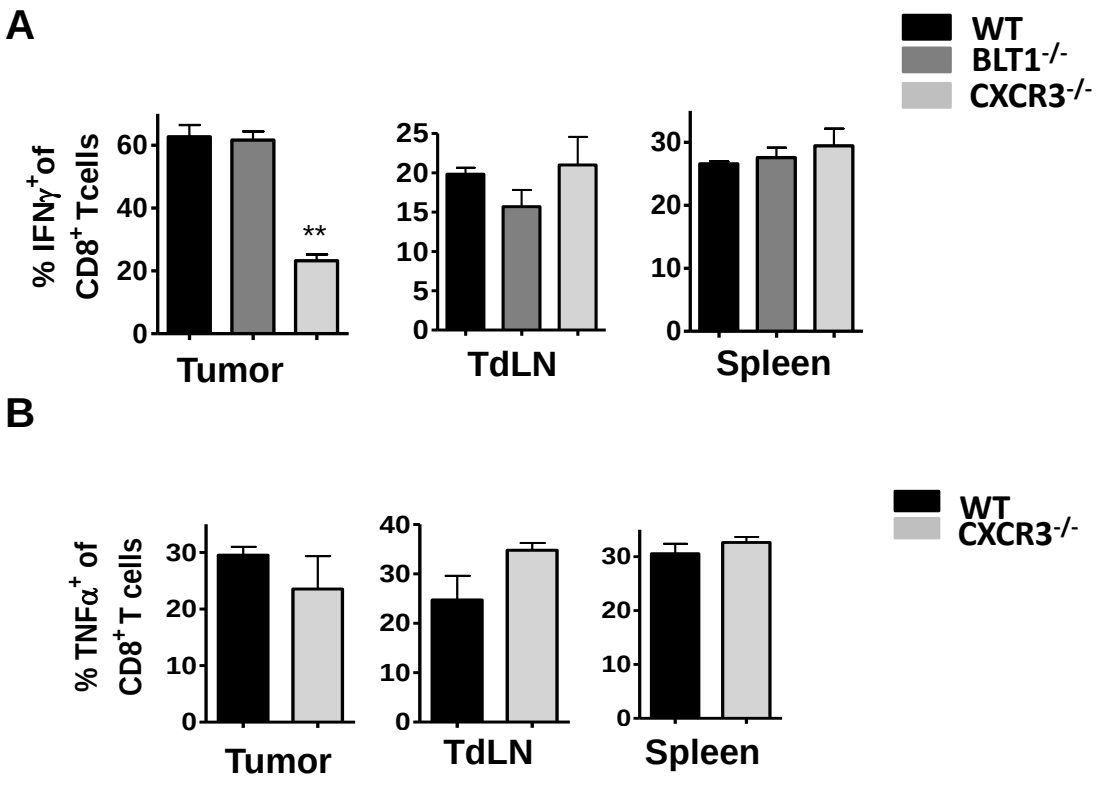

Figure 16: Effector functions of WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ CD8^{+} T cells in periphery versus tumor. A) Intracellular IFNγ staining was performed in WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ total tumor cell suspension by ex-vivo stimulation in the presence of PMA and ionomycin as mentioned in the methods. Percent IFNγ^{+} cells of total CD8^{+} T cells were analyzed in tumors (mid-sized tumors). CXCR3$^{-/-}$ CD8^{+} T cells in periphery have intact IFNγ secretion. Percent IFNγ^{+} cells of total CD8^{+} T cells were analyzed in TdLNs and spleens of tumor bearing WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ mice (mid-sized tumor). Data is representative of two independent experiments with n=4 animals in each group. **B)** Intracellular TNFα staining was performed in WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ total tumor cell suspension by ex-vivo stimulation in the presence of PMA and ionomycin as mentioned in the methods. Percent TNFα^{+} cells of total CD8^{+} T cells were analyzed in tumors, TdLNs and

spleens of tumor bearing WT and CXCR3$^{-/-}$ mice (mid-sized tumor). Data is representative of two independent experiments with n=4 animals in each group.

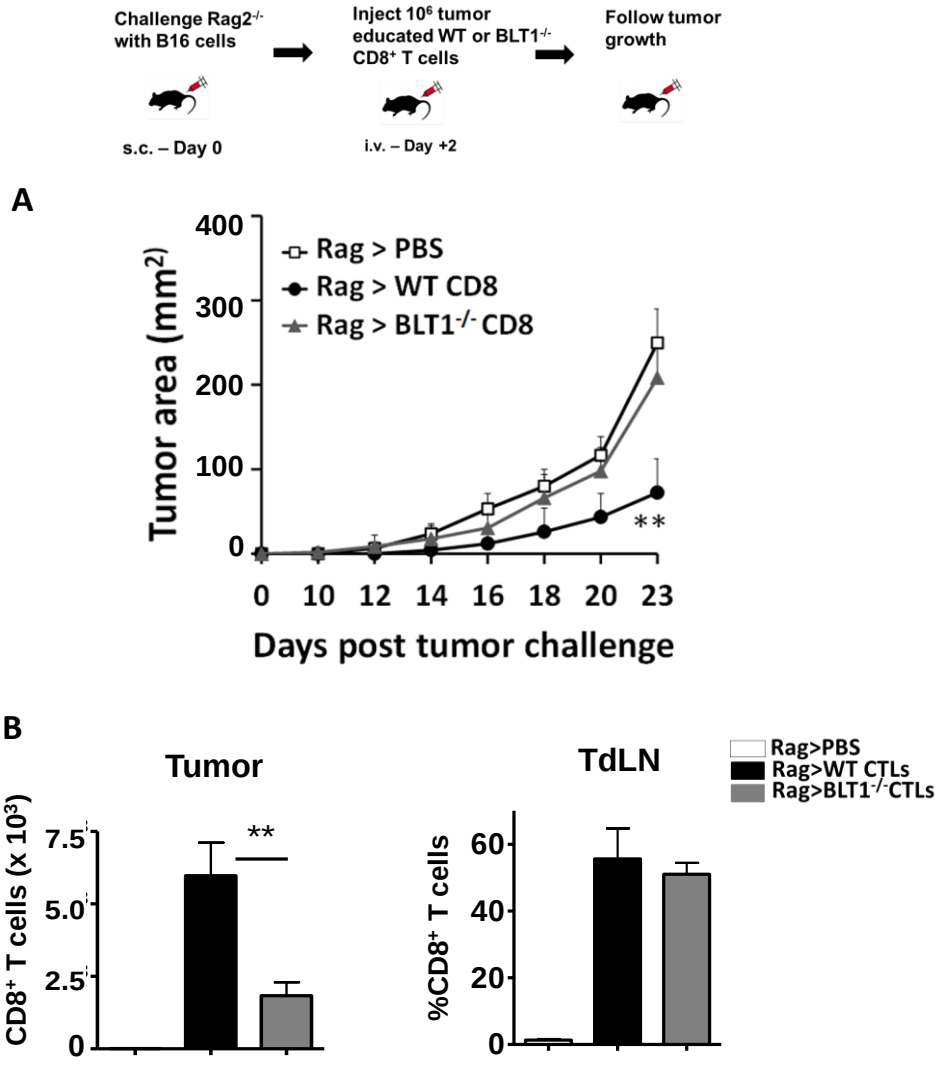

Figure 17: Adoptive transfer of WT but not BLT1$^{-/-}$ tumor experienced CD8^{+} T cells retarded tumor growth in Rag2$^{-/-}$ mice. Rag2$^{-/-}$ mice were challenged with 10^5 B16 cells. Two days later CD8^{+} T cells were isolated from the spleen and TdLN of B16 tumor bearing (3-5mm) WT or BLT1$^{-/-}$ mice by MACS technique and 1 million isolated CD8^{+} T cells (>98% purity) or PBS were injected i.v. in tumor inoculated Rag2$^{-/-}$ mice. **A,** Tumor growth kinetics for Rag2$^{-/-}$ mice transferred with either PBS (n=5), WT CD8^{+} T cells (n=5) or BLT1$^{-/-}$ CD8^{+} T cells

(n=5). **B**, Numbers of $CD8^+$ T cells (frequency of total) per million total tumor cells and percent $CD8^+$ T cells of total $CD45^+$ cells in TdLN for WT and $BLT1^{-/-}$ transferred $CD8^+$ T cells are shown as cumulative bar graphs. Data is representative of two independent experiments.

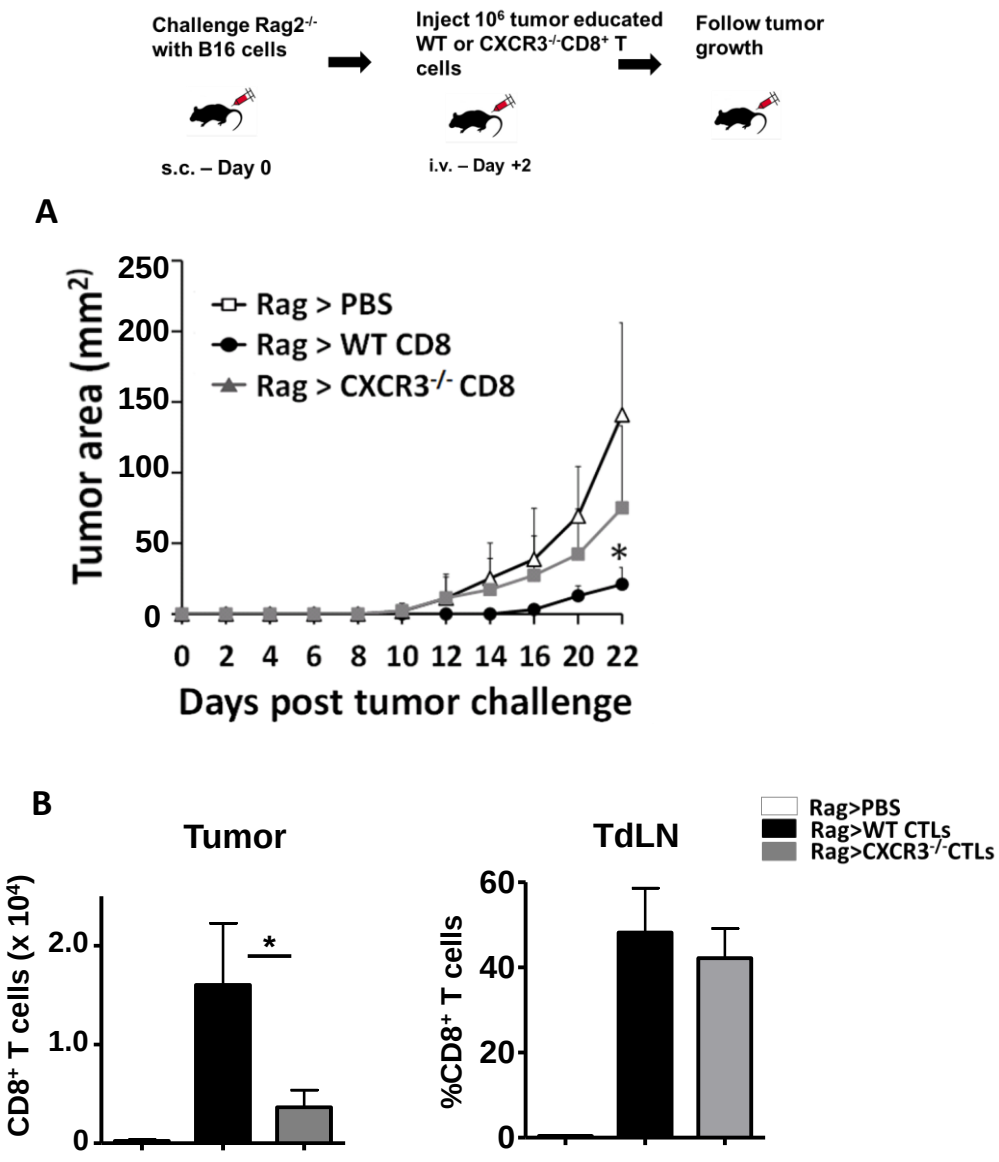

Figure 18: Adoptive transfer of WT but not CXCR3$^{-/-}$ tumor experienced CD8^{+} T cells retarded tumor growth in Rag2$^{-/-}$ mice. Rag2$^{-/-}$ mice were challenged with 10^5 B16 cells. Two days later CD8^{+} T cells were isolated from the spleen and TdLN of B16 tumor bearing (3-5mm) WT or CXCR3$^{-/-}$ mice by MACS technique and 1 million isolated CD8^{+} T cells (>98% purity) or PBS were injected i.v. in tumor inoculated Rag2$^{-/-}$ mice. **A**, Tumor growth kinetics for Rag2$^{-/-}$ mice transferred with either PBS (n=5), WT CD8^{+} T cells (n=5) or CXCR3$^{-/-}$ CD8^{+} T

cells (n=5). **B**, Numbers of $CD8^+$ T cells (frequency of total) per million total tumor cells and percent $CD8^+$ T cells of total $CD45^+$ cells in TdLN for WT and $CXCR3^{-/-}$ transferred $CD8^+$ T cells are shown as cumulative bar graphs. Data is representative of two independent experiments.

