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Type 3 T helper cell and myeloid derived suppressor cell population dynamics in a mammary carcinoma model

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TYPE 3 T HELPER CELL AND MYELOID DERIVED SUPPRESSOR CELL POPULATION DYNAMICS IN A MAMMARY CARCINOMA MODEL

A Thesis Submitted in Partial Fulfillment
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Master of Science

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ABSTRACT

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Immunotherapies that augment Type I immunity show robust responses in diffuse blood cancers yet remain relatively ineffective in breast and other solid tumor malignancies. Breast tumor resistance to immunotherapies is associated with polarization towards pro-tumor Type 2 immunity, as well as the expansion of a myeloid derived suppressor cell (MDSC) population that inhibits Type 1 T helper (T_h) and $CD8^+$ cytotoxic T cells. Does polarization toward Type 3 immunity play a role in mammary tumor formation? This question had not been investigated prior to these studies despite established relationships between MDSCs and Type 3 T_h cells in other inflammatory pathologies. Therefore, we investigated involvement of Type 3 T_h cells (T_h17 and T_h22) and their association with expanding MDSC populations in the 4T1 mouse mammary carcinoma model. When evaluated at multiple time points after 4T1 injection (days 7, 14, 21, and 28), tumor infiltration of T_h17 and T_h22 cells was first detected at d 14, and T_h17 populations declined after this time while T_h22 remained unchanged. In peripheral organs, T_h17 increased by d 7 before declining, while T_h22 were not elevated until later times. Only T_h17 and MDSC expansion in the bone marrow were positively correlated, suggesting further that T_h17 and T_h22 are functionally distinct lineages and that MDSCs may play a role in T_h17 fate determination in breast cancer. To further address a possible

relationship between MDSCs and Type 3 T_h cells in mammary carcinoma, we used CRISPR-Cas9 to knock out tumor cell-specific production of interleukin (IL) -6 (IL6-KO), which functions in T_h maturation, myelopoiesis, and MDSC recruitment. Tumor-resident T_h17, T_h22, and MDSCs did not change in IL-6 KO tumors, suggesting a limited role for IL-6 in local recruitment. However, induction of T_h22 and MDSCs in peripheral tissues was significantly reduced with IL6-KO tumors, while T_h17 cells were increased. These concomitant changes in peripheral Type 3 T_h and MDSCs suggests direct functional interactions between these populations, yet additional studies are required to confirm this. To conclude, we identify and characterize a pro-tumor Type 3 T_h immune response that accompanies MDSC expansion in a model of metastatic breast cancer. This is important because these populations are associated with reduced efficacy of cancer immunotherapies.

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CHAPTER I

INTRODUCTION

Resistance to Cancer Immunotherapy

In 2019 breast cancer was the leading cause of new cancer cases among American women and the second leading cause of cancer deaths in the US behind lung cancers (1). In the past decade, immunotherapies that involve training the patient's immune system to recognize and kill cancer cells have been investigated as treatment for breast and other cancers. While targeted immunotherapies, especially immune checkpoint (PD-1 and CTLA-4) antibody therapy, have been shown to be effective in some forms of lymphoma (2), leukemia (3), and melanoma (4), similarly dramatic results have not been observed for solid tumor malignancies like breast cancer (5). Even with the advent of immunotherapies, breast cancer mortality rates have remained relatively constant (1), and recent clinical observations have suggested rare instances in which immunotherapy treatment may have resulted in harmful breast tumor hyper-progression (6). Thus, there is a definitive clinical need to understand why immunotherapies are failing in these solid tumors.

Mechanisms of Resistance to Immunotherapy

Currently, this resistance to immunotherapy is in part thought to arise because breast and other solid tumors present a much more logistically complex location to access because they cloak themselves in a milieu of immunosuppressive factors. Additionally, solid tumors are not a diffuse easily accessible liquid environment like blood. Due to the

variety and breadth of immune countermeasures elicited by solid cancers (7), a complete understanding of immunotherapy failure has yet to be realized, and additional mechanisms remain to be explored. One well-described barrier to successful immunotherapy is the recruitment of myeloid derived suppressor cells (MDSC), a population of immature myeloid cells that directly mitigate cytotoxic T and T helper (T_h) cell potency – immune cell populations that checkpoint therapy hinges on for effectiveness. While breast cancers can recruit MDSCs, it is still unclear exactly how the tumor, host, and additional immune system components react to their expansion. It has been shown that breast tumors can cause polarization away from anti-tumor Type 1 toward pro-tumor Type 2 immunity (8). Previous work done on MDSC in autoimmunity and parasitic infections suggests an interaction between MDSC and Type 3 immunity (9), defined here as the accumulation of T_h17, T_h22, and transitional $T_h1/17$ cell populations. Surprisingly little has been done to investigate Type 3 immunity in the context of MDSC-expanding mammary carcinoma. Thus, the studies described in this thesis aim to: 1) Describe the response of Type 3 T_h immune cell populations- T_h17, $T_h1/17$ and T_h22- in a murine model of mammary cancer; 2) Correlate the Type 3 T_h responses with MDSC expansion; and 3) Determine the role of tumor derived interleukin (IL)-6 on MDSC and T_h cell accumulation and polarization. This work is important because it identifies immune polarization and interaction mechanisms that lead to immunotherapy resistance. A proposed working model of potential cellular interactions is shown in Figure 1.

Specific Aims and Research Hypothesis

A1 Characterize Type 3 T_h recruitment by 4T1 mammary tumors.

H1 Proportions of Type 3 Th immune cells (T_h17, T_h1/T_h17, and T_h22) will be elevated in the tumor and peripheral organs of 4T1 tumor-bearing mice compared to healthy controls.

A2 Explore the relationship between MDSCs and the Type 3 immune response.

H2 MDSC immune cell expansion will precede Type 3 expansion, suggesting MDSC involvement in systemic Type 3 recruitment.

A3 Determine the role of tumor derived IL-6 on MDSC, Type 3 immune, and total T_h recruitment

H3 4T1 tumors deficient in IL-6 will recruit fewer MDSCs and Type 3 T_h cells (T_h17, T_h1/T_h17, and T_h22) in tumors and peripheral tissues, while Type 1 T_h cell recruitment will correspondingly increase.

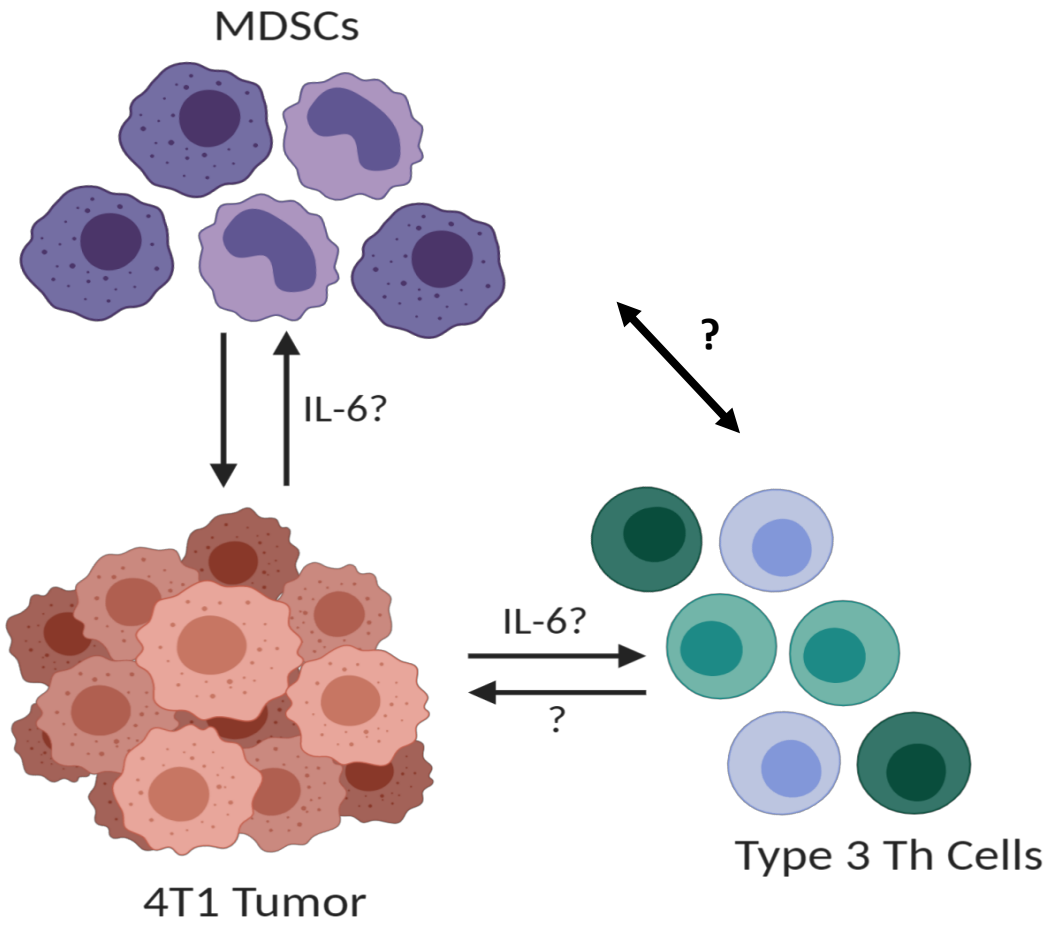


Figure 1: Proposed interactions between 4T1 tumor cells, type 3 T_h, and MDSCs; that are mediated by interleukin IL-6. In this predicted scenario, Type 3 T_h cells will be recruited from naïve T cells by the 4T1 mammary carcinoma cells. We hypothesize that tumor-derived IL-6 is partly responsible for recruiting and skewing naïve T cells into Type 3 T_h cells (T_h17 $T_h1/17$ and T_h22), effectively expanding their population and creating the opportunity to have Type 3 cells recruited into the tumor. IL-6 also plays a role in fate determination of myeloid cells, leading to the expansion of MDSC from immature myeloid cells in the bone marrow (10). However, it is unknown if specifically removing tumor-derived IL-6 will mitigate MDSC expansion. Lastly, the relationship between Type 3 T_h and MDSC expansion will be evaluated.

CHAPTER II

REVIEW OF THE LITERATURE

Normal Immunity

Adaptive and Innate Immunity

Adaptive immunity. T_h cells, cytotoxic T cells, and B cells are a part of the adaptive immune system and are characterized by eliciting antigen specific response through highly variable antigen receptors (11). Adaptive immunity can be broken down into two categories: humoral immunity and cellular immunity. Humoral immunity is often defined as B cell mediated formation and release of antigen specific immunoglobulins, also known as antibodies. Antibodies, among other functions, can opsonize pathogen for further recognition by innate phagocytotic immune cells. Cell mediated adaptive immunity generally does not involve antibody response and is often characterized by T_h and cytotoxic T cell activation (11, 12).

Innate immunity. Preceding adaptive immune response and thus T_h cell control of immunity, the innate immune system must first recognize danger. These molecular danger signals are known as pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPS). PAMPs are highly conserved regions on pathogen such as lipopolysaccharide, peptidoglycan, and unmethylated CpG regions of DNA, (13) while DAMPs are intracellular contents released from damaged cells such as ATP, histones, and mitochondrial DNA (14). Innate immune dendritic cells (DC) and macrophage, also known as professional antigen presenting cells (APC), do not rely on a

specific antigen to be activated but instead act through recognition of danger signals by pattern recognition receptors (PRR), such as toll-like receptors (TLR), nod-like receptors (NLR), and RIG-I-like receptors (RLR) (12). These innate immune cells are further equipped to aid in T_h activation and modulate the Type of immunity (15).