CHAPTER V

LACK OF BLT1 AND CXCR3 MEDIATED SYNERGISM TO FACILITATE CTL MIGRATION TO TUMORS AND ANTI-TUMOR IMMUNITY

INTRODUCTION

The data presented in the previous chapter indicated the individual importance of BLT1 and CXCR3 in regulating CTL migration to tumors and thus anti-tumor immunity. The data indicated that both BLT1 and CXCR3 deficient mice had similar significantly enhanced tumor growth kinetics, compared to WT mice. In this chapter we sought to investigate whether BLT1 and CXCR3 receptor mediated T cell homing acts in concert or is synergistic in function. For this purpose, $BLT1^{-/-}CXCR3^{-/-}$ double deficient mouse was generated by crossing $BLT1^{-/-}$ mouse to $CXCR3^{-/-}$ mouse.

Tumor growth in double knockout mice was compared to either of the single knockout to infer whether synergism exist with respect to regulation of anti-tumor immunity as well as $CD8^+$ T cell migration to tumors. **We hypothesize that BLT1 and CXCR3 mediated regulation of $CD8^+$ T cell migration to tumors and anti-tumor immunity acts in concert.** We expect to see a further enhancement of tumor growth in the absence of both BLT1 and CXCR3 receptors.

Also, the interdependence of BLT1 and CXCR3 mediated regulation of CTL migration to tumors was examined by employing co-transfer strategies of WT $CD8^+$ T cells with either of the knockout cells ($BLT1^{-/-}$, $CXCR3^{-/-}$ or $BLT1^{-/-}$ $CXCR3^{-/-}$ $CD8^+$ T cells) in equal proportion in the adoptive transfer model employed in previous experiments. WT CTLs facilitating either of $BLT1^{-/-}$ or $CXCR3^{-/-}$ $CD8^+$T cell infiltration but not $BLT1^{-/-}CXCR3^{-/-}$ $CD8^+$ T cells to tumors would mean a possible interdependence between BLT1 and CXCR3 pathways. **We hypothesize that WT $CD8^+$ T cells could facilitate additional BLT1 deficient $CD8^+$ T cells via CXCR3 receptor.** This hypothesis stems from previously published study where BLT1 mediated infiltration of WT neutrophils in $BLT1^{-/-}$ mice facilitated infiltration of endogenous $BLT1^{-/-}$ neutrophils to the inflamed joint suggesting that BLT1 expression on neutrophils is essential only for the initial recruitment and other chemokines could then perpetuate the disease progression [180]. We expect that the initial infiltration of WT CTLs to tumors would enable IFNγ > CXCL9/10 > CXCR3 mediated T cell homing loop, to enhance the infiltration of additional $BLT1^{-/-}$ CTLs via CXCR3 receptor.

These experiments would provide us the plausible combinatorial regulatory mechanisms for BLT1 and CXCR3 mediated T cell homing to tumors as well as anti-tumor immunity.

RESULTS

Generation of BLT1$^{-/-}$CXCR3$^{-/-}$ mice

BLT1 receptor gene, Ltb4R, is localized to chromosome 14 in mice as well as in humans (autosomal) [179]. On the other hand, CXCR3 gene Cxcr3 is located on the sex-chromosome and is an X-linked gene both in mice and humans [133]. Hence, males are hemizygous for CXCR3 gene and females are homozygous. BLT1$^{-/-}$ female mice were bred with CXCR3$^{-/-}$ male mice to generate BLT1-CXCR3 heterozygous female mice. BLT1-CXCR3 heterozygous females were then bred with BLT1$^{-/-}$ males to generate BLT1$^{-/-}$CXCR3$^{+/-}$ females and BLT1$^{-/-}$ CXCR3$^{-/-}$ males. The BLT1$^{-/-}$CXCR3$^{+/-}$ females were then bred with BLT1$^{-/-}$ CXCR3$^{-/-}$ males to generate BLT1$^{-/-}$CXCR3$^{-/-}$ female mice. The BLT1$^{-/-}$CXCR3$^{-/-}$ male and female mice were then bred with each other for several rounds of breeding to generate BLT1$^{-/-}$CXCR3$^{-/-}$ male and female breeders. The representative breeding scheme is shown in **Table 1.** The BLT1$^{-/-}$CXCR3$^{-/-}$ mice were born at the expected Mendelian ratios and displayed normal developmental and morphological features.

Accelerated tumor growth and reduced survival in BLT1$^{-/-}$CXCR3$^{-/-}$ mice

To examine the interdependence of BLT1 and CXCR3 receptor mediated regulation in anti-tumor immunity, BLT1$^{-/-}$CXCR3$^{-/-}$ double knockout mouse (DKO) was generated as discussed. At the sub-lethal dose of B16 cells ($4x10^4$), BLT1$^{-/-}$CXCR3$^{-/-}$ mice showed significantly enhanced tumor growth as well as

significantly reduced survival as compared to WT mice (**Figure 19 A and B**). There was 100% mortality of BLT1$^{-/-}$CXCR3$^{-/-}$ mice by day 22, while 50% of WT mice still survived at day 35-post tumor challenge **(Figure 19B)**.

To compare the tumor growth kinetics in double knockout mice versus either of the single knockout mice; WT, BLT1$^{-/-}$, CXCR3$^{-/-}$ and BLT1$^{-/-}$CXCR3$^{-/-}$ mice were challenged with 10^5 B16 cells followed by tumor growth kinetic analysis. The BLT1$^{-/-}$CXCR3$^{-/-}$ mice displayed significantly enhanced tumor growth kinetics as compared to WT mice but similar growth kinetics as compared to BLT1$^{-/-}$ or CXCR3$^{-/-}$ mice at lethal tumor dose **(Figure 20A)**. No significant difference was observed in the survival **(Figure 20B)** or the tumor growth (data not shown) between either of the single knockout mice and double knockout mice even at the sub-lethal dose. Therefore, these results suggest that BLT1 and CXCR3 mediated regulation of anti-tumor immunity may be co-dependent but not additive or synergistic.

WT CTLs do not facilitate knockout CTL infiltration to tumors

The migration patterns of the WT and knockout CD8$^+$ T cells co-transferred into Rag2$^{-/-}$ mice were determined to explore whether WT CTLs can facilitate the trafficking of knockout CTLs into tumors. Equal proportions of tumor experienced WT CD8$^+$ T cells and BLT1$^{-/-}$, CXCR3$^{-/-}$ or BLT1$^{-/-}$CXCR3$^{-/-}$ (DKO) CD8$^+$ T cells were introduced intravenously in tumor bearing Rag2$^{-/-}$ mice. UBC-GFP mice were used as WT mice to differentiate between WT (bright green fluorescence) and the knockout CD8$^+$ T cells **(Figure 21)**. CD8$^+$ T cells were stained in tumor,

blood, spleen and TdLN once the tumor reached 7-9 mm diameter and the ratio of WT to knockout $CD8^+$ T cells was assessed. The ratios of WT to knockout CTLs in TdLN were similar across all the groups and close to 1-1.5 **(Figure 22)**. WT $CD8^+$ T cells infiltrated into tumors around 3 fold more as compared to $BLT1^{-/-}$ $CD8^+$ T cells, around 8 fold more as compared to $CXCR3^{-/-}$ $CD8^+$ T cells and around 5.5 fold more as compared to $BLT1^{-/-}CXCR3^{-/-}$ $CD8^+$ T cells (**Figure 23**). In contrast, the numbers of $BLT1^{-/-}$, $CXCR3^{-/-}$, and $BLT1^{-/-}CXCR3^{-/-}$ $CD8^+$ T cells were 1.5 to 3 fold higher than the WT cells in the blood and spleen **(Figure 24)**. These studies suggest that WT cells do not facilitate additional knockout CTL infiltration to tumors.

BREEDING SCHEME

Cross	Parent (Male)	Parent (Female)	Selected Progeny
1.	$CXCR3^{-/-}$	$BLT1^{-/-}$	$BLT1^{+/-}$ $CXCR3^{+/-}$ Female
2.	$BLT1^{-/-}$	$BLT1^{+/-}$ $CXCR3^{+/-}$	a) $BLT1^{-/-}CXCR3^{-/-}$ Male b) $BLT1^{-/-}$ $CXCR3^{+/-}$ Female
3.	$BLT1^{-/-}CXCR3^{-/-}$	$BLT1^{-/-}$ $CXCR3^{+/-}$	a) $BLT1^{-/-}CXCR3^{-/-}$ Male b) $BLT1^{-/-}CXCR3^{-/-}$ Female
4.	$BLT1^{-/-}CXCR3^{-/-}$	$BLT1^{-/-}CXCR3^{-/-}$	$BLT1^{-/-}CXCR3^{-/-}$ male and female pups.

Table 1: Breeding scheme for generation of $BLT1^{-/-}CXCR3^{-/-}$ mice: Breeding scheme to generate $BLT1^{-/-}CXCR3^{-/-}$ double deficient mice. $BLT1^{-/-}$ females were bred with $CXCR3^{-/-}$ males to generate BLT1-CXCR3 heterozygous females. BLT1-CXCR3 heterozygous females were then bred with $BLT1^{-/-}$ males to generate $BLT1^{-/-}CXCR3^{+/-}$ females and $BLT1^{-/-}CXCR3^{-/-}$ males. The $BLT1^{-/-}CXCR3^{+/-}$ females were then bred with $BLT1^{-/-}CXCR3^{-/-}$ males to generate $BLT1^{-/-}CXCR3^{-/-}$ females. The $BLT1^{-/-}CXCR3^{-/-}$ males and females were then bred with each other for several rounds of breeding to generate $BLT1^{-/-}CXCR3^{-/-}$ male and female breeders. The representative breeding scheme is shown.

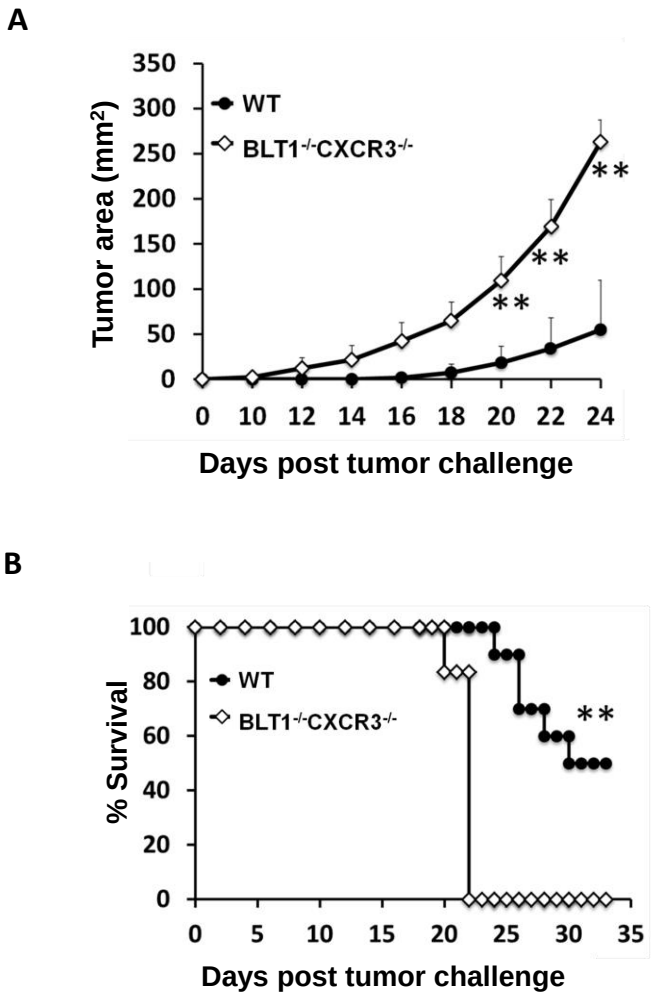

Figure 19: Defective immune surveillance and anti-tumor immunity in BLT1$^{-/-}$CXCR3$^{-/-}$ double knockout (DKO) mice. A, WT (n=10) and BLT1$^{-/-}$CXCR3$^{-/-}$ (n=6) mice were challenged subcutaneously with 4x10^4 B16 cells (sub-lethal dose) and the tumor area calculated. Tumor area was measured by multiplication of two perpendicular diameters (LxW). **B,** Survival in mice with sub-lethal dose of tumor challenge was monitored till day 35 post tumor challenge. Log-rank test and Kaplan-Meier methods were used for survival analyses and student t tests

were used for tumor sizes. Data is representative of two independent experiments.

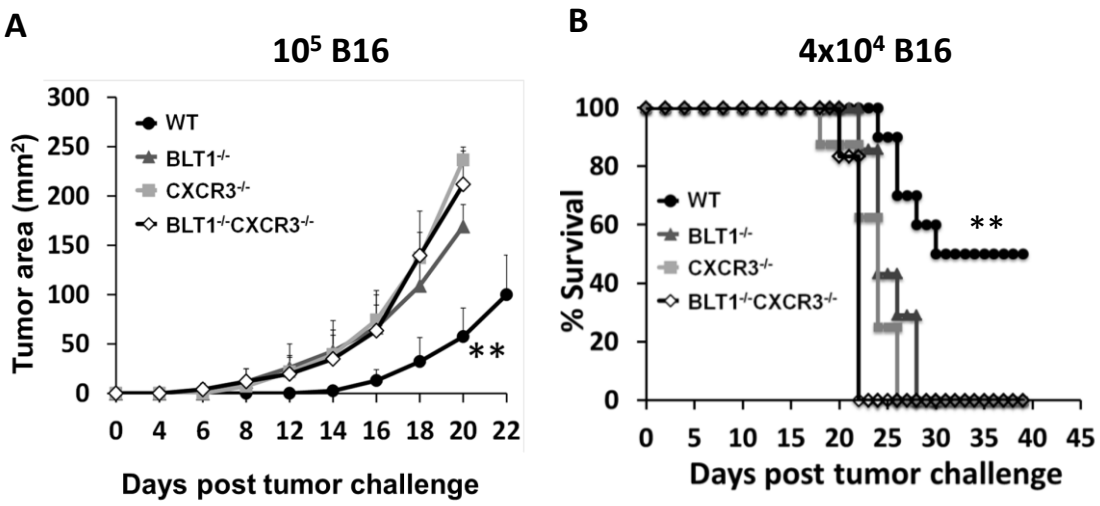

Figure 20: Lack of synergism in BLT1 and CXCR3 mediated regulation in anti-tumor immunity. A) WT (n=4), BLT1$^{-/-}$ (n=5), CXCR3$^{-/-}$ (n=4) and BLT1$^{-/-}$CXCR3$^{-/-}$ (n=6) mice were challenged subcutaneously with 10^5 B16 cells (lethal dose). Tumor area was measured by multiplication of two perpendicular diameters (LxW). **B)** WT (n=10), BLT1$^{-/-}$ (n=7), CXCR3$^{-/-}$ (n=7) and BLT1$^{-/-}$CXCR3$^{-/-}$ (n=6) mice were challenged subcutaneously with $4x10^4$ B16 cells (sub-lethal dose). Survival in mice with sub-lethal dose of tumor challenge was monitored till day 40 post tumor challenge Data is representative of two independent experiments. Log-rank test and Kaplan-Meier methods were used for survival analyses and student t tests were used for tumor sizes.

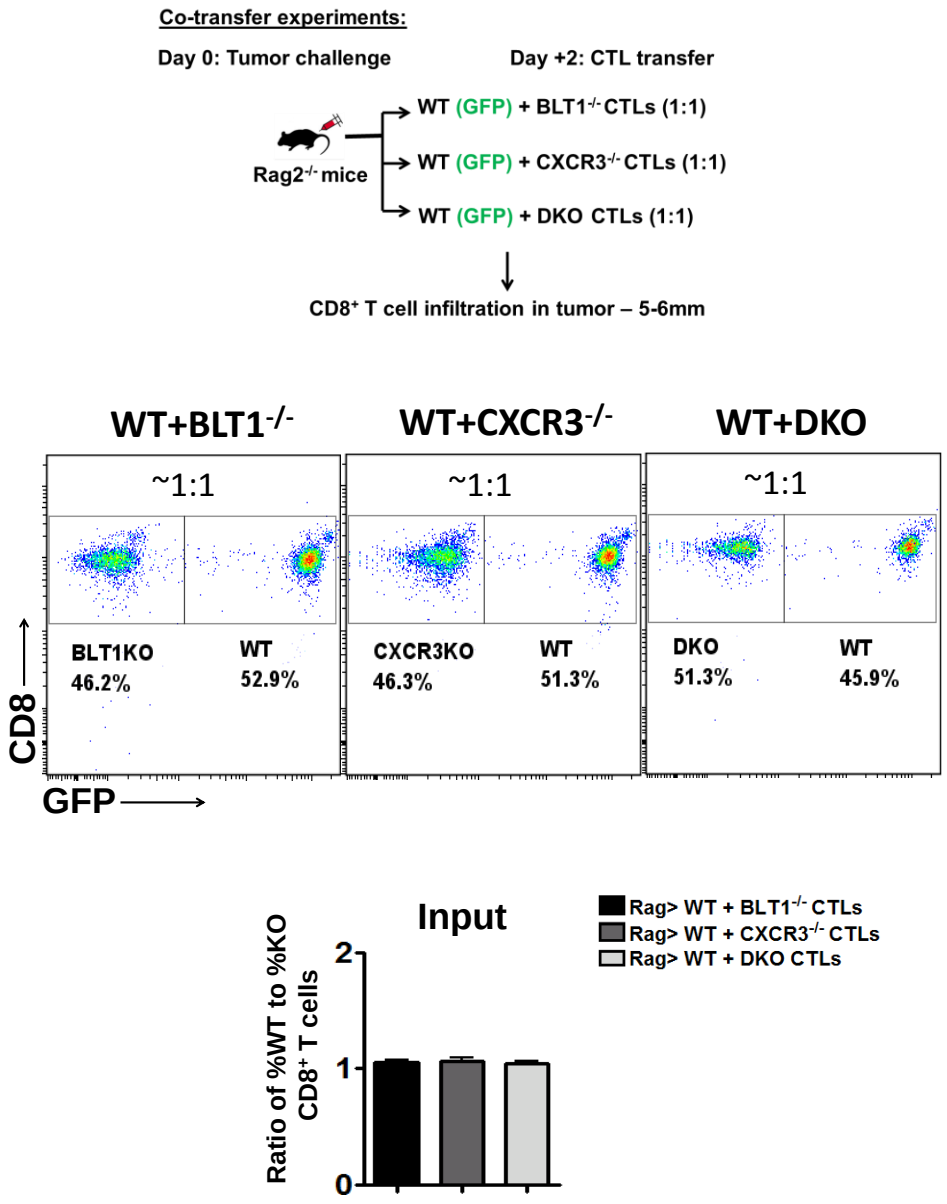

Figure 21: Equal proportions of WT (GFP) and knockout CD8$^+$ T cells adoptively transferred in tumor bearing Rag2$^{-/-}$ mice. Co-transfer experiments were performed as mentioned in methods. Briefly, CD8$^+$ T cells were isolated from tumor bearing (3-5 mm) WT (GFP), BLT1$^{-/-}$ (non-GFP), CXCR3$^{-/-}$ (non-GFP) mice and BLT1$^{-/-}$CXCR3$^{-/-}$ (non-GFP) mice. WT and KO CD8$^+$ T cells were equally mixed and 1 million cells were injected i.v. in tumor bearing Rag2$^{-/-}$ mice.

Representative dot plots of GFP^+ (WT) and GFP^- (KO) CTLs transferred in tumor inoculated $Rag2^{-/-}$ mice and corresponding cumulative bar graph of ratio of %WT to %KO $CD8^+$ T cells (gated on $CD3^+CD8^+$ cells) injected which is equal to 1. Bar in black represents WT + $BLT1^{-/-}$ CTL mix; grey bars represent WT + $CXCR3^{-/-}$ CTL mix and light grey bar represents WT + DKO CTL mix. Data is representative of two independent experiments.

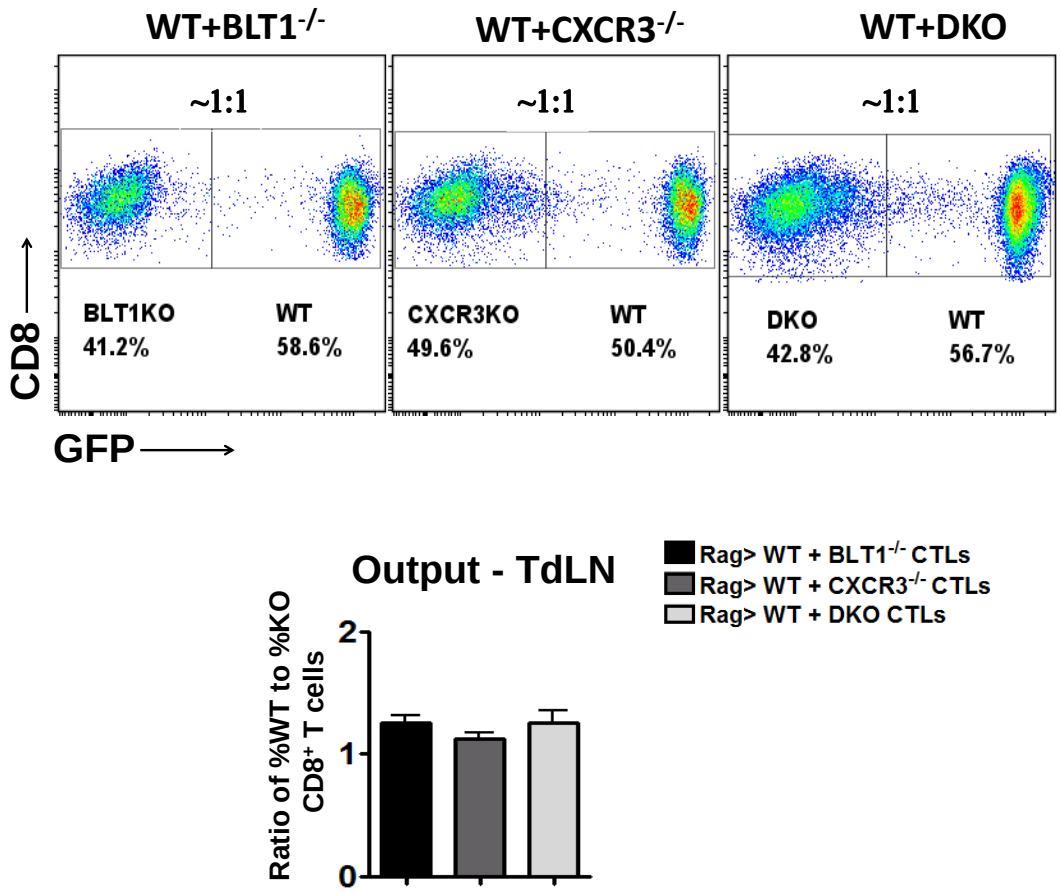

Figure 22: Equal proportions of WT and knockout CD8$^+$ T cells in the TdLN of Rag2$^{-/-}$ mice. Co-transfer experiments were performed as mentioned in methods. Briefly, CD8$^+$ T cells were isolated from tumor bearing (3-5mm) WT(GFP), BLT1$^{-/-}$ (non GFP), CXCR3$^{-/-}$ (non-GFP) mice and BLT1$^{-/-}$CXCR3$^{-/-}$ (non-GFP) mice. WT and KO CD8$^+$ T cells were equally mixed and 1 million cells were injected i.v. in tumor bearing Rag2$^{-/-}$ mice. Animals were sacrificed when the tumors reached 7-8mm tumor diameter. %WT and KO CD8$^+$ T cells were determined upon gating on live CD3$^+$CD8$^+$ T cells and looking for GFP$^+$ and GFP$^-$ populations respectively. Representative dot plots of %WT and %KO CTLs