T Cell Activation and T Cell Receptor Biology

Unlike B cells that recognize antigen in its native form to activate (11), T cells with traditional α/β T cell receptor (TCR) require presentation of antigen peptide loaded on major histocompatibility complex (MHC), which is expressed on the surface of APC. There are two classes of MHC on which peptide antigen can be presented: MHC I and MHC II. The MHC class restricts T cell response to either cytotoxic T or T_h recognition. Cytotoxic T cells recognize peptide loaded onto MHC I due to cluster of differentiation (CD) 8 co-receptor expression, while T_h cells recognize peptide loaded MHC II due to CD4 co-receptor expression. MHC I and MCH II differ in cellular expression patterns, with MHCI expressed on the surface of all nucleated cells, allowing for cytotoxic T cell and natural killer (NK) cell monitoring of self-cells and subsequent deletion of self when an abnormal antigen is presented (15, 16). Due to the widespread expression of MHC I, cytotoxic T cell-mediated cell death is vital for surveillance and removal of virally infected cells and pre-malignant cells with mutated antigen, also termed cancer neo-antigen (17, 18). T_h1 cells play a further role in enhancing cytotoxic T cell accumulation and function through signaling of relevant cytokines such as interferon gamma (IFN-γ) (19). MHC II is concerned mostly with extracellular-sourced antigens and is only expressed on professional APC (B cells, dendritic cells, and macrophages) with a functional phagolysosome. B cell antigen presentation to T cells in the lymphoid follicles

(T_{fh}) is necessary for the development of a hyper-specific and effective antibody response, while presentation via the myeloid cells, especially DC, is a necessary bridge between innate and adaptive immunity required for the full resolution of a response to immunological threat (16).

TCR interaction with peptide-loaded MHC accompanied by CD4 or CD8 co-receptor stimulation is necessary but not sufficient to initiate T cell clonal expansion. Co-stimulation must accompany TCR:MHC interaction for clonal expansion to ensue. This occurs through adequate upregulation of costimulatory molecules CD80 (B7.1) and CD86 (B7.2) on APCs, which stimulate CD28 on naïve T cells. Adequate co-stimulation, TCR engagement, and APC production of IL-2 are each necessary and altogether sufficient for T cell activation, and expansion. Without proper co-stimulation, T cells often undergo anergy (20). Anergy mechanisms involving immune checkpoints cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are covered later more extensively in the Immune Tolerance section.

Types of Immunity Based on T Helper Cell Phenotype

Broadly, there are three main types of immunity to threats/pathogens (with examples) the immune system is equipped to respond to: Type 1- intracellular (viruses), Type 2-large extracellular (helminth) and Type 3- small extracellular pathogens (bacteria and fungi). Each immunity type is ultimately mediated and maintained by the activation and polarization of specific adaptive $CD4^+$ T_h cells. Once activated, T_h cells undergo clonal expansion (with respect to their specific TCR sequence) and produce a milieu of distinct cytokines that program and dictate further immune response specificity for a

given pathogen (21). Indeed, type of immunity was characterized by the response initiated following specific pathogen infection; however, the polarization of different types of immunity may occur in other contexts, an example being aberrant Type 3 activation leading to autoimmunity (22). Characterizing the type of immune response instigated by cancer and other pathologies will be important in understanding the inappropriate immune responses that often accompany these disease states.

T Helper Cell Polarization Amongst Type 1, 2, and 3

T_h polarization follows naïve T_h cell recognition of antigen and activation. Contingent on the specific danger signal, professional APCs produce a specific cytokine profile, which is necessary to polarize T_h cells down an immunological lineage. Indeed, this encourages fate determination by promoting one type of immunity, which aligns with lineage restricted overexpression of specific transcription factors and cytokines. The clonal expansion of all T_h broadly requires IL-2 stimulation, while distinct polarization of T_h cells enabling the more pathogen specific responses requires specific stimulation as will be described below. As a general rule though, the cytokines that promote one category of polarized immunity will inhibit other T_h subset formation, which leads to specialized T_h cell mediated immunity following a specific immune challenge (23). The process of normal polarization will be described in the sections immediately following, while a potential role of polarization in cancer will be described in a later section.

Type 1 Immunity

Following a Type 1 challenge such as a viral infection, T_h cells are stimulated and polarized to become T_h1 cells by IL-12 and IFN-γ secreting dendritic cells and macrophages (23) allowing for the upregulation of transcription factors T-bet, signal

transducer and activator of transcription (STAT) 4 and STAT1, which further polarize T_h cells to the T_h1 phenotype. T_h1 cells then respond by producing more IFN-γ facilitating (23) immunoglobin (IgG) 1, IgG2a and IgG3 B cell (clarify/rephrase) class switching (19, 23); and recruitment of $CD8^+$ cytotoxic T cells, M1 macrophages, and NK cells. These cellular responses further direct and specify Type 1 immunity (23). In addition to its viral target, Type 1 immunity is the major immune response required to clear pre-malignant, mutated 'self'-cells (24).

Type 2 Immunity

Type 2 immunity is mediated by T_h2 polarization dependent on local concentrations of IL-2 and IL-4 dendritic cells and macrophages (23), which leads to T_h cell expression of transcription factors STAT6 and GATA-3, ultimately allowing for increased production of IL-4, IL-5, and IL-13 (25). This response is accompanied by IgE B cell class switching and recruitment of mast cells, eosinophils, basophils, and M2 macrophages (19, 25, 26). Type 2 immunity promotes a targeted response to large multicellular parasites, but in the context of cancer, it appears to foster tumor growth, likely because resources are misdirected away from Type 1 immunity (24).

Type 3 Immunity

T helper cell 22 versus T helper cell 17. Type 3 immunity is mediated by T_h17 and T_h22 cells. In the late 1980s, T_h cells were initially classified into two groups T_h1 and T_h2 (27). It was not until the mid-2000s that T_h17 were identified as a distinct lineage subset (28, 29). In 2009 T_h22 were identified as yet another unique T_h subset (30); however, ever since the distinction between T_h22 and T_h17 cells has been hotly debated. This is in part due to limited studies highlighting functionally divergent roles of T_h22 and

T_h17 (31). To date, most studies of T_h22 function have explored the immunomodulatory effects of IL-22 (32). Confoundingly, T_h17 are also a source of IL-22, and therefore it remains unclear whether there is a distinct T_h22 function. Additionally, many studies use *in vitro* T_h22 polarization protocols that also stimulate T_h17 differentiation, making interpretation of these results difficult. Recently, however, researchers have developed an *in vitro* differentiation protocol for T_h22 that excludes T_h17 differentiation. Studies evaluating T_h17 versus T_h22 functionality are sure to follow (31).

Polarization towards type 3 T helper cells. Following a bacterial challenge, T_h17 or T_h22 fate determination is dictated chiefly by expression of transcription factor retinoic acid receptor related orphan nuclear receptor gamma t (RORγt). The T_h17 phenotype requires T_h expression of RORγt promoting the ability to produce Type 3 immune cytokines IL-17A and IL-17F. Polarization toward the T_h22 lineage requires inhibition of RORγt expression, and as a consequence T_h22 do not express IL-17A or IL-17F (23). The polarization toward T_h17 requires transforming growth factor beta-1 (TGFβ-1), IL-6, and IL-23; alternatively, T_h22 requires the combination of IL-6, IL-23, and IL-1β (31). T_h22 express IL-22, which is regulated in part by transcription factors aryl hydrocarbon receptor (AHR) and STAT3 (33). Interestingly, T_h17 retain expression of IL-22. T_h22 and T_h17 mediated immunity to allow for the recruitment of neutrophils and MDSC. Type 3 immunity is elicited to target small extracellular pathogens; however, in the context of solid tumors, this response may be tumor protective (34). Again, this may arise because it draws finite resources from Type 1 immunity but could also directly stimulate IL-22 receptor (IL-22R) expressing epithelial cancers like breast cancer.

Interleukin 17 and interleukin 22 Synergy. T_h cell co-expression of IL-22 and IL-17 may have additive or synergistic effects. In microbial infection, co-expression of IL-17 and IL-22 enhanced expression of anti-pathogen, pro-inflammatory markers CXCL8, IL-6, S100A8 and S100A9 (35, 36). Additionally, IL-22 and IL-17 are known to work in conjunction to protect against mucocutaneous infection (22). In airway inflammation, for example, IL-22 alone did not lead to a pro-inflammatory state, but the addition of both IL-22 and IL-17 incited airway inflammation. Specifically, this was mediated by T_h17 and allowed for neutrophil recruitment to the airway (37). These additive properties of IL-17 and IL-22 may explain the perceived conflicting pro- and anti-inflammatory nature of IL-22.

Resolution of Inflammation

The polarization of T_h to the proper category focuses the immune response to a given immunological challenge and is pertinent for timely resolution of inflammation. If an immune response is polarized toward the wrong type of immunity for a given pathogen, the immune system may not be properly equipped to fight it, thus prolonging inflammation and increased disease severity. A great example of this is following *Mycobacterium leprae* (*M. leprae*) infection, which causes leprosy. *M. leprae* is a small intracellular pathogen. Within cases of leprosy, there is a more severe illness known as lepromatous leprosy and less severe illness known as tuberculoid leprosy. In both instances leprosy is caused by *M. leprae,* however, the pathology of the disease is determined by whether the host elicits a type1 immune response as seen in tuberculoid leprosy or a misguided type 2 response as seen in lepromatous leprosy (38).

Following termination of an adaptive immune response, a select group of adaptive T cells and B cells, with a receptor repertoire specific to the antigen cleared, are maintained in the greater lymphocyte population. This adaptive memory allows for faster activation of the T cell and B cell response if a secondary challenge occurs (39).

Following clearance of the pathogen, the immune responses must be dialed back and actively suppressed. In large, this occurs through recruitment of the $CD4^+$ T regulatory (T_{reg}) cells that can quell cytotoxic and T_h expansion through the release of inhibitory cytokines (TGFβ, IL-10, and IL-35) (40), cell lysis, metabolic disruption and targeting of dendritic cells to inhibit T_h expansion (41, 42). MDSCs are also known to support T_{reg} expansion, and consequently, both T_{reg} and MDSCs are tumor protective (43). T_{reg} and MDSC are necessary to maintain peripheral tolerance to self and prevent autoimmune reactions (41, 44).

Immune Tolerance

Immunological tolerance prevents inappropriate immune responses targeted to self-antigen and occurs by either central or peripheral tolerance selection mechanisms. Central tolerance occurs in the thymus after T cells undergo VDJ rearrangement of the TCR. VDJ rearrangement results in a broad repertoire of antigen specificity. Due to the extensive repertoire of TCR specificity, some T cells may be inherently auto reactive. When naïve T cells are challenged by thymic epithelium or myeloid cell with self-peptide loaded MHC. Tissue specific self-peptide expression is mediated by autoimmune regulator in thymocytes. If TCR high affinity binding occurs the T cell is either deleted by apoptosis, undergoes further receptor editing, or is induced to become a T_{reg}. Since not

all self-antigens are presented in the thymus, some auto-reactive T cells will eventually escape central tolerance (45).

Thus, peripheral tolerance mechanisms outside the thymus exist to prevent further T cell autoimmunity most often mediated by immunosuppressive T_{reg} cells, lack of co-stimulatory molecules, and immune checkpoints. The process and outcome of peripheral tolerance and may result in T cell apoptosis, anergy or T_{reg} formation (45). A specific mechanism of T cell peripheral tolerance or inflammatory response resolution is referred to as "Immune Checkpoints." This mechanism relies on inhibitory receptors expressed by T cells and APC that impede adequate T cell co-stimulation and TCR signaling. The most common checkpoint receptors are CTLA-4 and PD-1 both expressed on T cells. CTLA-4 is upregulated on T cells after activation and binds to costimulatory molecules CD80 and CD86 on APCs, which reduces co-stimulation and eventually may leads to T cell anergy. Indeed anergy occurs because of the lack of proper co-stimulatory molecules for T cell to interact with upon MHC:TCR interaction as a control mechanism to avoid an unsolicited autoimmune response. PD-1 is expressed on hyperactivated and thus exhausted T cells. It binds programmed death ligand-1 (PD-L1), which is constitutively expressed and active on a variety of myeloid and tumor cells. The interaction of these two proteins stimulates T cell apoptosis, which again eliminates potentially auto-reactive T cells in the periphery (20).