obtained from TdLNs of Rag2$^{-/-}$ mice when the tumor reaches 7-8mm tumor diameter. Cumulative bar graph demonstrating the ratio of %WT to %KO CTLs in TdLN is also shown. Data is representative of two independent experiments for each transferred combination with n=5 in each experimental group.

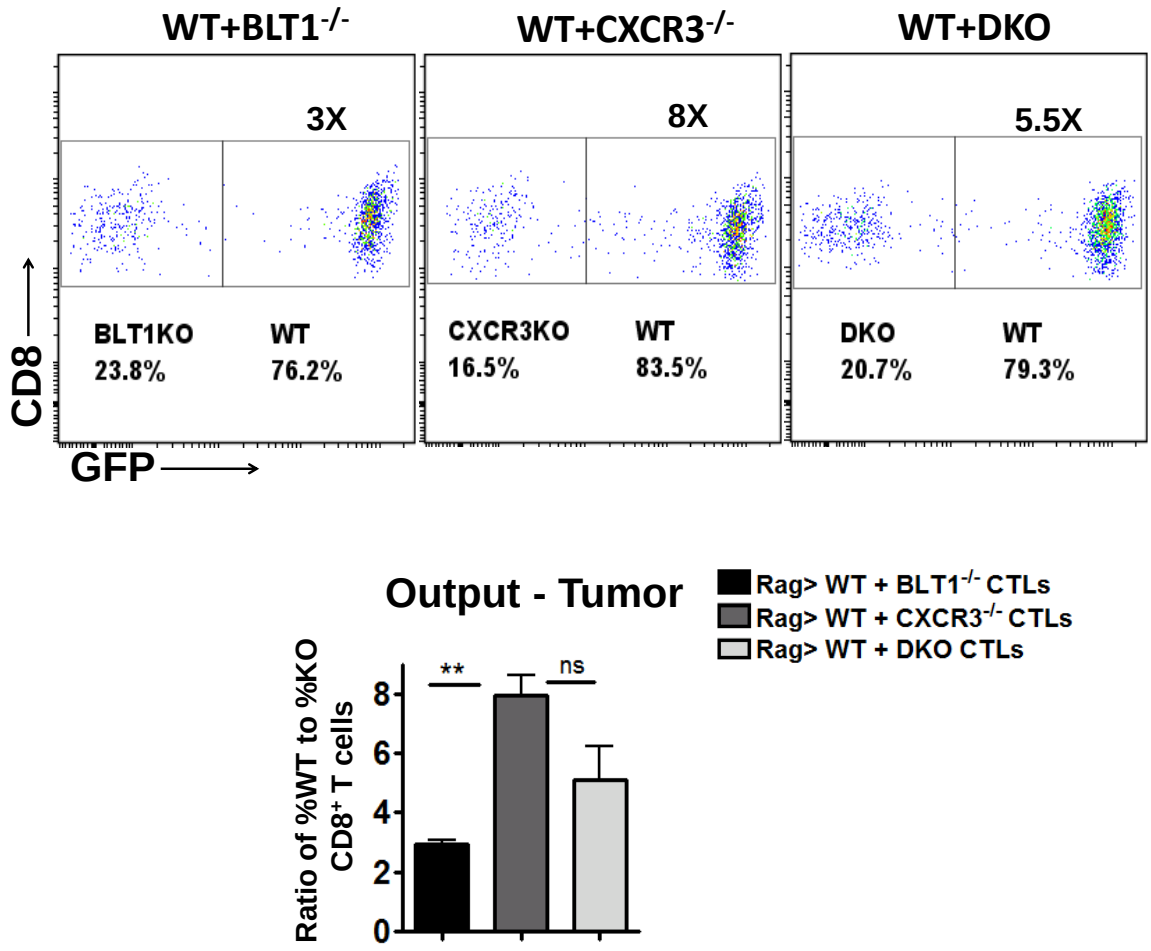

Figure 23: WT CD8$^+$ T cells do not facilitate knockout CD8$^+$ T cell infiltration to tumors. Co-transfer experiments were performed as mentioned in methods. **A)** Representative dot plots of %WT and %KO CTLs obtained from tumor of Rag2$^{-/-}$ mice when the tumor reaches 7-8mm tumor diameter. The %GFP+ and %GFP- is shown after gating on CD8$^+$ T cells. **B)** Cumulative bar graph demonstrating the ratio of %WT to %KO CTLs in TdLN is also shown. **C)** Cumulative bar graph demonstrating total live CD8$^+$ T cells in tumors of Rag2$^{-/-}$ mice adoptively transferred with equal proportions of WT and knockout CD8$^+$ T cells. Bar in black represents WT + BLT1$^{-/-}$ CTL mix; grey bars represent WT +

CXCR3$^{-/-}$ CTL mix and light grey bar represents WT + DKO CTL mix. Data is representative of two independent experiments for each transferred combination with n=5 in each experimental group.

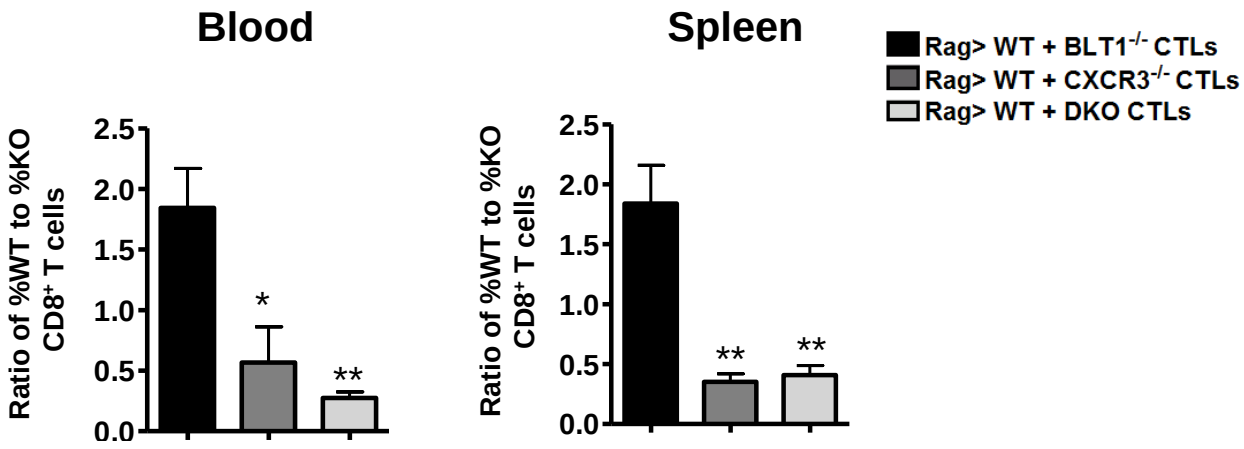

Figure 24: **CXCR3$^{-/-}$ and DKO CD8^{+} T cells are significantly more as compared to WT cells in blood and spleen of tumor bearing Rag2$^{-/-}$ mice.** Co-transfer experiments were performed as mentioned in methods. Briefly, CD8^{+} T cells were isolated from tumor bearing (3-5mm) WT(GFP), BLT1$^{-/-}$ (non GFP), CXCR3$^{-/-}$ (non-GFP) mice and BLT1$^{-/-}$CXCR3$^{-/-}$ (non-GFP) mice. WT and KO CD8^{+} T cells were equally mixed and 1 million cells were injected i.v. in tumor bearing Rag2$^{-/-}$ mice. Animals were sacrificed when the tumors reached 7-8mm tumor diameter. %WT and KO CD8^{+} T cells were determined upon gating on live CD3^{+}CD8^{+} T cells and looking for GFP^{+} and GFP^{-} populations respectively. Cumulative bar graph representing ratio of %WT to %KO CTLs (frequency of CD8^{+} T cells) is shown. Data representative of two independent experiments with n=5 per group in each experiment.

CHAPTER VI

PD-1 BLOCKADE BASED VACCINE FAILS IN BLT1$^{-/-}$, CXCR3$^{-/-}$ AND BLT1$^{-/-}$ CXCR3$^{-/-}$ MICE

INTRODUCTION

Data from previous chapters demonstrated the role of BLT1 and CXCR3 receptor in regulating endogenous anti-tumor responses. In this chapter we sought to investigate the role of BLT1 and CXCR3 receptors in regulating a vaccine-induced immune response. Whether BLT1 and CXCR3 receptor mediated CTL recruitment to tumors and anti-tumor immunity could be bypassed under the presence of an external immune stimulation was tested. We tested PD-1 blockade based immunotherapy to answer this question.

PD-1 is an immunosuppressive molecule present on T cells that have been activated against an antigen. PD-1 based immunosuppression is involved in restraining or attenuating an inflammatory response after an infection or cause for inflammation has been eliminated. However, in the context of anti-tumor immunity, as discussed before, PD-L1-PD-1 based immunosuppression leads to tumor mediated immunosuppression of CTLs enabling tumor immune evasion. Blocking PD-1 expression on T cells enhances anti-tumor immunity and has recently revolutionized the field of immunotherapy.

In order to conduct this experiment, we employed PD-1 antagonistic antibody based vaccine formulation consisting of Trp-2 melanoma peptide. The vaccine was administered as a therapeutic regimen upon tumor challenge. The data presented here indicate an obligate requirement for BLT1 and CXCR3 receptors in obtaining vaccine efficacy. CTL infiltration studies were carried out in tumors, spleen, TdLN and blood of unvaccinated and vaccinated mice. This chapter would provide essential information on the requirement of BLT1 and CXCR3 pathways in mediating efficacy of PD-1 blockade based immunotherapy.

RESULTS

Obligate requirement for BLT1 and CXCR3 in anti-PD-1 Ab based vaccine efficacy

The importance of BLT1 and CXCR3 in vaccine induced anti-tumor immune response was determined. WT, $BLT1^{-/-}$, $CXCR3^{-/-}$ and $BLT1^{-/-}CXCR3^{-/-}$ mice were challenged with 10^5 B16 cells subcutaneously on Day 0 followed by vaccine administration on day +5 and day +15. Although the vaccine did not completely eradicate the tumors in WT mice, there was a significant reduction in the tumor growth kinetics in WT mice upon vaccination. However, the vaccine completely failed to delay tumor growth in the $BLT1^{-/-}$, $CXCR3^{-/-}$ and $BLT1^{-/-}CXCR3^{-/-}$ animals **(Figure 25).**

The vaccine efficacy correlated with $CD8^+$ T cell infiltration to tumors, with significantly reduced $CD8^+$ T cell infiltration in tumors of knockout mice upon vaccination. As expected the vaccine decreased $CD8^+$ T cell numbers in the blood of WT mice as a reflection of the concurrent increase in the $CD8^+$ T cells in tumors of WT mice (**Figure 26)**. The percentages of $CD8^+$ T cells in the TdLNs and spleens of the knockout animals were comparable to the WT animals in both the vaccinated and unvaccinated cohorts (**Figure 27**). The ability of the vaccine to enhance $CD8^+$ T cell migration in WT tumors but not the knockout tumors was also confirmed by confocal microscopy **(Figure 27 A).** Granzyme-B an effector molecule secreted by $CD8^+$ T cells was significantly enhanced in WT mice upon vaccination, the increase was absent in knockout animals upon vaccination

suggesting reduction in effector responses in tumors of knockout mice with or without vaccination **(Figure 27 B).** $CD8^+$ T cells derived from blood and TdLN of tumor bearing mice showed increased BLT1 expression (increased mean fluorescent intensity) and a moderate CXCR3 upregulation (more counts of cells expressing CXCR3) upon vaccination **(Figure 28A and 28 B).** These results suggest that optimum efficacy of anti-PD-1 antibody based vaccine requires the expression of both BLT1 and CXCR3 for effective CTL infiltration to tumors and anti-tumor immunity.

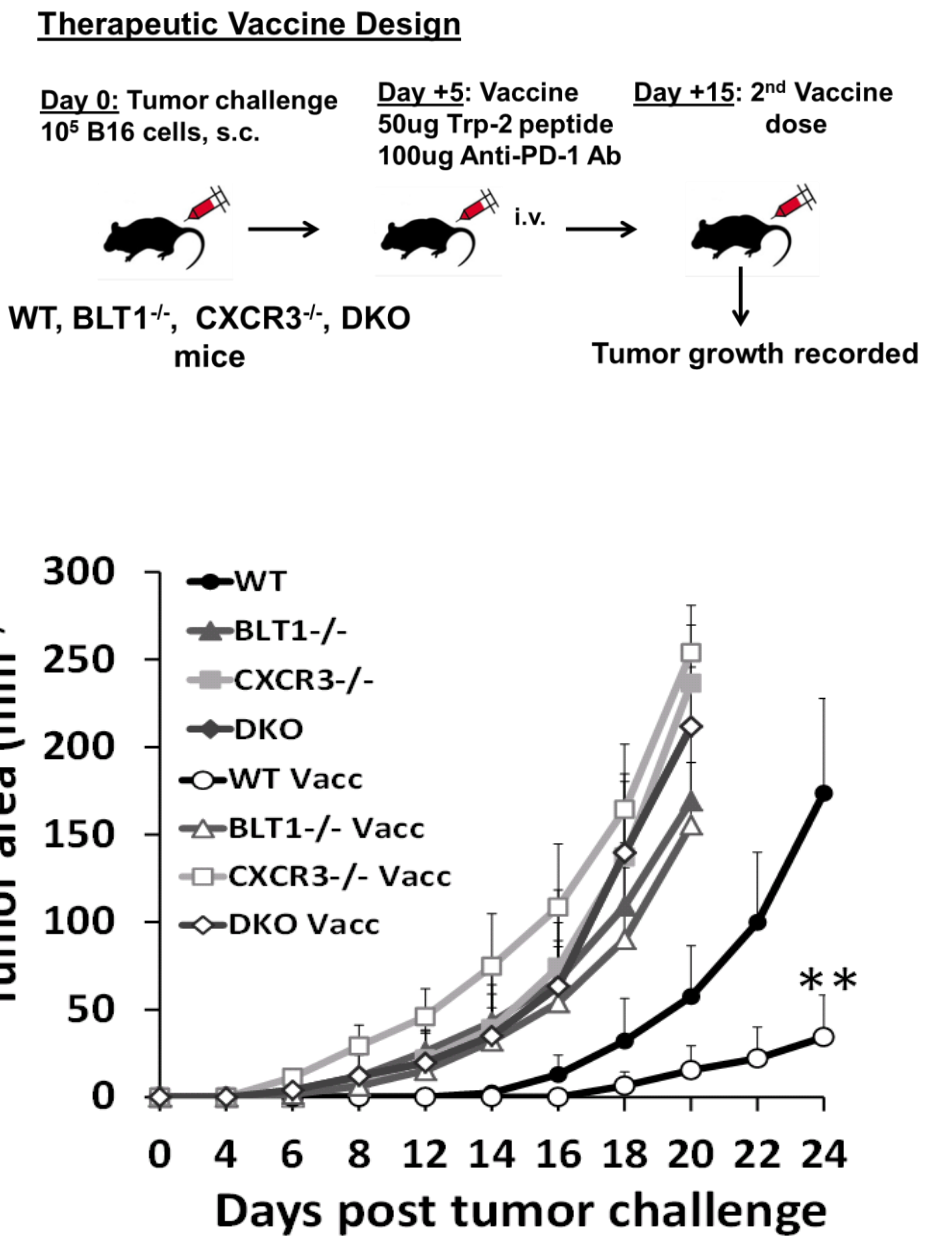

Figure 25: Obligate requirement of BLT1 and CXCR3 for optimum efficacy of anti-PD-1 antibody based immunotherapy. WT (Unvacc: n=4, Vacc: n=6), BLT1$^{-/-}$ (Unvacc: n=5, Vacc: n=5), CXCR3$^{-/-}$ (Unvacc: n=3, Vacc: n=5) and DKO (Unvacc: n=6, Vacc: n=5) mice were subcutaneously challenged with 10^5 B16 cells and left either unvaccinated (PBS) or vaccinated with Trp-2 peptide (50 µg)

and anti-PD-1 Ab (100 µg) twice intravenously on day +5 and +15 post tumor inoculation. Tumor area measured by multiplication of two perpendicular diameters in unvaccinated and vaccinated WT, BLT1$^{-/-}$, CXCR3$^{-/-}$ and DKO mice is shown. Data is representative of two independent experiments.

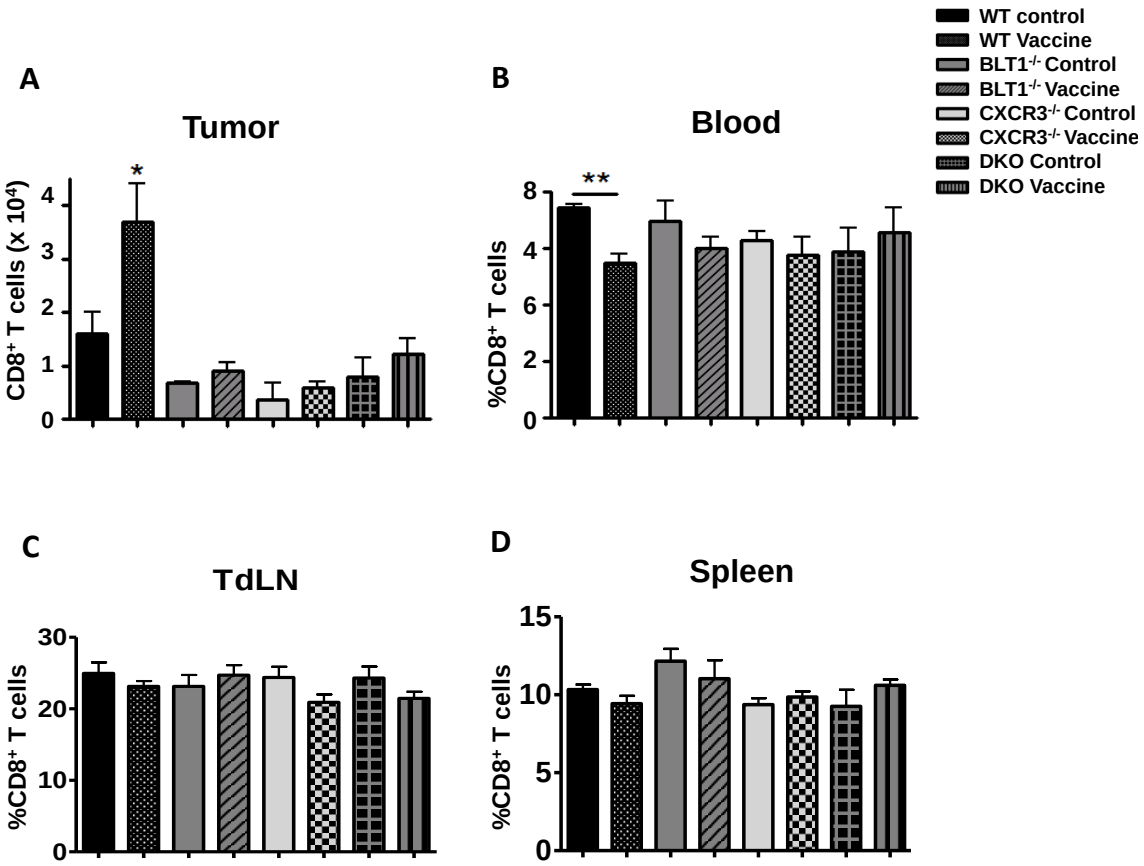

Figure 26: Vaccine enhances CD8$^+$ T cell infiltration in tumors of WT mice but not knockout mice. WT (Unvacc: n=4, Vacc: n=6), BLT1$^{-/-}$ (Unvacc: n=5, Vacc: n=5), CXCR3$^{-/-}$ (Unvacc: n=3, Vacc: n=5) and DKO (Unvacc: n=6, Vacc: n=5) mice were subcutaneously challenged with 10^5 B16 cells and left either unvaccinated (PBS) or vaccinated with Trp-2 peptide (50 µg) and anti-PD-1 Ab (100 µg) twice intravenously on day +5 and +15 post tumor inoculation. A. Enhanced CD8$^+$ T cell numbers in tumors of WT mice but not knockout mice upon vaccination. Cumulative bar graph representing CD8$^+$ T cell numbers per million total tumor cells (frequency of total) in unvaccinated and vaccinated WT,

BLT1$^{-/-}$, CXCR3$^{-/-}$ and DKO mice is represented. **B,C.** Cumulative bar graph representing %CD8^{+} T cells (frequency of CD45^{+} cells) in spleen **(B)** and TdLN **(C)** of unvaccinated and vaccinated WT, BLT1$^{-/-}$, CXCR3$^{-/-}$ and DKO mice is shown. **D.** Cumulative bar graph representing %CD8^{+} T cells (frequency of CD45^{+} cells) in blood of unvaccinated and vaccinated WT, BLT1$^{-/-}$, CXCR3$^{-/-}$ and DKO mice is shown. Data is representative of two independent experiments.