Immune Surveillance

As alluded to above, a primary function of the immune system is to surveil 'self' cells for abnormalities. Thus, the immune system is the primary defense mechanism against transformed tumor cells that may emerge following the failure of cellular-level,

innate, tumor suppressor mechanisms. Immune recognition of tumor neoantigen is hypothesized to be the primary route of direct cancer cell elimination. This response is initiated by Type I MHC damage signals followed by an IFN-γ-driven Type 1 immune response mediated by T_h1 cells (46). Macrophages then acquire a pro-inflammatory M1 phenotype with the hallmark of increased antigen presenting capability and increased production of cytokines IL-6, IFN-γ, and tumor necrosis factor alpha (TNF-α) (47). Cytotoxic T cells and NK cells are then recruited and can facilitate direct malignant cell death via perforin membrane pore formation, TNF-related apoptosis inducing ligand (TRAIL), FAS/FASL, and granzyme b (46). Due to these responses, cancerous cells are often killed before they pose a major concern. However, clearly, some tumor cells are not deleted, which can be followed by further tumor immune escape.

Tumor Immune Escape

Immune surveillance is protective against pre-malignant cells, and thus for carcinoma cells to successfully form a growing tumor mass, they must overwhelm or evade the body's immune response resulting in 'tumor escape' (48–53). Tumor escape is often driven by stochastic misappropriation of surface molecule expression. Specific examples include T cell anergy resulting from the upregulation of co-inhibitory checkpoint molecules CTLA-4 and PD-1 described above, or downregulation of MHC I leading to an inability for cytotoxic T cells to surveil for abnormal self-antigen. In addition to surface marker regulation, tumor cells also manipulate their local milieu of cytokines to preferentially attract tumor promoting immune cells such as M2 macrophages, MDSC and T_{reg}. Tumoral cytokines such as TGF-β1 and IL-6 enhance pro-tumor immune cell phenotype while aiding in tumor growth, local but disorganized

angiogenesis, and eventual metastases (54). This recruitment is often initiated by polarization away from an anti-tumor type 1 response toward an ineffectual type 2 or type 3 response (55). Again, many of the studies detailed in this thesis aim to better understand these tumor escape mechanisms in mammary carcinoma to improve immunotherapy treatments.

Cancer Immunity

Checkpoint Immunotherapy Cancer

Checkpoint inhibitor antibodies have been developed to target CTLA-4 and PD-1/ PD-L1 described above. These antibodies have shown clinical success in hematological tumors and solid tumors with a high neoantigen burden, leading to tumor regression and considerable gains in patient survival (56–60). Unfortunately, not all patients have benefited equally from checkpoint inhibition as non- or poor responders, adverse immune events, and tumor hyper-progression have also been reported (6, 61–64). These reports of hyper-progression suggest, paradoxically, that releasing inhibitory control of the CTLA-4 and PD-1/PD-L1 axes on the immune system can aid in tumor progression under certain immunological circumstances. These disparate outcomes clearly indicate that we need more information on a cancer patient's immunological status to ensure that checkpoint and other immunotherapies are delivered into an optimally effective immune context. Case in point the only predictors of therapeutic tumor hyper-progression across all tumor types following checkpoint inhibitor treatment is female sex, which is concerning when treating breast cancer patients with checkpoint immunotherapy (61).

The variable tumor responses to checkpoint immunotherapy are likely explained primarily by the phenotype and quantity of tumor infiltrating lymphocytes (TIL)- the

immune cells that directly occupy the tumor mass (65). However, several other systemic immune factors may also play a role in checkpoint therapy effectiveness. Upregulation of immune inhibitory cells such as MDSCs can impair $CD8^+$ cytotoxic T cell activation, and more broadly, inhibit effector T_h cell function (66). Another important factor is tumor immune polarization as a means of immune evasion by reducing an anti-tumor type 1 response and amplifying a pro-tumor type 2 or type 3 response (67–70). Thus, it is important to understand the Type of T cell-mediated immunity a tumor elicits before administering checkpoint inhibitors that preserve or amplify T cell responses.

Immune Polarization in Breast and Other Carcinomas

Breast cancer-mediated polarization away from an effective type 1 response toward an ineffective, helminth-targeting type 2 response was previously characterized (71, 72), yet no investigations have addressed possible polarization toward type 3 immune responses normally geared for microbial infection. T cell polarization toward a type 3 immune response involving T_h17 or T_h22 cells has been defined in mouse models and human cases of cervical, ovarian, prostate, and gastric cancers (34, 68, 73–77), indicating that there is precedent for its exploration in models of breast cancer. Further, increased expansion of type 3 T_h cells has been observed in autoimmune diseases such as lupus erythematosus, autoimmune encephalomyelitis, and autoimmune arthritis, as well as in *H. pylori* infections. Intriguingly, type 3 responses in these inflammatory conditions are also associated with a significant MDSC response (9, 44, 78). As indicated previously, many breast malignancies lead to the recruitment of MDSCs (43). Is there is a link between this MDSC response and polarization toward type 3 T_h cells that has largely been ignored?

Type 3 Immunity and Cancer: T Helper Cell 22 and Interleukin 22

T helper cell 22. T_h22 cells were first described as a terminally differentiated T_h subset in inflammatory skin diseases: psoriasis, atopic dermatitis, and contact dermatitis (30). To our knowledge, T_h22 have not been explored in the context of breast cancer, though they have been characterized to some extent in a handful of other malignancies. Reinforcing the clinical relevance of this cell type, T_h22 were significantly elevated in the blood and tumors of human patients in gastric, cervical and ovarian cancer (77, 79–81). T_h22 accumulation in blood was correlated with poor prognosis and lymph node metastasis in patients with stage III and IV gastric and ovarian cancer (79–81). In a study investigating T_h22 in human colon cancer, it was found that T_h22 cells supported tumor formation, being positively correlated with cancer proliferation and loss of cell cycle control in adjacent target cells through polycomb repressive complex 2 (82). In the above examples and elsewhere, T_h22 cells have been generally implicated a shaping an aggressive tumor microenvironment through secretion of IL-22 and potentially other undefined factors.

Under normal physiological conditions, T_h22 cells express CCR10 in both human and mouse (30, 83), which is a receptor for CCL27 and CCL28 expressed in skin and mucosal tissues (84, 85). Since these chemokine receptors and their ligands are essential for cell migration and where they congregate, T_h22 cells tend to reside in epithelial barriers (skin and gut) (30). As such, in humans, T_h22 typically comprise of about 1-2% of peripheral blood mononuclear cells and the majority are thought to reside in epithelial barrier tissues and aid in wound healing (86). CCR10 has been shown to be

overexpressed on breast cancer and melanoma tumor cells activated by elevated levels of CCL27 and CCL28, which aids in enhanced tumor invasion and migration (84, 87, 88). Given that normal epithelial tissues express the CCL27 and CCL28 ligands, if an alternative tumor source of these chemokine ligands are present, does this attract T_h22 cells to the tumor? It is clear now from our studies that T_h22 are recruited in the 4T1 mammary carcinoma model, and we believe (though have not yet tested) that tumor derived CCL27/CCL28 ligands may underlie this recruitment and local delivery of pro-tumor IL-22.

Interleukin 22 ligand. Cellular sources of IL-22 are limited mostly to immune cells: T_h1, T_h17, T_h22, type 3 innate lymphoid cells 3 (ILC3) and NK cells (33). STAT3 and AHR are important drivers in *IL-22* transcription initiation and c-maf and suppressor of cytokine signaling 3 (SOCS3) downregulate IL-22 production (33). IL-22 is recognized by a heterodimeric receptor IL-22R1 and IL-10Rb, and although immune cells produce IL-22, they do not express IL-22R1 and therefore, are unresponsive to IL-22 stimulus (89).

IL-22 is a dual-function cytokine that appears to be both immune protective or pro-inflammatory depending on the context. IL-22 acts on epithelial target tissues to enhance survival and proliferation, increase expression of antimicrobial peptide, and aid in wound healing. The anti- or pro-inflammatory nature of IL-22 may be a consequence of target tissue specific response or acute versus chronic inflammation (33). In ulcerative colitis, a chronic inflammatory disease of the colon, hypomorph mutations in the gene loci encoding IL-22 and IL-10Rb are disease risk factors (90, 91), suggesting that IL-22 is critical for maintaining an anti-inflammatory state in the colon. However, IL-22 can

promote inflammation in psoriatic lesions in the skin (92) and is upregulated in the serum of patients with granulomatous mastitis (93).

In breast carcinoma, IL-22 has been investigated for its pro-tumor effects including: promotion of epithelial cell transformation (94, 95), increased invasiveness and migration (96), chemotherapy resistance (97), and uncoincidentally, stimulation of epithelial-mesenchymal transition (EMT) (98). However, the role of IL-22 in shaping the tumor immune environment remains undefined. In normal epithelial target tissues, IL-22 signaling leads to increased activation of Janus kinase (JAK)/STAT pathways specifically activation of STAT1, STAT3 and STAT5 followed by transcription of target genes (99). In keratinocytes, IL-22 stimulates expression of the alarmins S100A8 and S100A9 that have been implicated in MDSC recruitment in other tissues (100–102). Further, IL-22 has been shown to induce the expression of various chemokines, CXCL2 (103), CXCL3 (104), and CXCL5 (101), which aid in MDSC chemotaxis to various target tissues (105–107). Indeed, IL-22 in other tissues has been shown to alter the immune milieu in favor of MDSC recruitment. Does T_h22 and thus IL-22 presence in breast cancers ultimately support MDSC recruitment?

Type 3 Immunity and Cancer: T Helper Cell 17 and Interleukin 17

T helper cell 17. T_h17 cells are a heterogeneous cell type that are similar to T_h22 cells in that they exhibit both pro-inflammatory or anti-inflammatory functions depending on the context. Pro-inflammatory, or pro-pathogenic T_h17 cells are characterized by expression of IL-17A, IL-17F, IL-21, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-22; whereas anti-inflammatory, or non-pathogenic T_h17 cells primarily

express IL-17A, IL-17F, and IL-10. Pathogenic T_h17 are thought to be important drivers of various autoimmune diseases, while conversely, non-pathogenic T_h17 are that to play a regulatory, immunomodulatory role in autoimmune prevention. The development of the pro- versus anti-inflammatory T_h17 phenotype is dependent on the cytokine profile present during T_h17 polarization. Absence of IL-23 drives non-pathogenic T_h17 differentiation while the presence of IL-23, IL-1β or TGF-β3 favors pathogenic T_h17 development (108, 109). In carcinomas, the cytokine profile of T_h17 resembles a non-pathogenic phenotype that may assist in shaping a pro-tumor microenvironment.

Not only are T_h17 a heterogeneous population, but they are among the most 'plastic' of T_h cells in that T_h17 may repolarize following recruitment to a target tissue. The most common repolarization events that T_h17 undergo are reprograming to a T_{reg} which may give rise to an IL-17^+ T_{reg} (110, 111), or reprogramming to a T_h1 phenotype leading to an intermediate IFN-γ^+, IL-$17A^+$ phenotype referred to as $T_h1/17$ (108, 112, 113). To our knowledge, the $T_h1/17$ phenotype preferentially occurs by T_h17 repolarization, but this is not to say that T_h1 reprogramming to T_h17 via the $T_h1/17$ intermediate does not occur. T_h17 cell presence in a solid tumor can also have pro- or anti-tumor effects, depending on the context of the cytokine milieu and tumor microenvironment. This duality likely stems from T_h17 plasticity: in an anti-tumor scenario, T_h17 can transdifferentiate to a $T_h1/17$ IFNγ^+ producing cell to further promote Type 1 immunity (112, 114). $T_h1/17$ may also support anti-cancer immunity by increasing TIL populations through the expression of CXCL9 and CXCL10 (115). In a pro-tumor state, T_h17 may differentiate into a T_{reg}, or even a T_h2 cell phenotype, thus promoting immune suppression and ineffective Type 2 immunity (112). Ultimately, T_h17

repolarization toward a pro or anti-tumor phenotype is largely dependent on the cytokine profile provided by the tumor microenvironment.