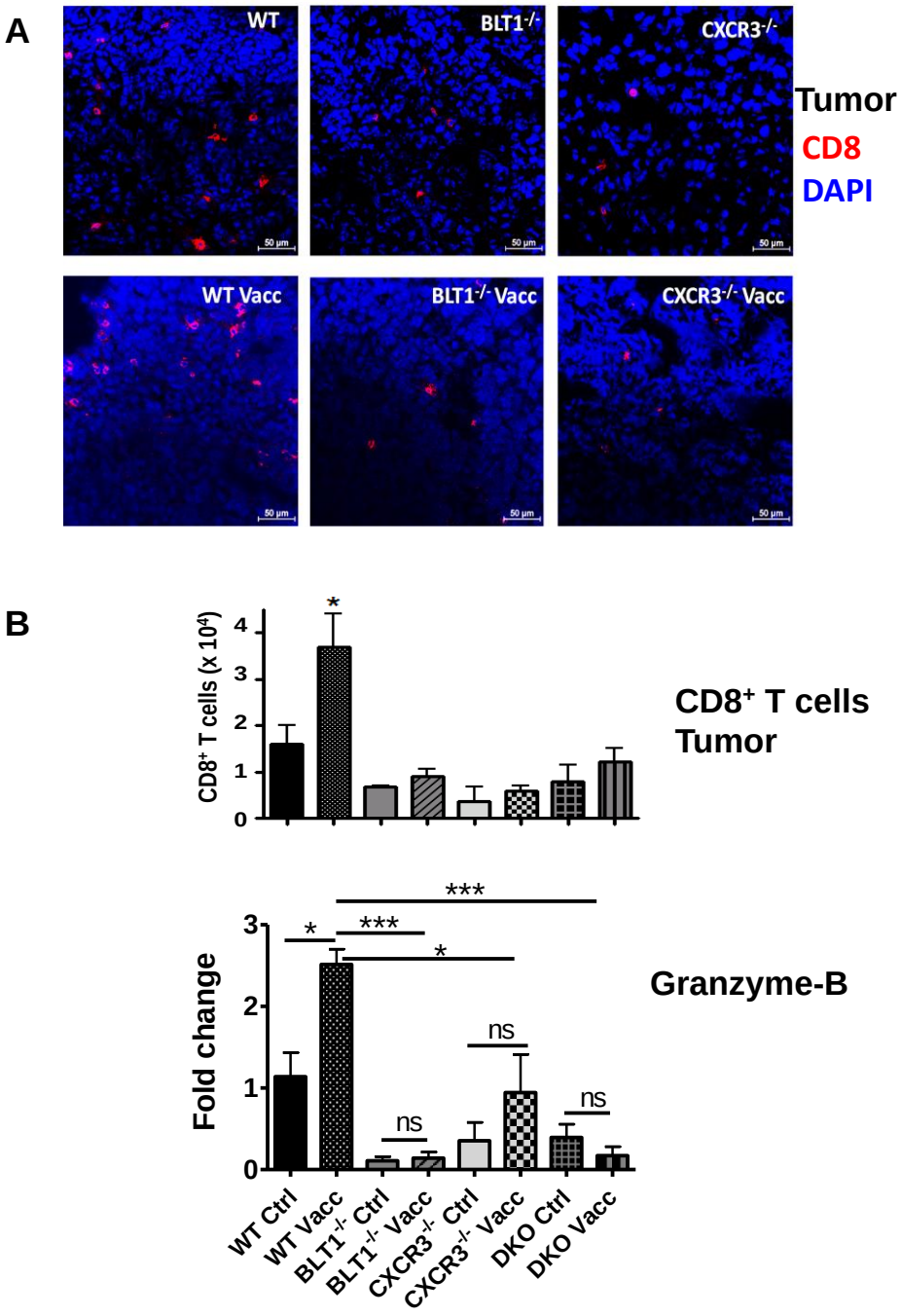

Figure 27: Vaccine enhances CD8$^+$ T cell infiltration in tumors of WT mice but not knockout mice. A) Representative immunofluorescence staining images of CD8$^+$ T cells in WT, BLT1$^{-/-}$ and CXCR3$^{-/-}$ tumors from vaccinated or unvaccinated mice. Tumors harvested were frozen, sectioned and stained as

described in Methods, CD8 represented in Red, DAPI in blue. The images were captured using Nikon A1R confocal microscope. The scale represents 50 µM. Data representative of two independent experiments. **B)** Enhanced $CD8^+$ T cell numbers in tumors of WT mice but not knockout mice upon vaccination. Cumulative bar graph representing $CD8^+$ T cell numbers per million total tumor cells (frequency of total) in unvaccinated and vaccinated WT, $BLT1^{-/-}$, $CXCR3^{-/-}$ and DKO mice is represented. Also, fold change of granzyme-B transcript in RNA isolated from WT, $BLT1^{-/-}$ and $CXCR3^{-/-}$ tumors of vaccinated and unvaccinated mice when the knockout tumors reach 15mm tumor diameter is shown. GAPDH was used as the housekeeping gene. Relative fold change to WT tumors is shown. n=4 in each group.

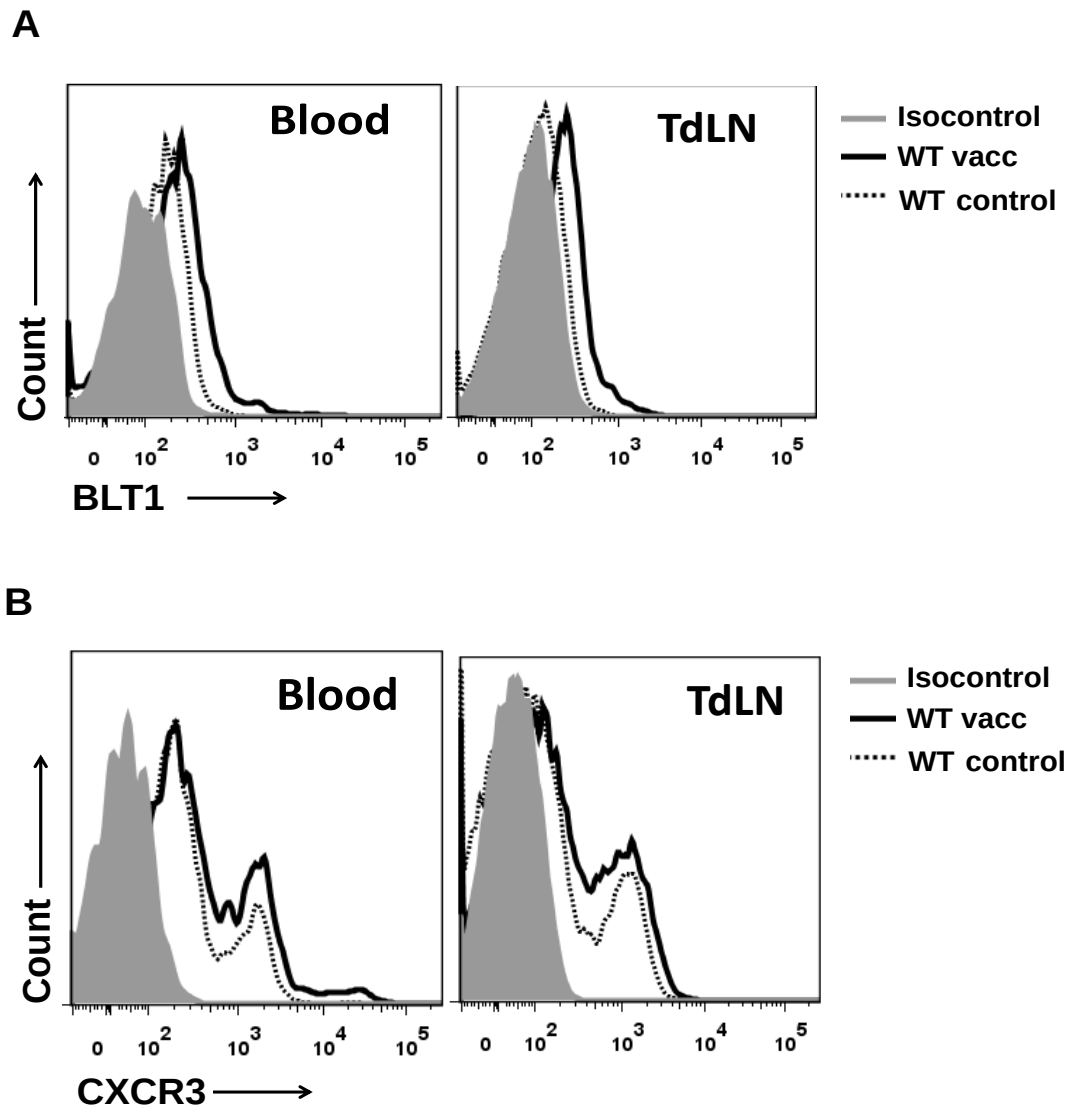

Figure 28: Enhanced expression of BLT1 and CXCR3 on $CD8^+$ T cells upon vaccination. $CD8^+$ T cells from blood and tumor draining lymph nodes of unvaccinated and vaccinated tumor bearing WT mice were analyzed for BLT1 Antibody developed in the lab (unpublished study) and CXCR3 as mentioned in the methods section**.** n=5 in each group. **A)** Representative histogram plots for BLT1 expression on $CD8^+$ T cells (gated on $CD3^+CD8^+$ T cells) from blood and

TdLN of WT vaccinated and unvaccinated control mice. Dotted line represents WT unvaccinated $CD8^+$ T cells and solid black line represents WT vaccinated $CD8^+$ T cells. **B)** Representative histogram plots for CXCR3 expression on $CD8^+$ T cells (gated on $CD3^+CD8^+$ T cells) from blood and TdLN of WT vaccinated and unvaccinated control mice. Dotted line represents WT unvaccinated $CD8^+$ T cells and solid black line represents WT vaccinated $CD8^+$ T cells.

CHAPTER VII

DISCUSSION

Cancer immunotherapies rely on achieving stronger and long lasting effector $CD8^+$ T cell responses in the tumor. A major obstacle for attaining this goal is the inefficient migration of $CD8^+$ T cells into the tumor [40, 75, 144]. The results presented here suggest that both BLT1 and CXCR3 are independently required for $CD8^+$ T cell migration to tumors and sustained anti-tumor immunity. In the absence of either of these receptors, there is a breach in achieving an effective anti-tumor response.

Although BLT1 is expressed on a variety of leukocytes, there is a preferential BLT1 mediated recruitment of certain cells under specific disease condition. For example, Th2 and $CD8^+$ T cells cell infiltration is preferred during airway hyperresponsiveness in asthma, T cells in autoimmune uveitis, macrophages in diet induced obesity and atherosclerosis and neutrophils in silica induced lung cancer promotion; in all of these models genetic deletion of BLT1 was shown to be protective [181, 183, 186, 189-192]. Recently, we demonstrated a crucial anti-tumor role of BLT1 in mediating $CD8^+$ T cell recruitment to tumors using TC-1 cervical cancer model wherein BLT1 deficient mice showed enhanced tumor growth and reduced survival compared to WT mice [176]. Lack of CTL infiltration to tumors can delay an anti-tumor response in other types of cancers as well.

Using an autologous melanoma tumor model expressing self-antigens [193], herein we corroborated that BLT1 mediates CTL recruitment to tumors and thus plays an important role in anti-tumor immunity; consistent with the TC-1 cervical cancer model. This suggests that BLT1 mediated regulation of CTL infiltration may be true across a variety of immunogenic solid tumor types. Hence, BLT1 mediated recruitment of various immune cells at specific locations in different tumor models is key to the type of inflammation (pro-tumor or anti-tumor) that accrues. In melanoma, BLT1 mediated $CD8^+$ T cell infiltration to tumors is a crucial mechanism for effective anti-tumor immunity. Consistent with this observation, anti-tumor response in spontaneous $Apc^{Min/+}$intestinal model of tumorigenesis also requires the expression of BLT1 (unpublished data).

Previous studies have reported a pro-tumorigenic role for BLT1-LTB_4 pathway, in cancer [187, 194-197]. Our studies highlight the importance of BLT1 on immune cells (CTLs) in achieving an effective anti-tumor response. Moreover, the data presented here suggests that in the absence of BLT1, major CTL chemokine receptors like CXCR3 that have been demonstrated to be indispensable for T cell trafficking at the tumor vasculature [142], cannot achieve optimum CTL infiltration to tumors.