Blood and tumor T_h17 populations have been associated with poor prognosis as in colorectal, pancreatic and hepatocellular carcinoma; however, ovarian cancer patients with elevated T_h17 showed enhanced survival (114). In breast cancer patients specifically, increased numbers of T_h17 have been associated with more aggressive subtypes including luminal B ($HER2^+$ luminal) and triple-negative breast cancer (estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative) (116). T_h17 cells have been shown to promote the upregulation of tumor growth-stimulating CXCL1 (117), and they also express the CD39 and CD73 ectonucleotidases, which hydrolyze ATP to adenosine (118). Extracellular adenosine has immunosuppressive functions; it acts to halt effector T cell functions while promoting differentiation of T_{reg}. Thus CD39, CD73, and associated adenosine are now recognized as an immune checkpoint and coincidently, CD39 and CD73 are also highly expressed on MDSCs (119).

Interleukin 17 ligand. While few studies have detailed the effects of T_h17 cells on breast cancer, their hallmark chemokine IL-17 has been scrutinized extensively and predictably has been described as having both pro- and anti-tumor effects. On the pro-tumor side, breast cancer cells exposed to IL-17 upregulate expression of CXCL8, MMP2, and MMP9, which drive tumor angiogenesis and tumor cell invasion into surrounding tissue (120). In the 4T1 mouse model of mammary carcinoma, knocking out IL-17 receptor heightened apoptosis and slowed tumor cell proliferation (121). Additionally, IL-17 has been shown to promote tumor growth and metastasis by

recruitment of neutrophils through CXCR2 chemokine ligands and IL-6 (114, 121). On the anti-tumor side, two studies have now identified IL-17 mediated inhibition of MDSC expansion. MDSC were found to be inhibited by IL-17 in three ways, it: 1) inhibited MDSC proliferation by suspending cells in G0/G1 of the cell cycle, 2) triggered MDSC apoptosis, and 3) stimulated further differentiation to a mature myeloid cell phenotype (122, 123).

Interestingly, MDSC are thought to be immature cells arrested in an early stage of myeloid development, and while the stage of development is unclear, MDSCs have the potential to differentiate into mature myeloid lineages including (potentially anti-tumor) macrophage, neutrophils or dendritic cells (124, 125). Because of this differentiation potential, and the fact that very similar marker profiles are used to distinguish MDSCs from mature macrophage, neutrophils or dendritic cells; there is often confusion as to whether MDSCs or a mature myeloid cell is being identified. Preforming a functional T cell suppression is a good way to distinguish the difference between MDSC and other myeloid cells (126). Nonetheless, IL-17-stimulated MDSC differentiation seems to be in direct opposition to two studies where IL-17 mediated recruitment of neutrophils correlated with poorer breast cancer outcomes (127, 128). This may also be partly due to the multifaced neutrophil phenotypes where N1 neutrophils possess anti-tumor effects versus N2 neutrophils that have pro-tumor effects (129). Additionally, a high neutrophil-to-lymphocyte ratio has been correlated in many studies as an indicator of metastatic potential and poor prognosis (130–134). Therefore, IL-17 stimulated MDSC-to-neutrophil differentiation could, in fact, stimulate metastatic potential. Of course, the

question may be posed are neutrophils correlated breast cancer metastases or are these neutrophils truly just misidentified MDSC?

Myeloid Derived Suppressor Cells in Cancer

MDSCs have long been known to facilitate tumor progression, promote tumor resistance to therapy, and are generally associated with poor prognosis for cancer patients (10). MDSCs can be classified into two distinct subsets: monocytic (M-MDSC) and granulocytic or polymorphonuclear (PMN-MDSC). M-MDSC are more potently suppressive than PMN-MDSC, however, the PMN-MDSC population is observed to be more readily expanded in breast cancer patients. The most prominent forms of direct T_h suppression associated with MDSC include: upregulation of reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) and oxidative NO production, arginine substrate depletion by arginase-1, and local release of inhibitory molecules such as prostaglandin E2 and adenosine (43, 119). Moreover, MDSC facilitate immunosuppression indirectly by TGF-β1 expression (135), which supports the production of T_{reg} (43, 136).

Many systemic growth factors have been implicated in the development and expansion of the MDSC population, with the most prominent candidates being macrophage colony-stimulating factor (M-CSF), GM-CSF, and IL-6 (137). For some time, M-CSF and GM-CSF were presumed to be necessary and sufficient to drive all of MDSC biology, suggesting that altering IL-6 expression alone would not significantly reduce MDSC development in the cancer setting (10). Recently, however, IL-6 has been shown to be involved in MDSC recruitment and may underlie the poor prognosis in many IL-6 overexpressing malignancies including breast cancer (138–140). In therapeutic

models, inhibiting IL-6 signaling in murine squamous cell carcinoma led to a reduction in MDSCs by initiating a shift toward anti-tumor type 1 IFN-γ production (141). Additionally, IL-6 further aids in type 3 T_h polarization (31). Considering its role in cancer and other disease states, IL-6 has also become a sought-after therapeutic target in cancer due to the appearance of FDA approved tocilizumab in 2010 to treat autoimmune arthritis (142).

Interleukin 6 and Clustered Regularly Interspaced Short Palindromic Repeats

Interleukin 6. There are two types of inflammation- acute and chronic. Acute inflammation occurs when the immune system recognizes damaged tissue, but there is minimal damage or stimuli, so the acute inflammatory response mediated by neutrophils will initiate healing and resolve quickly. Chronic inflammation ensues when significant, prolonged exposure to inflammatory stimuli exists over months of time as would likely occur in cancer, autoimmunity, or sepsis. The persistent activation of adaptive and innate immune cells, coupled with continuous tissue repair and remodeling, leads to polarization of an immune response that is often initially correct but may progressively drift or change to actually further exacerbate inflammation (143).

IL-6 is a pleiotropic cytokine that plays a role in the initiation of both acute and chronic inflammatory responses. Acutely, IL-6 is a major upstream regulator of acute phase, damage-response proteins (144). Chronically, IL-6 helps promote an adaptive immune response and further shapes and defines the innate immune response. IL-6 signal transduction begins when IL-6 ligand enables dimerization of the IL6R and gp130 membrane receptors, which are coupled to a JAK/STAT signaling cascade, and IL-6

activation of STAT3 transcription factor is most notable (145). gp130 is ubiquitously expressed, while IL6R expression appears to be limited to hepatocytes, leukocytes, and megakaryocytes; however, IL-6 signaling is not limited to these few cell types. In a phenomenon known as IL-6 'trans-signaling,' a metalloprotease specifically ADAM17 (146) cleaves IL6R from the cell surface freeing soluble IL6R that can stimulate cells expressing gp130- which would explain IL-6's ability to signal systemically to a variety of tissues (145). In cancer, IL-6 plays a role in manipulating both immune landscape and tumor growth by promoting cell proliferation and survival through STAT3 activation (147).

Although IL-6 was initially thought of as an anti-tumor cytokine due to its conventional role in initiating an acute inflammatory response (148), the prolonged, chronic expression of IL-6 appears to directly support the implementation and differentiation of pro-tumor immunity. For example, IL-6 directly polarizes T_h mediated immunity away from an anti-tumor type 1 IFN-γ T_h1 response (149) toward a pro-tumor type 3 T_h17 or T_h22 phenotype (31). Moreover, chronic IL-6 expression in sepsis, cancer, autoimmune disease, traumatic injury and now obesity has been correlated with MDSC accumulation (139, 150–152). For some time, GM-CSF and M-CSF were solely credited with MDSC formation, with little attention paid to IL-6 (10). Indeed, M-CSF and GM-CSF are important factors in the promotion of general myelopoiesis, but their expression alone does not account for the arrested development of the myeloid lineage that spawns MDSCs (10, 153).

Indeed, it is the presence of chronic IL-6 signaling that prompts immature myeloid accumulation with a suppressive, MDSC phenotype. Prolonged IL-6 signal

transduction results in altered myelopoiesis within the bone marrow, which has been linked to suppression of SOCS3, which under steady state conditions feeds back to inhibit JAK/STAT activation. A decrease in SOCS3 allows for continuous phosphorylation and activation of STAT3 by chronic IL-6 stimulation, and it also promotes GM-CSF and M-CSF signaling that further enhances myelopoiesis (139).

However, there remain many unresolved questions in the interactions between IL-6 and MDSCs. For example, how does chronic IL-6 affect myelopoiesis such that myeloid development is arrested at the 'MDSC stage'? Does IL-6 affect myelopoiesis by systemic delivery of IL-6 to the bone marrow? Or are MDSCs suppressive when they leave the bone marrow, or do they require further 'education', *i.e.*, prolonged exposure to IL-6 or splenic 'education' to become suppressive? Do MDSCs react to acute bouts of IL-6 expression, such as exercise or physical activity? Is this a concentration-, source specific- or simply chronic expression-dependent effect of IL-6 on MDSC? Further questions emerge regarding T_h populations and chronic IL-6 exposure. Does chronic IL-6 affect polarization to T_h17 versus T_h22? Is it possible that reducing tumor IL-6 increases an IFN-γ mediated immune response? Does IL-6 affect T_h17 and T_h22 systemically or locally within the tumor? Is tumor IL-6 necessary for T_h recruitment to the tumor?

Clustered regulatory interspersed short palindromic repeats. Considering the significant number of questions circulating around about IL-6 listed above, we targeted IL-6 for knockout using CRISPR-Cas9 in the 4T1 model of mammary carcinoma. Clustered regulatory interspersed short palindromic repeats (CRISPR) were originally discovered as a bacterial adaptive immune system against bacteriophage virus infection. In short, bacteria store DNA sequence from previous viral encounters in between

clustered regulatory interspersed short palindromic repeats of DNA. These segments then get transcribed into RNA know as a guide RNA (gRNA) and loaded into a Cas endonuclease. gRNA loaded Cas seek out the specific viral DNA target and induce a double stranded break when proper base pairing is formed (154–156). In 2013, the Zhang lab was the first to successfully take advantage of the CRISPR-Cas9 system for genome editing in eukaryotic cells. In eukaryotic cells, the principal is the same: a gRNA is designed for a gene of choice and is either 1) loaded directly into a Cas (Cas9 being the most common) or 2) coded into a plasmid containing Cas coding region. This is injected or transfected into a cell, then the Cas9 gRNA complex unwinds genomic DNA probing the entire genome for base pairing matches. Each Cas has a protospacer adjacent motif (PAM) requirement, which must be recognized in the adjacent strand to the gRNA target in DNA before cutting will occur. When a complete match occurs the Cas endonuclease induces a double stranded DNA cut. Once a double stranded break is induced the cell will repair DNA by non-homologous end joining, often leaving small insertions or deletions (157).Thus rendering a knock out of the particular target gene.

Rationale for Study

As alluded to above, the classical view of the MDSC suppressive activity is direct cell-to-cell inhibition of the effector cytotoxic and T_h populations. This model of MDSC suppression has overshadowed other potential immune interactions, specifically the interplay between type 3 T_h17 and T_h22 cells (9). It is thought that solid tumors of epithelial origin benefit from recruitment of type 3 T_h helper cells due to amplified levels growth and survival-promoting IL-22 and IL-17A. The chronic presence of these Type 3 cytokines has been linked to tumor promotion as well as increased metastatic potential

(33, 76, 158). In addition, IL-6 is implicated in both MDSC recruitment and T_h17/ T_h22 polarization (31, 139), yet few studies have logically linked these phenomenon and explored whether chronic expression of IL-6 may, in fact, elicit both MDSC and Type 3 T_h expansion in the context of mammary carcinoma (31, 139).

These studies examine the response of canonical type 3 T helper populations: T_h17, and non-canonical populations: T_h22, in the 4T1 model of mouse mammary carcinoma. Additionally, we characterize the response of $T_h1/17$, which are T_h17 cells transiting to a T_h1 phenotype and are an indicator of immune polarization toward an effective, anti-tumor type 1 immune response (159). The 4T1 model was chosen for this study because it is representative of a triple negative breast cancer that does not have high levels of TIL, with a low mutational load rendering it unresponsive to immune checkpoint therapy (160, 161). 4T1 cells had also been shown by others to elicit a pronounced myeloid reaction in the form of MDSCs (162). Our findings provide further insight into the interactions among MDSC and type 3 immune cells, as well as IL-6, in a murine mammary cancer model (Figure 1). These results may help clarify some of the failures of immunotherapies in human breast cancers and inform future attempts aimed at improving immune and other therapies.

CHAPTER III

METHODS

Mice and 4T1 Mammary Tumors

4T1 cells were purchased from the ATCC (Manassas, VA) and cultured in RPMI 1640 (ThermoFisher) supplemented with, 1% pen/strep, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 10 mM HEPES, 0.05 mM β-mercaptoethanol, and 10% (by volume) FB Essence (VWR), the latter which is a mixed serum supplement. To grow syngeneic mammary tumors, female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) between four to six weeks of age were inoculated with 1.0×10^4 4T1 cells. Cells were washed and suspended in 100 µL 1X HBSS (Ca^{2+}/Mg^{2+} free, ThermoFisher) before injection through the nipple of the upper right mammary fat pad using an insulin syringe. Mice were monitored daily for availability of food and water and any signs or symptoms of peripheral infection or inflammation. Palpable tumors were routinely detected 2 weeks after injection and tumor sizes were monitored by calipers. Final tumor volumes were calculated by (volume=(3.14/6)width×length^2)(163). On the day of tissue harvest, mice were euthanized by CO_2 asphyxiation and tumors weighed. All animal procedures were performed according to the Institutional Animal Care and Use Committee protocol 1906CE-RH-RM-22, (previously 1511CE-RH-RM-18) and 1702C-NP-M-20. Additionally, the 4T1 tumor time course study was conducted, and tissue harvested at days 0, 7, 14, 21, and 28 post injection time point (Figure 2).

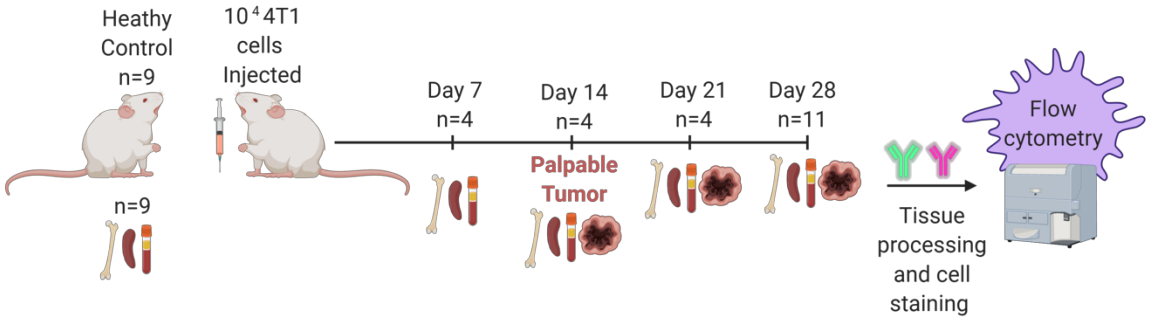


Figure 2: 4T1 wild type time-course study. Wild Type 4T1 (4T1-WT) were injected into non-tumor bearing mice. Spleen, blood, bone marrow, and tumor were harvested on days 7, 14, 21 and 28 post 4T1-WT injection. Tissue was processed, stained, and analyzed by flow cytometry. Heathy non-tumor bearing mice were included as a control. Mice form palpable tumor day 14 after injection.

Interleukin 6 Gene Knock-Out in 4T1 Cells

The CRISPR/Cas9 system was used to create 4T1 cells deficient in IL-6 (4T1-IL6-KO). Guide RNAs were designed with CRISPOR software (http://crispor.tefor.net/) to target exon 2 of the *Mus musculus il6* gene (GenBank: M24221.1) at sequence TATACCACTTCACAAGTCGG. The gRNA oligos (Invitrogen) were annealed, phosphorylated, and cloned into the pX458 vector following the Zhang lab protocol (157). pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138; http://n2t.net/addgene:48138 ; RRID:Addgene_48138) (Figure 3). A transfection protocol using Lipofectamine 3000 (ThermoFisher) was optimized for 4T1 at 50% confluency in 12 well dishes by growing cells overnight in a 100 µl of additive-free RMPI 1640 containing transfection lipid and plasmid. Lipid and plasmid volume remained the same as suggested in the ThermoFisher protocol. The vector includes a green fluorescent protein (GFP) reporter, thus GFP^+ cells were single-cell sorted into 96-well plates using a Sony SH800 Cell Sorter. Subclones were cultured and IL-6 deficiency

was verified by fixed-cell flow cytometry with IL-6 antibody (BioLegend, #504504) (Figure 3). Two independent subclones verified as IL-6 knockouts were pooled to create the 4T1-IL6-KO line used in these studies.

Figure 3: pSpCas9(BB)-2A-GFP (PX458) vector. gRNA with sequence TATACCACTTCACAAGTCGG was cloned into pX458 vector. pX458 plasmid map shown here from Addgene (Addgene #48138).

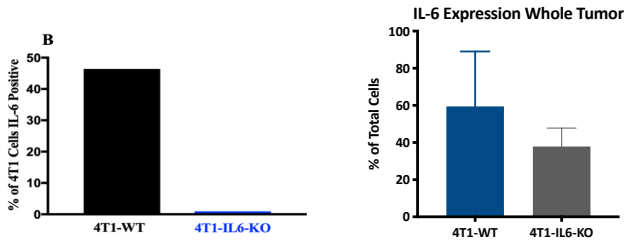


Figure 4: CRISPR/Cas9 knockout of IL-6 in the 4T1 cell line. Wild Type 4T1 (4T1-WT) versus IL-6 knock out 4T1 (4T1-IL6-KO) expression of IL-6 via intracellular flow cytometry. (**A**) Histogram demonstrating relative fluorescence, and (**B**) Percent of total 4T1 that are IL-6 positive. (**C**) Total cells in a 4T1-IL6-KO tumor positive for IL-6.

Tissue Processing

Spleens were weighed, dissected, and dissociated using a rubber policeman in 500 µL of 1X HBSS. Blood was collected from the chest cavity following a cut to the aorta and local heparin infusion. Blood was centrifuged at 0.2 rcf for 10 min and buffy coat and plasma transferred to fresh vials and any residual red blood cells lysed with ACK lysis buffer. Bone marrow was flushed from excised femurs and tibias using HBSS. Final tumor sizes were measured prior to excision, and tumors were weighed after excision and removal of extraneous tissue. Tumor tissue was minced and mechanically separated using a cell dissociation sieve fitted with a 100 µm mesh screen and resuspended in 1 ml HBSS. Cells were resuspended in Type IV collagenase (2mg/1mL) and DNase (0.1 mg/mL; Worthington) and incubated at 37 ºC rocking at 225 rpm for 1 hour. Remaining erythrocytes from various tissues were cleared with ACK lysis buffer (Quality Biological).

Flow Cytometry and Antibodies

Cells were stained in flow cytometry buffer: 0.5% BSA in Ca^{2+}/Mg^{2+} free 1X Dulbecco's PBS (ThermoFisher). Flow reagents and antibodies were purchased from Biolegend (San Diego, CA) unless stated otherwise. Fc block (101302) was used prior to staining with extracellular markers following the manufacturer's protocol. Any surface marker staining was performed prior to fixation in fixation buffer (420801) per the manufacturer's recommendation. For intracellular markers, prior to cell surface staining and fixation, cells were treated with 1X brefeldin A (420601) for 3-4 h at 37 ºC to block intracellular protein trafficking. Cells were then stained for surface markers, fixed, then permeabilized with permeabilization buffer (421002), and finally stained for intracellular targets according to manufacturer's protocols. Samples were analyzed using an Attune NxT cytometer (ThermoFisher); raw data were processed in FCS Express (De Novo Software). The biomarker profiles and antibodies used to define cell types in these studies outlined in Table 1 and 2. Example of flow cytometric gating strategy Figure 5A, B.

Table 1: Cell biomarker profiles

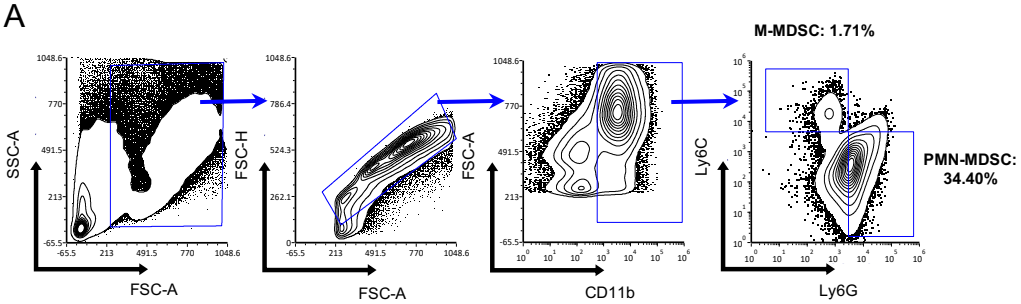
Cell Type	Biomarker Profile
M-MDSC	$CD11b^{+}Ly\text{-}6G^{-}Ly\text{-}6C^{hi}$

PMN-MDSC	$CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{low}$
T_h	$CD3^{+}CD4^{+}$
T_h17	$CD3^{+}CD4^{+}ROR\gamma t^{+}IFN\text{-}\gamma^{-}IL\text{-}17A^{+}IL\text{-}22^{+/-}$
$T_h1/17$	$CD3^{+}CD4^{+}ROR\gamma t^{+}IFN\text{-}\gamma^{+}IL\text{-}17A^{+}IL\text{-}22^{+/-}$
T_h1	$CD3^{+}CD4^{+}IFN\text{-}\gamma^{+}ROR\gamma t^{-}IL\text{-}17A^{-}IL\text{-}22^{+/-}$
T_h22	$CD3^{+}CD4^{+}IFN\text{-}\gamma^{-}ROR\gamma t^{-}IL\text{-}17A^{-}IL\text{-}22^{+}$

Table 2: Antibodies and Catalog Numbers

Biomarker	Antibody Catalog Number
CD3	100237 Biolegend
CD4	100438 Biolegend
IFN-γ	505806 Biolegend
IL-22	516409 Biolegend
IL-17A	506904 Biolegend
RORγt	46698180 ThermoFisher
MDSC antibody cocktail	77496 Biolegend

A



B

Figure 5: Flow cytometry gating strategies. Representative samples of (**A**) MDSC and (**B**) T_h gating strategies. Blood represented.

Statistical Analysis

All data are presented as mean ±SEM and statistical tests used $\alpha = 0.05$. Grubbs' test was used to identify any outliers. All multiple comparisons were calculated using

one-way ANOVA with Turkey's multiple comparisons test. Single comparisons were calculated using Student's t-test. A simple linear regression and Pearson's correlation were used to assess the relationship between cell types in the 4T1-WT time course study for each of these regressions animals across all time points were included. A confidence interval (CI) of 95% is represented as well as Pearson r and r^2 values, with significance set by $\alpha = 0.05$. All statistical analyses were conducted in Prism 8 (GraphPad). R^2 (coefficient of determination) and r (Pearson correlation coefficient) values are used to predict the relationship between independent and dependent variables in a linear model. R represents the overall strength and direction of a correlation, while r^2 is indicative of how well the data fit to a regression line. R values are measured from -1 to +1; 0 indicating no relationship. R^2 are measured from 0 to +1 while 0 indicates the model explains none of the variability (164).