In a GM-CSF gene transduced leukemia model, Yokota et.al. showed that $BLT1^{-/-}$ mice showed similar primary anti-tumor response but enhanced recall memory response. The better recall response induced by GM-CSF in $BLT1^{-/-}$ mice was attributed to enhanced DC maturation and function, reduced MDSC numbers,

enhanced NK cells in the knockout tumors and an enhanced Th1 and predominantly skewed Th2 response. In contrast to this crucial finding, our results suggest that BLT1$^{-/-}$ mice have a significant defect in primary anti-tumor response due to defective CD8^{+} T cell infiltration in the tumors of BLT1$^{-/-}$ mice; the other immune cell subsets remaining similar to those in WT mice. We also did not find any difference in the cytotoxic function in BLT1$^{-/-}$ mice as evident through in vivo-killing assay by immunization using peptide as well as tumor cells (data not shown). The divergence in the results observed can be accounted to the differences in the mouse strains (BALB/c) and induction of GM-CSF in the tumor cells [187]. GM-CSF is long known to induce DC maturation and T helper responses [198]. Also, the BALB/c mice model has been reported to be biased towards Th2 response [199] and Th2 responses are crucial for memory anti-tumor responses [200]. Although we did not find any difference in the numbers of CD4^{+} T cells, NK cells or myeloid cells in the tumors of BLT1$^{-/-}$ mice, the helper contributions of CD4^{+} T cells and NK cells in CD8^{+} T cell mediated anti-tumor immunity cannot be ruled out. Further studies are essential to determine the role of LTB_4-BLT1 pathway in CD4^{+} T cell dependent primary and memory anti-tumor responses.

The role of CXCR3 and the ligands CXCL9 and CXCL10 in anti-tumor immunity is well established [81, 142, 143, 201]. In a recent study, *Mikuchi et.al* elegantly demonstrated that CXCR3 mediated signalling to be a critical and an indispensable checkpoint for tumor antigen specific CD8^{+} T cells to traffic across the tumor vasculature for carrying out effective tumoricidal activity in mice and

human melanoma [142]. CTL chemokine receptors CCR5 and CCR2 were not essential for CXCR3 mediated CTL extravasation across tumor vessels despite the presence of CCL2 and CCL5 chemokines in the tumor mileu. Using an antigen specific B16Ova - OT-I system and ACT setting, they showed that WT OT-I but not CXCR3$^{-/-}$ OT-I CTLs were able to significantly reduce tumors; with 50% of WT OT-I transferred mice showing complete tumor regression. Consistent with their study, our data from tumor kinetics in WT versus CXCR3$^{-/-}$ mice and adoptive transfer of tumor educated WT or CXCR3 $^{-/-}$ CTLs demonstrated a crucial indispensable role for CXCR3 in mediating CTL recruitment to tumors and endogenous anti-tumor immunity. Moreover, in a therapeutic vaccine model based on PD-1 blockade in WT or CXCR3$^{-/-}$ tumor bearing mice suggested an obligate role for CXCR3 in the success of immune checkpoint blockade based therapies. Additionally, we demonstrated an equally indispensable role for BLT1 for endogenous anti-tumor immunity and therapeutic efficacy. *Mikucki et. al.* demonstrated an essential role for CXCR3 in mediating firm adhesion of tumor Ag specific CD8^{+} T cells at the tumor vessels while not affecting the rolling property of the CTLs. However, at what juncture of the multi-step trafficking process is BLT1 on CD8^{+} T cells required for homing into tumors, remains to be determined.

With respect to the functionality of the CD8^{+} T cells in the tumor microenvironment, CXCR3 expression plays a crucial role in interferon gamma secretion as CXCR3$^{-/-}$ CTLs in the tumors have a defect in interferon gamma production. Various other studies have shown similar defects in IFNγ production

of $CXCR3^{-/-}$ T cells [89, 202, 203]. Perturbed amplification loop in IFNγ production due to reduced Th1 and NK cells in $CXCR3^{-/-}$ or increased suppressive function of M2 macrophages in $CXCR3^{-/-}$ mice may be the reasons for defective IFNγ production in $CXCR3^{-/-}$ $CD8^+$ T cells [92, 204]. Another explanation of perturbed IFNγ in $CXCR3^{-/-}$ CTLs in tumor microenvironment may be due to significantly reduced $CD4^+$ T cells, NK cells and IL-2 (data not shown) in those tumors. $CD4^+$ T cells support $CD8^+$ T cell responses not only by IL-2, production which is required for survival and expansion of effector T cells but also by licensing of APCs [205]. NK cells are also a crucial part of anti-tumor immunity. NK cells by secreting IL-2, IFNγ, CXCL10 aids in the maintenance of T-helper and CTL population [206]. It is also conceivable that due to less IL-2 production in the tumors, the survival and effector functions of the $CXCR3^{-/-}$ CTLs cells is affected. Importantly, CXCR3 is a major player in the recruitment of T cells and NK cells and their survival and effector functions in the tumors. A recent study demonstrated that adenosine in the tumor milieu suppressed the production of CXCL10 followed by suppression in T cell infiltration; and partial reversion was seen upon adenosine receptor blockade [143]. Another study suggested that adenosine receptor blockade therapy failed to reduce tumor growth in $CXCR3^{-/-}$ mice [207]. Hence, expression of CXCR3 on CTLs in the tumor seems crucial for CTL effector function. Also, it is essential to understand the roles of BLT1 and CXCR3 on $CD4^+$ T cells and NK cells as well as their crosstalk with $CD8^+$ T cell survival and effector functions.

In general various leukocyte subtypes express several chemo-attractant receptors in overlapping patterns (eg. BLT1 is expressed on both activated T cells and myeloid cells) and one cell-type express various receptors that can respond to multiple chemo-attractant cues that may be present at the target tissue. Using a simplified 2D agarose based system where neutrophils encountered two different chemoattractant signals in a defined spatial array, neutrophils were shown to chemotax sequentially, first migrating up a primary gradient of IL-8 into a disorienting concentration which later could effectively retain capacity to resume migration to a secondary distant chemoattractant gradient of LTB_4, suggesting a potential for step-by-step navigation of immune cells from one chemo-attractant gradient to another in complex chemo-attractant fields [195]. Hence, two attractant pathways specific for the same cell may function together rather than being redundant in order to effectively recruit immune cells. Our studies suggest that BLT1 and CXCR3 seem to play an essential, non-redundant, cell-autonomous role in $CD8^+$ T cell infiltration to tumors and anti-tumor immunity.

We attempted to study the combinatorial regulation of CTL infiltration to tumors by BLT1 and CXCR3 via generation of BLT1/CXCR3 double knockout mice (DKO). The data presented here suggest a lack of synergism in BLT1 and CXCR3 but a probable occurrence of interdependence since there was no further enhancement of tumor growth in the DKO mice compared to either of the single knockout mice. Studies with the model of arthritis revealed that BLT1 mediated infiltration of WT neutrophils in $BLT1^{-/-}$ mice facilitated infiltration of endogenous

BLT1$^{-/-}$ neutrophils to the inflamed joint suggesting that BLT1 expression on neutrophils is essential only for the initial recruitment and other chemokines could then perpetuate the disease progression [180]. However, our co-transfer experiments with WT and individual knockout CTLs revealed that WT CTLs do not facilitate additional BLT1$^{-/-}$ or CXCR3$^{-/-}$ CTL infiltration to tumors suggesting that BLT1 and CXCR3 mediated signalling cannot be bypassed by other chemoattractant systems for CTL migration in to tumors.

The magnitude of importance of BLT1 and CXCR3 signalling pathways in vaccine-induced immune response was tested using immune-checkpoint blockade based vaccine. Blockade of Programmed cell Death-1 (PD-1) pathway has been recently FDA approved and is a promising anti-tumor immunotherapy in humans as it releases the brakes on the T cells thereby enhancing their function [208-211]. Anti-PD-1 antibody therapy was shown to enhance T cell infiltration of adoptively transferred T cells [211]. The data presented herein shows that while anti-PD-1 based vaccine enhanced T cell infiltration to tumors thereby reducing tumor growth in WT mice; the vaccine lost its efficacy in the absence of either or both BLT1 and CXCR3 receptors, a phenotype ascribed to the failure of the knockout CTLs to infiltrate the tumors. We hereby show an obligate role for both BLT1 and CXCR3 expression on CTLs in achieving optimum anti-PD-1 based vaccine efficacy.

In lung metastatic melanoma model, melanoma cells were shown to be a source for CXCL9 and CXCL10 production. Among the immune cells, CD4^{+} T cells were considered the major producers of CXCL9 as well as IFNγ in the metastatic

nodules in lung [143]. While most myeloid cells can readily make LTB_4, the source of this BLT1 ligand in the B16 tumors remain to be determined.

Taken together, these findings suggest potential ways to improve the current ACT therapies, including Chimeric Antigen Receptor (CAR) based therapies by employing BLT1 as well as CXCR3 receptor up-regulation strategies on tumor Ag specific $CD8^+$ T cells or receptor specific agonists to facilitate increased CTL trafficking into tumors. Since studies show that the cells used for ACT already have considerable CXCR3 expression on them [142], BLT1 can be upregulated on these cells to achieve better infiltration. Alternatively enhancing the CXCR3 chemokine levels as well as LTB_4 levels in the tumor may help achieve increased CTL infiltration to tumors. These findings also have an important implication in probing the potential efficacy of PD-1 blockade based treatment in patients bearing CXCR3 receptor polymorphisms (for e.g. CXCR3rs2280964), which results in an altered receptor that fails to be expressed on cell surface [212]. Taken together, our studies suggest that, in melanoma tumor, LTB_4-BLT1 pathway, is equally essential as CXCL9/CXCL10 - CXCR3 for CTL migration to tumors and anti-tumor immunity and can be targeted for therapy.

SUMMARY

Immunotherapies have revolutionized the field of cancer therapy and have shown considerable efficacy in the clinic. However, the efficacy of the current immunotherapy is restricted to a few patients while many patients remain unresponsive to treatment. A major obstacle recognized only recently in the success of these immunotherapies is defective $CD8^+$ T cell recruitment to tumors which significantly impairs the anti-tumor response. Chemokine-chemokine receptor signaling is a crucial T cell homing mechanism. Herein, we investigated the role of leukotriene B_4 receptor BLT1 and CXCL9 and CXCL10 receptor CXCR3 in regulating $CD8^+$ T cell migration and anti-tumor immunity using an autologous B16 melanoma model.

The results in this thesis suggest an important role for chemoattractant receptors BLT1 and CXCR3 in anti-tumor immune regulation **(Figure 29**). Both $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice demonstrated significantly enhanced tumor growth to a similar extent that reduced survival compared to WT mice **(Figure 9)**. Investigating cellular mechanisms to this phenotype revealed that BLT1 and CXCR3 both are essential for $CD8^+$ T cell migration to tumors, myeloid cell infiltration levels remaining similar **(Figure 10)**. Analysis of the effector functions of WT and knockout $CD8^+$ T cells revealed no intrinsic defect in killing ability, IFNγ and TNFα secretion in the periphery **(Figures 15 and 6).** However, compared to WT CTLs from tumors, CXCR3 deficient CTLs had a significant defect in IFNγ levels **(Figure 16).** This suggests that CXCR3 but not BLT1 signaling is essential in the tumor microenvironment for IFNγ production. Using adoptive transfer model, the

data demonstrated that tumor educated WT but not BLT1 and CXCR3 deficient $CD8^+$ T cells reduced tumor growth significantly in $Rag2^{-/-}$ mice. In fact the control $Rag2^{-/-}$ mice without any transferred CTLs had similar tumor growth kinetics as mice transferred with either of the knockout CTLs. The tumor growth in mice that received BLT1 and CXCR3 deficient cells correlated with defective tumoral but not TdLN $CD8^+$ T cell infiltration. This reinforced the importance of both BLT1 and CXCR3 receptor signaling in T cell recruitment into tumors **(Figure17 and 18).**

Analysis of tumor growth in BLT1-CXCR3 double knockout mice suggested similar tumor kinetics between $BLT1^{-/-}$, $CXCR3^{-/-}$ and $BLT1^{-/-}CXCR3^{-/-}$ mice indicating a lack of synergism in regulation of anti-tumor immunity **(Figure 21)**. The possible inter-dependence was determined by co-transfer of WT with either of the knockout $CD8^+$ T cells in tumor bearing $Rag2^{-/-}$ mice. The results obtained suggested that WT CTLs did not facilitate additional $BLT1^{-/-}$, $CXCR3^{-/-}$ or $BLT1^{-/-}$ $CXCR3^{-/-}$ CTLs into tumors **(Figure 24).**

We next sought to investigate the immunotherapy induced responses in $BLT1^{-/-}$, $CXCR3^{-/-}$ and $BLT1^{-/-}CXCR3^{-/-}$ mice. Interestingly, PD-1 blockade based vaccine efficacy was completely abolished in the absence of BLT1 and CXCR3 signaling. TIL analysis of vaccinated and unvaccinated controls suggested that the vaccine enhanced $CD8^+$ T cell infiltration into tumors significantly in WT mice but not the knockout mice. The results presented here suggest that BLT1 and CXCR3 mediated anti-tumor immunity cannot be bypassed. This suggests an obligate

requirement for both BLT1 and CXCR3 in mediating successful checkpoint blockade based vaccine efficacy.

Taken together, the data presented in this thesis suggest an equally crucial role for both BLT1 and CXCR3 receptors for efficient $CD8^+$ T cell trafficking to tumors and regulation of endogenous as well as immune checkpoint blockade based vaccine response. The data obtained from tumor growth in double knockout mice and co-transfer experiments indicated that BLT1 and CXCR3 probably are components of a single pathway involved in T cell homing processes. Therefore, ablation of any one of the receptors or both the receptors has similar end result and deficiency in BLT1 is not compensated by CXCR3, making both the genes equally crucial in anti-tumor immunity.

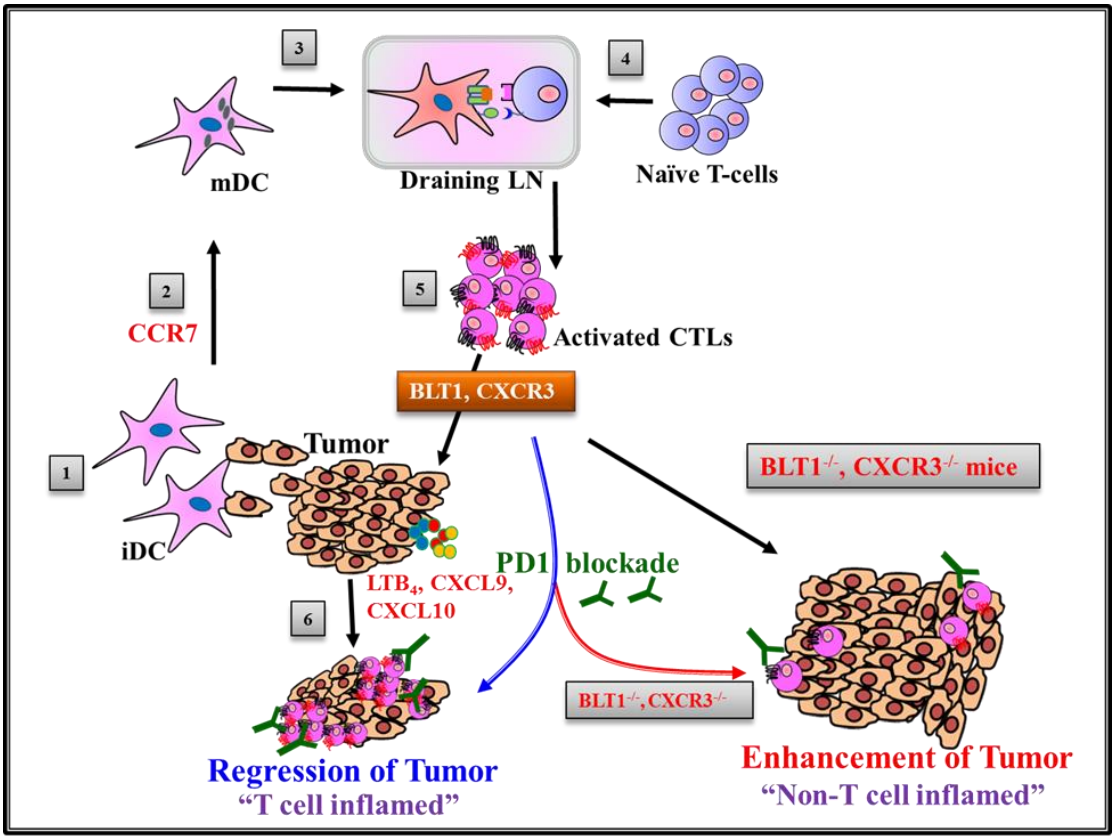

Figure 29: A model for BLT1 and CXCR3 mediated regulation of anti-tumor immunity. DCs phagocytose the tumor cells, undergo maturation and migrate to the tumor draining lymph nodes where they present the tumor antigen to the T cells. The T cells specific for the antigen get activated. Through BLT1 and CXCR3, the activated $CD8^+$ T cells then migrate to tumors in response to LTB4 and CXCL9/10 in tumor microenvironment. The present study suggests that BLT1 and CXCR3 are equally important for CTL migration to tumors and anti-tumor immunity. PD-1 blockade immunotherapy also requires the presence of BLT1 and CXCR3 that gives a "T cell inflamed" phenotype in tumor. In the absence of BLT1 and CXCR3, the PD-1 blockade therapy completely fails and leads to "non- T cell inflamed" phenotype that fails to reduce the tumor size.

IMPLICATIONS AND FUTURE DIRECTIONS

The data presented in this thesis suggest an equally crucial role for both BLT1 and CXCR3 in mediating efficient $CD8^+$ T cell migration to melanoma tumors and achieving effective anti-tumor immunity. We demonstrate an important role for LTB_4-BLT1 axis in mediating immune surveillance in both viral Ag derived TC-1 cervical cancer as well as autologous B16 melanoma model, suggesting that BLT1 mediated CTL recruitment to tumors is true probably across various other solid tumors.

Anti-PD-1 antagonistic antibodies, Nivolumab and Pembrolizumab were recently approved by the FDA for treating inoperable end stage melanoma and non-small cell lung carcinoma and have shown promising results in the clinic. Our study has contributed in the understanding of the factors required for the successful efficacy of these therapies. Recent studies have shown that a cohort of patients remain unresponsive to this treatment and that the treatment works best in T cell inflamed tumor (presence of T cells and T cell chemokines) versus non-T cell inflamed tumor (absence of T cells and T cell chemokines). These findings also have an important implication in probing the potential efficacy of PD-1 blockade and possibly even CTLA4 blockade in patients bearing CXCR3 receptor polymorphisms (for e.g. CXCR3rs2280964), which results in an altered receptor that fails to be expressed on cell surface. Also, with respect to BLT1 pathway, it would be crucial to understand if certain patient specific polymorphisms in BLT1 receptor gene or even genes involved in LTB_4 production like 5-Lipoxygenase

and LTA_4 hydrolase are associated with unresponsiveness to PD-1 therapies or associated with non-T cell inflamed tumors.

How to redirect the peripheral tumor antigen specific T cells into tumors by targeting chemoattractant-chemoattractant receptor interactions is an important question requiring research in the area. Improving CTL recruitment to tumors by enhancing LTB_4, CXCL9 and CXCL10 ligand levels can potentiate the anti-tumor responses. Alternatively, our study has potential implications in improvement of CAR-based ACT approaches. Antigen specific chimeric antigen receptors with BLT1 or CXCR3 could be designed to improve tumor infiltration of the transferred cells and probably reduce toxicities associated with off-target effects of CAR therapies.

It was recently demonstrated by Mikuchi. et.al using intra-vital microscopy that CXCR3 is crucial for enabling adhesion and extravasation of antigen specific T cells through the tumor endothelium. At what juncture is BLT1 required in this process could be answered.

Future directions of this project could be aimed at understanding the specific cell types involved in BLT1 and CXCR3 production in tumors. We currently believe that myeloid cells including macrophages and mast cells are the major producers of LTB_4 at the sites of inflammation. Investigating the same in the context of tumors would be important.

While it is well demonstrated in various studies that immunotherapy including PD-1 blockade strategies significantly induce CXCR3 ligands CXCL9 and

CXCL10 in the tumors to aid additional effector cells to migrate in response to the increased chemokine gradient in tumors, it would be interesting to understand whether PD-1 blockade therapy in melanoma patients induces LTB_4 production in tumor biopsies. This would add a new mechanism in PD-1 blockade mediated chemoattractant induction in the tumor.

We demonstrated that Anti-PD-1 based vaccine completely lost its efficacy in $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice. The question still remains whether defective CTL migration is the only cause for the loss in efficacy of the vaccine in knockout mice. Possible future experiments must be directed to understand whether tumor mutation based neo-antigen-specific T cells in WT versus knockout tumors is different. The hypothesis here is that in the absence of CXCR3 and BLT1 receptors, clonal diversity of neo-antigen specific T cells would be diminished. TCR-repertoire sequencing in tumor versus periphery of WT as well as $BLT1^{-/-}$ and $CXCR3^{-/-}$ mice would be essential in answering this question.

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APPENDIX

LIST OF ABBREVIATIONS

ABBREVIATION	STANDS FOR
1. ACT	Adoptive cell therapy
2. AOM-DSS	Azoxymethane – Dextran sulfate sodium
3. CAR	Chimeric Antigen Receptor
4. CTL	Cytotoxic T lymphocyte or $CD8^+$ T cells
5. CTLA4	Cytotoxic T lymphocyte associate protein 4
6. COX2	Cyclooxygenase 2
7. DC	Dendritic Cells
8. DKO	Double Knockout mice
9. ECM	Extra cellular matrix
10. IL	Interleukin
11. IFN	Interferon

12.	IFNγ	Interferon gamma
13.	IDO	Indoleamine-2,3-dioxygenase
14.	LTB_4	Leukotriene B_4
15.	MDSC	Myeloid Derived Suppressor Cells
16.	MHC	Major histocompatibility complex
17.	MSI	Microsatellite Instable
18.	NSAIDs	Non-steroidal anti-inflammatory drugs
19.	NK	Natural Killer
20.	NF-κβ	Nuclear Factor kappa beta
21.	PD-1	Programmed cell Death – 1
22.	PD-L1	Programmed cell Death Ligand - 1
23.	PD-L2	Programmed cell Death Ligand – 2
24.	Rag2	Recombination activating gene 2
25.	ROS	Reactive oxygen species
26.	RNI	Reactive nitrogen intermediates
27.	STAT	Signal Transducer and Activator of transcription
28.	TAM	Tumor Associated Macrophages

29. TdLN — Tumor draining Lymph Node
30. Trp2 — Tyrosinase related protein-2
31. TIL — Tumor infiltrating leukocytes
32. TNFα — Tumor Necrosis Factor alpha
33. Treg — Regulatory T cells
34. Th — T-helper
35. WT — Wild Type

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PEER REVIEWED PUBLICATIONS:

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PUBLICATIONS IN REVIEW:

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2. *Sharma RK, Chheda Z., et.al.* A Spontaneous Metastasis Model Reveals the

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MANUSCRIPTS UNDER PREPARATION:

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ORAL PRESENTATIONS IN BLOCK SYMPOSIUMS:

1. *Chheda Z, Sharma RK, Jala VR, Haribabu B.* Regulation of CTL trafficking into tumors and anti-tumor immunity by chemo-attractant receptors BLT1 and CXCR3. *American Association of Immunologists (AAI) annual meeting, New Orleans, 2015.*

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POSTER PRESENTATION:

1. *Chheda Z., Sharma R.K, V.R. Jala, B. Haribabu*. Regulation of CTL trafficking into tumors and anti-tumor immunity by chemo-attractant receptors BLT1 and CXCR3. *American Association of Immunologists (AAI), New Orleans, May 10th, 2015.*
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