CHAPTER IV

RESULTS

T Hepler Cell Immune Populations in Response to 4T1 Wild Type Tumors

Tumor and Spleen

We assessed the T_h immune response including type 1, type 3, and transitional T_h populations in animals bearing 4T1 mammary tumors at 0 (no tumor control), 7, 14, 21 or 28 days (d) after tumor cell injection. When we inject 10^4 4T1 carcinoma cells into the mammary gland of our BALB/c mice, small but palpable tumors form at the injection site by d 14 (Figure 6A, B). Others have also reported increased spleen size, or splenomegaly, in this model (165). Interestingly, in d 7 animals we observed a significant reduction in spleen mass relative to controls, but by days 21 and 28 the tumor-bearing animals show a pronounced increase in spleen size as has been reported elsewhere (Figure 6C). To our knowledge, this is the first report of this early reduction in spleen size in this 4T1 tumor model.

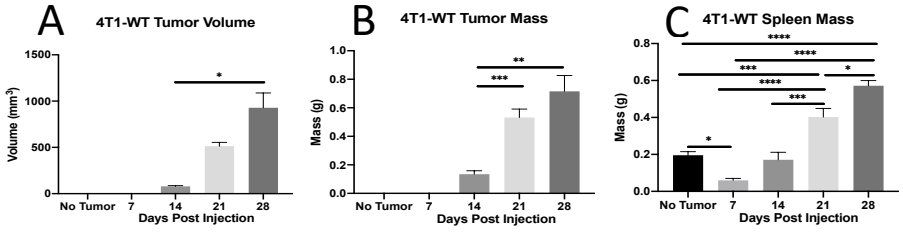


Figure 6: Tumor and spleen sizes in 4T1 tumor bearing animals. 4T1-WT carcinoma cells (10^4) were injected into the mammary gland of BALB/c mice and harvested at 0 (no tumor control), 7, 14, 21 or 28 d post injection. (**A**) Tumor volume, (**B**) tumor mass and (**C**) spleen mass were determined at the indicated times. Data are represented as mean ± SEM; n= 4-11 mice per time point. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001.

Total T Helper Cell Populations

Various T_h cell phenotypes were assessed in spleen, blood, bone marrow, and tumor at each time point. The percentage of total T_h cells remained relatively unchanged in tumor, blood, and other peripheral tissues with modest increases detected in spleen and bone marrow at days 14 and 21, respectively (Figure 7A, B).

Type 3 T Helper Cell Populations

Within the total T_h cell population, however, we observed dynamic changes in the T_h cell subtype. In the spleen, the T_h17 population increased significantly by d 7 post-injection compared to no tumor controls and declined thereafter such that the percentage of T_h17 recruited/remaining by d 28 was significantly lower than d 7. This same trend in T_h17 recruitment was observed in the blood and bone marrow (Figure 7, G-H). Notably, unlike T_h17, T_h22 cells in peripheral organs were not expanded initially at d 7 compared to no tumor controls. T_h22 increased significantly over time in blood compared to heathy controls (Figure 7I). In the tumors, T_h17 and T_h22 are present initially at d 14, T_h17 significantly decline by d 28 whereas T_h22 significantly increase at d 28 compared to d 14 tumors (Figure 7H, J). Due to the inverse pattern of T_h17 and T_h22 recruitment, we computed the Pearson correlation between T_h22 and T_h17 polarization over time. There was no correlation between T_h22 and T_h17 in the blood or bone marrow; however, there was a significant negative correlation (r^2=0.14) in the spleen, and a significant positive correlation in tumors (r^2=0.25; Figure 8A-D).

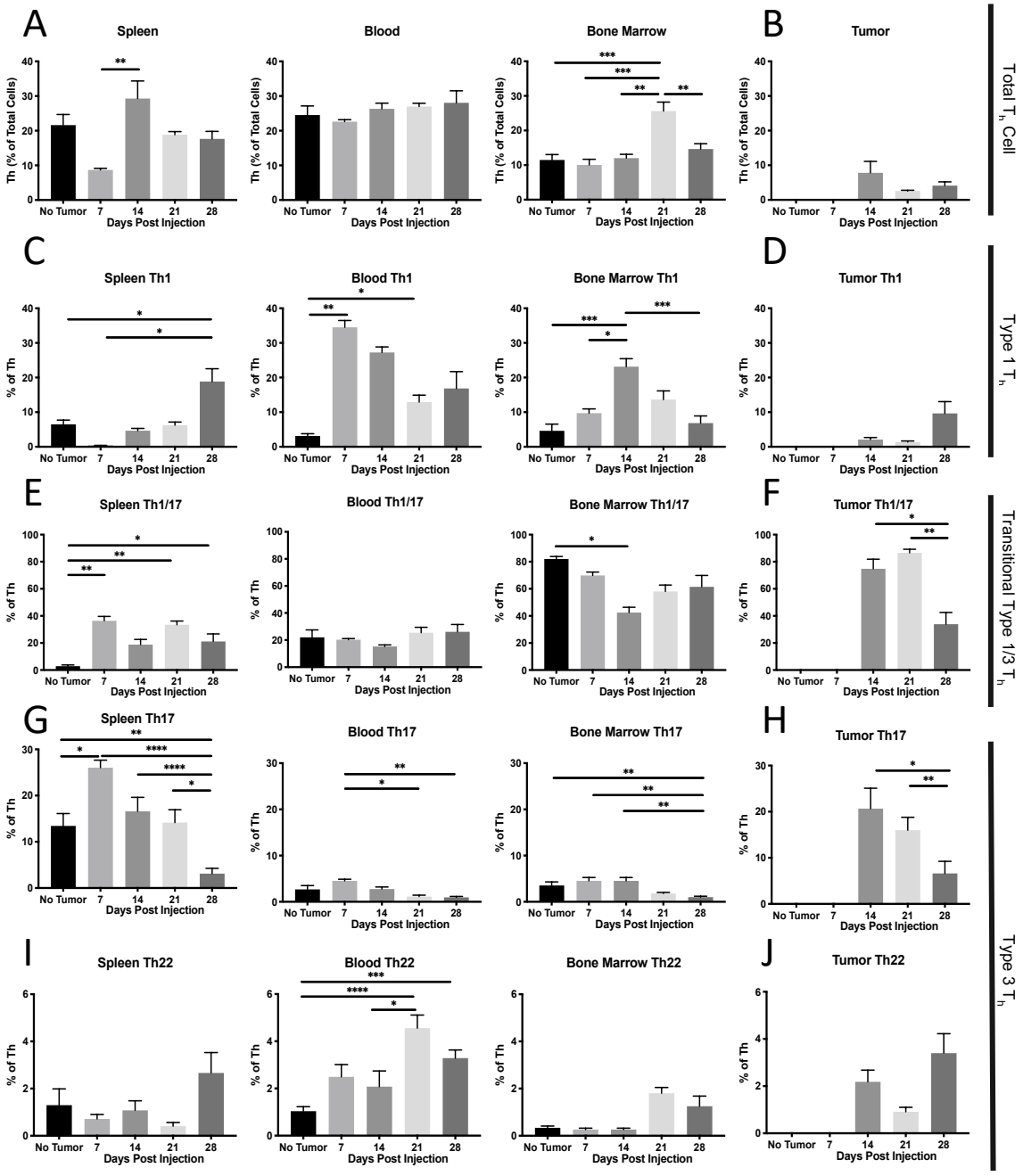


Figure 7: T_h populations over time in 4T1-WT tumor bearing mice. Flow cytometric measurement of total T_h, T_h1, $T_h1/17$, T_h17, and T_h22 in peripheral tissues (**A, C, E, G, I**) or tumors (**B, D, F, H, J**) at the indicated times. Data are represented as mean ± SEM and n= 4-11 mice per time point. **p*<0.05, ***p*<0.001, ****p*<0.001 and ****p*<0.0001.

T Helper Cell 1 Polarization

T_h1 recruitment was also assessed. The spleen was the only tissue where T_h1 remained statistically elevated compared to healthy animals (Figure 7C). In the blood, T_h1 were significantly expanded at all times post injection consistent with a mobilization of this T_h subtype. In bone marrow, T_h1 were significantly elevated compared to control and d 28 tumor-bearing mice (Figure 7C). Although T_h1 are present in the tumor, there was no statistical significance seen over time suggesting that increased circulating T_h1 in the blood did not result in a pronounced infiltration of these cells into the tumor microenvironment (Figure 7D).

We also examined correlations between T_h1, T_h17, and T_h22 and found significant negative correlations between T_h1 and T_h17 in the spleen and tumor (Figure 8E, H), but a significant positive correlation in the bone marrow (Figure 8G). When assessing T_h1 versus T_h22, there was a significant negative correlation between these two cell types in the tumor (Figure 8L). These findings suggest that in the spleen or tumor there is potential for polarization away from type 1 toward a type 3 T_h phenotype. In addition, it may be that spleen and tumor are conducive to initial T_h activation and polarization (or repolarization), which may explain the relationships in these tissues, but not in blood (Figure 8F, J).

Transitional Type 1 to 3 T Helper Cell 1/17 Populations

$T_h1/17$ were relatively unchanged in blood and bone marrow throughout the time course. However, in the spleen $T_h1/17$ were significantly increased compared to d 0 at every time point except d 14 (Figure 7E). The same trend was not seen in the tumor.

$T_h1/17$ were initially detectable in the tumor at d 14 but were then significantly reduced at d 28 (Figure 7F). Interestingly, the pattern of recruitment of $T_h1/17$ and T_h17 to the tumor were similar during the various time points. Due to the $T_h1/17$ differentiation plasticity that exists between T_h1 and T_h17, we looked at Pearson correlations and found that $T_h1/17$ and T_h17 were positively correlated over time (r^2=0.62, Figure 8P). However, no correlation was observed in peripheral organs (Figure 8, M-O). When examining $T_h1/17$ and T_h1 we saw significant negative correlations in the spleen (r^2=0.19), blood (r^2=0.36) and bone marrow (r^2=0.76; Figure 7, Q-S) but a minimal relationship in the tumor (Figure 8T). These correlation patterns are consistent with differentiation away from the type I T_h1 cells toward the type 3 T_h17 phenotype.

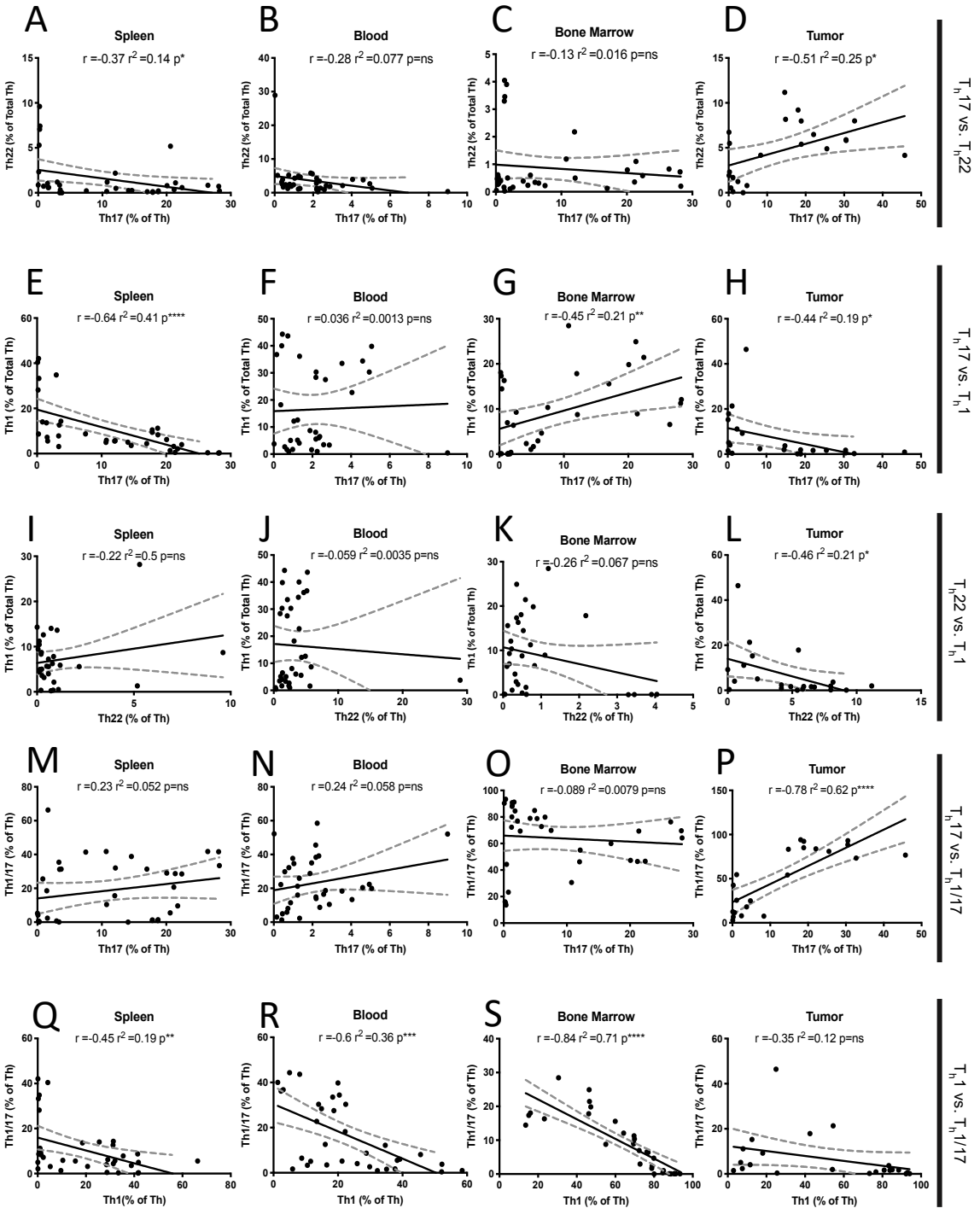


Figure 8: Correlations among T_h subsets in 4T1-WT tumor bearing mice. Pearson correlations between (**A-D**) T_h17 and T_h22 (**E-H**) T_h17 and T_h1 (**I-L**) T_h17 and T_h1 (**M-L**) T_h17 and $T_h1/17$ (**Q-T**) T_h1 and $T_h1/17$ levels in spleen, blood, bone marrow and tumors in 4T1-WT tumor bearing mice based on data shown in Figure 7. Simple linear regression and 95% CI are included to help visualize relationships. **p*<0.05, ***p*<0.001, ****p*<0.001, ****p*<0.0001, and *****p*<0.00001

Myeloid Derived Suppressor Cell Expansion and Relationship to Type 3 T Helper Cell 17 and T Helper Cell 22

Myeloid Derived Suppressor Cells

MDSCs were measured in the spleen, blood, bone marrow and tumor, and both M-MDSC and PMN-MDSC exhibited expansion in the peripheral organs, though each displayed a distinct pattern of MDSC expansion (Figure 9, A-C). In the spleen, MDSCs progressively increased over time, while blood MDSCs were not significantly elevated until late stage (d 28) of tumor development. In the bone marrow, MDSCs peaked early on at d 7, and then declined at later time points, however, they remained significantly elevated above baseline. Recruitment to the tumor differed between M-MDSC and PMN-MDSC yet were both present by d 14 (Figure 9B, D). M-MDSC did not change significantly over time, whereas PMN-MDSC progressively increased in the 4T1 tumors.

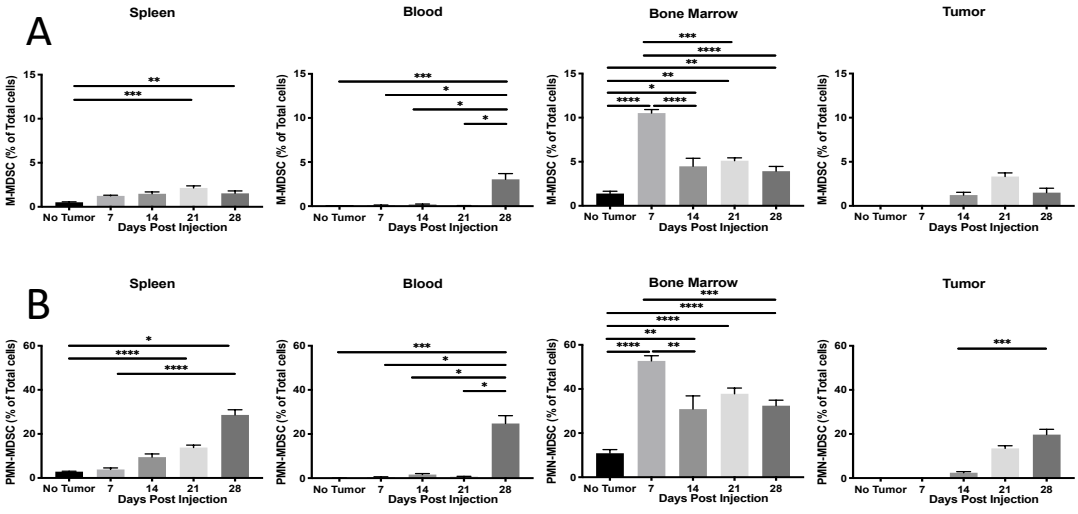


Figure 9: 4T1-WT tumor-induced MDSC expansion. Flow cytometric measurement of (**A**) M-MDSC ($CD11b^{+}Ly\text{-}6G^{-}Ly\text{-}6C^{hi}$) and (**B**) PMN-MDSC ($CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{low}$) in response to 4T1-WT mammary carcinoma over time. Data are represented as mean ± SEM and n= 4-11 mice per time point. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001.

Correlating Myeloid Derived Suppressor Cell and Type 3 T Helper Cell

Due to a positive MDSC and type 3 T_h correlation in other systems (lupus erythematosus, autoimmune encephalomyelitis, rheumatoid arthritis, and autoimmune arthritis, *H. pylori* infections, and colon cancer (9, 44, 78) suggesting that perhaps MDSCs may be directly stimulating the type 3 immune response, we wanted to determine if similar associations exist in mammary carcinoma. Again, we used Pearson's correlations to compare the patterns of various T_h cell types described in Figure 7 across the time course of MDSC proportions in Figure 9 (Figure 10). No significant correlation was observed in the spleen or tumor between MDSCs and any of the T_h cell types. There were significant negative correlations in blood between T_h17 and PMN- or M-MDSCs, r^2=0.21 and r^2=0.42 respectively (Figure 10J, N). In the bone marrow, we observed no relationship between T_h22 and MDSC (Figure 10C, G). When looking at the correlation between T_h17 and MDSC in bone marrow we saw a positive association between PMN-MDSC and T_h17 with r^2=0.41 as well as M-MDSC and T_h17 r^2=0.67 (Figure 10K and O). Cumulatively this suggests that MDSC likely do not elicit type 3 T_h in mammary cancer specifically in tumors. MDSC may, however, recruit T_h17 in the bone marrow of 4T1 tumor bearing mice.

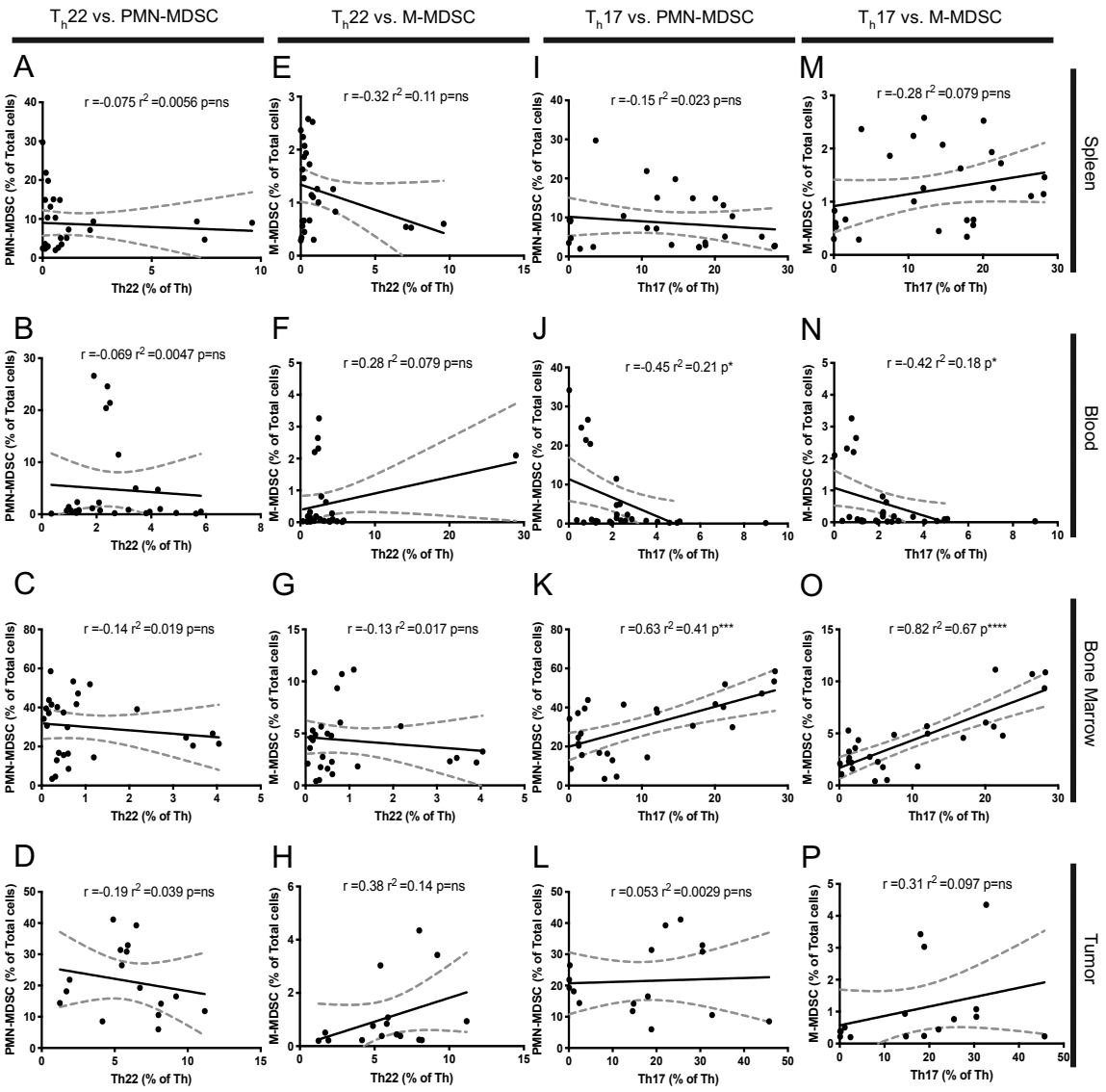


Figure 10: Correlations between Type 3 T_h and MDSC accumulation. Pearson correlation assessing the association between Type 3 T_h percentages shown in Figure 7, and MDSC percentages shown in Figure 9. (**A-D**) T_h22 and PMN-MDSC, (**E-H**) T_h22 and M-MDSC, (**I-J**) T_h17 and PMN-MDSC, and (**M-P**) T_h17 and M-MDSC recruitment in the spleen, blood, bone marrow and tumor of 4T1 tumor-bearing animals. Simple linear regression included to visualize relationship and 95% CI are represented. *p<0.05, **p<0.001, ***p<0.001, ***p<0.0001, and ****p<0.00001.

The Role of Tumor Interleukin 6 in Type 3 T Helper Cell Recruitment

Following our initial time course study, we chose to knock out IL-6 using CRISPR-Cas9 (Figure 3, (166)) to generate a 4T1 IL-6 knock out (4T1-IL6-KO) cell line. Unlike other studies in which IL-6 is knocked out in the entire animal (167), this approach narrows the IL-6 deficiency to the 4T1 tumor cells. Indeed, in whole tumors there are still stromal and immune cells recruited that produce IL-6 (Figure 4). We did not observe a significant difference in tumor volume over time or day 28 tumor mass between 4T1-WT and 4T1-IL6-KO tumors (Figure 11A-B). Day 28 spleen mass was significantly elevated in 4T1-IL6-KO compared to healthy no tumor controls (Figure 11C).

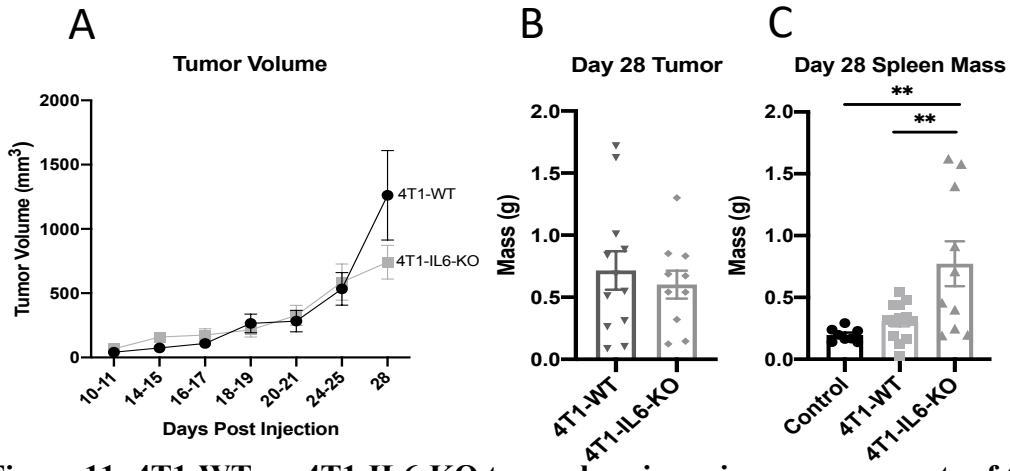


Figure 11: 4T1-WT vs. 4T1-IL6-KO tumor bearing mice measurements of tumor and spleen. (**A**) 4T1-WT and 4T1-IL6-KO tumor volumes were measured over the course of progression. 4T1-WT and 4T1-IL6-KO tumor volumes were not statistically significant at any time point. (**B**) Final tumor mass comparing 4T1-WT and 4T1-IL6-KO tumors at a day 28 post injection time point. Tumors were not statistically different in mass. Measurements in (**A**) and (**B**) should likely be repeated as many animals developed two tumors and is likely a result of miss injection. (**C**) Spleen mass at a day 28 time point. Healthy non-tumor bearing spleens included for reference. 4T1-IL6-KO spleens have a significantly greater mass than 4T1-WT and healthy controls. ANOVA with Tukey's multiple comparisons. Data are represented as means ± SEM and n= 6-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001.

Based on the results obtained in the earlier time course, we decided to look at type 3 accumulation at the d 28 time point with the 4T1-IL6-KO cells. While 4T1-WT tumors significantly reduced T_h17 at d 28 in the periphery compared to healthy controls, T_h17 remained unchanged with the 4T1-IL6-KO tumors in all peripheral organs (Figure 12A). T_h22 were expanded in blood and bone marrow of 4T1-WT mice yet remained unchanged in spleen and blood in 4T1-IL6-KO. In the bone marrow, T_h22 were significantly reduced in 4T1-IL6-KO compared to 4T1-WT tumor-bearing mice (Figure 12C). In tumors, T_h17 are expanded (Figure 12B) and T_h22 are significantly reduced (Figure 12D) in 4T1-IL6-KO compared to 4T1-WT. These data suggest that T_h17 are affected by tumoral IL-6 local to the tumor. This observation may be in line with the tumor microenvironment ability to re-polarize the type of T_h immunity (68). We saw little systemic effect on T_h17 due to the reduction of IL-6 but we see a significant reduction of T_h22 in the bone marrow. This observation may suggest that 4T1-IL6-KO have a lower metastatic potential to the bone marrow than 4T1-WT due to the reduction in T_h22 in the bone marrow.

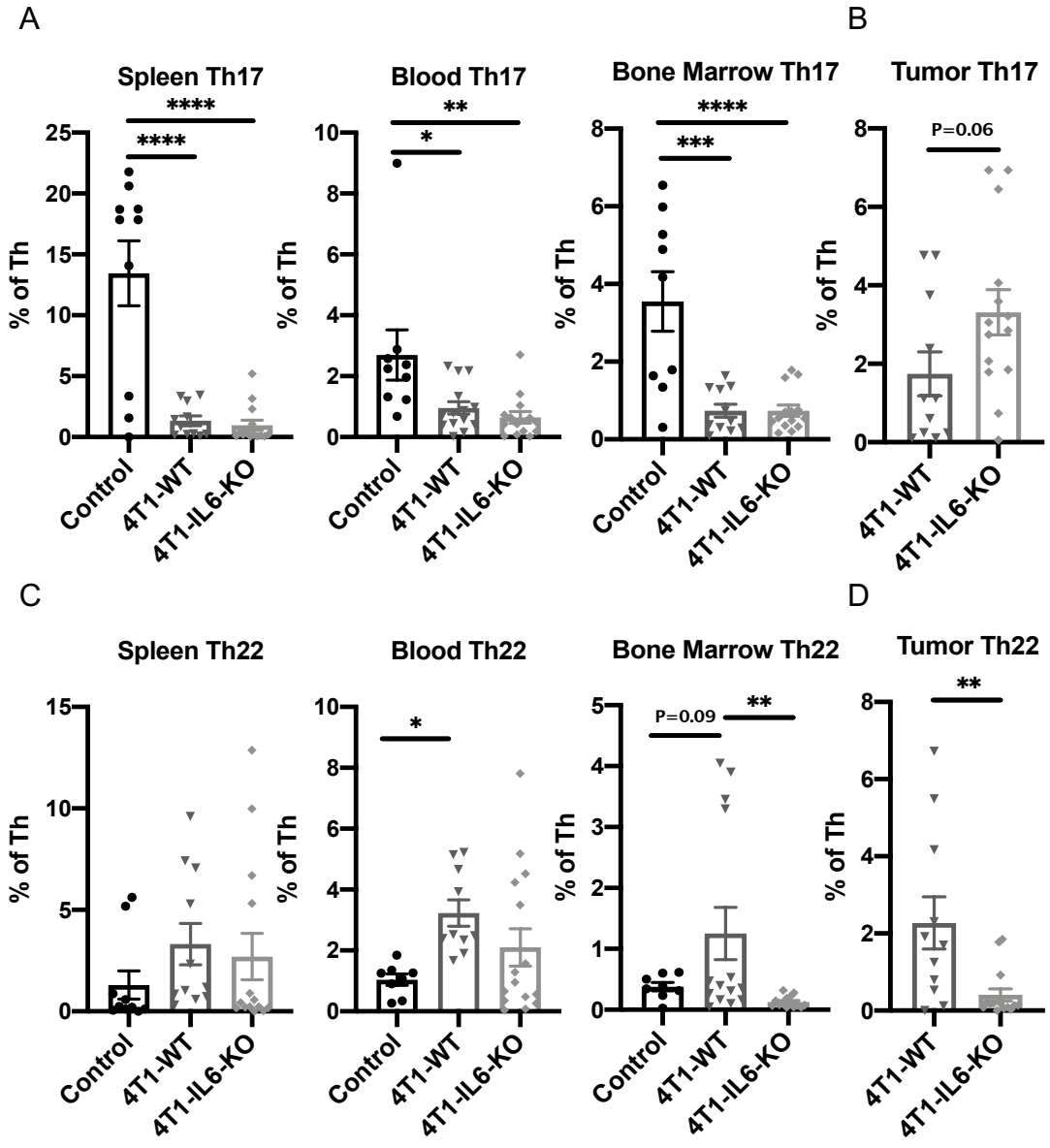


Figure 12: Tumor IL-6 affects type 3 T_h recruitment to the tumor at day 28. Day 28 recruitment of (**A-B**) T_h17 and (**C-D**) T_h22 in healthy non-tumor controls, 4T1-WT and 4T1-IL6-KO in periphery and tumors of tumor bearing mice. 4T1-IL6-KO is 4T1 specific IL-6 knockout. Statistical significance was measured using one-way ANOVA with Tukey's multiple comparisons or students t-test. Data are represented as means ± SEM and n= 4-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001

Effects of Tumor Interleukin 6 on T Helper Cell 1, T Helper Cell 1/17, T Helper Cell, and Myeloid Derived Suppressor Cell

We also examined $T_h1/17$, T_h1, total T_h, and MDSC recruitment in the 4T1-IL6-KO model as these cell populations could be viewed as indicators of a 'pro-immunotherapeutic' environment assuming tumor IL-6 could be similarly depleted in the clinic. In 4T1-IL6-KO tumor bearing mice, the percentage of total Th cells remained unchanged in spleen and bone marrow but were significantly increased in blood and tumors (Figure 13). We found that $T_h1/17$ were significantly increased in blood and tumor in 4T1-IL6-KO compared to 4T1-WT tumor bearing mice. Compared to 4T1-WT where $T_h1/17$ polarization was readily apparent, in 4T1-IL6-KO tumors and $T_h1/17$ were significantly increased (Figure 14A). T_h1 significantly increased in the spleen and significantly decreased in the blood in 4T1-IL6-KO tumor bearing mice compared to 4T1-WT counterparts suggesting that removal of tumoral IL-6 may be therapeutically beneficial (Figure14B).

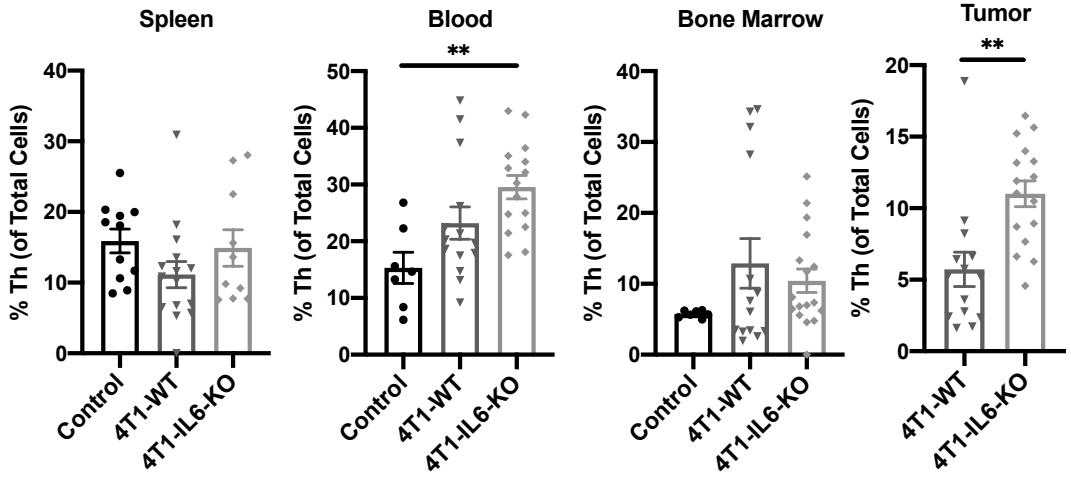


Figure 13: Tumor IL-6 depletion increases tumor-infiltrating T_h Cells. T_h cell recruitment at d 28 by 4T1-WT and 4T1-IL6-KO tumors. Data are represented as means ± SEM and n= 4-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001

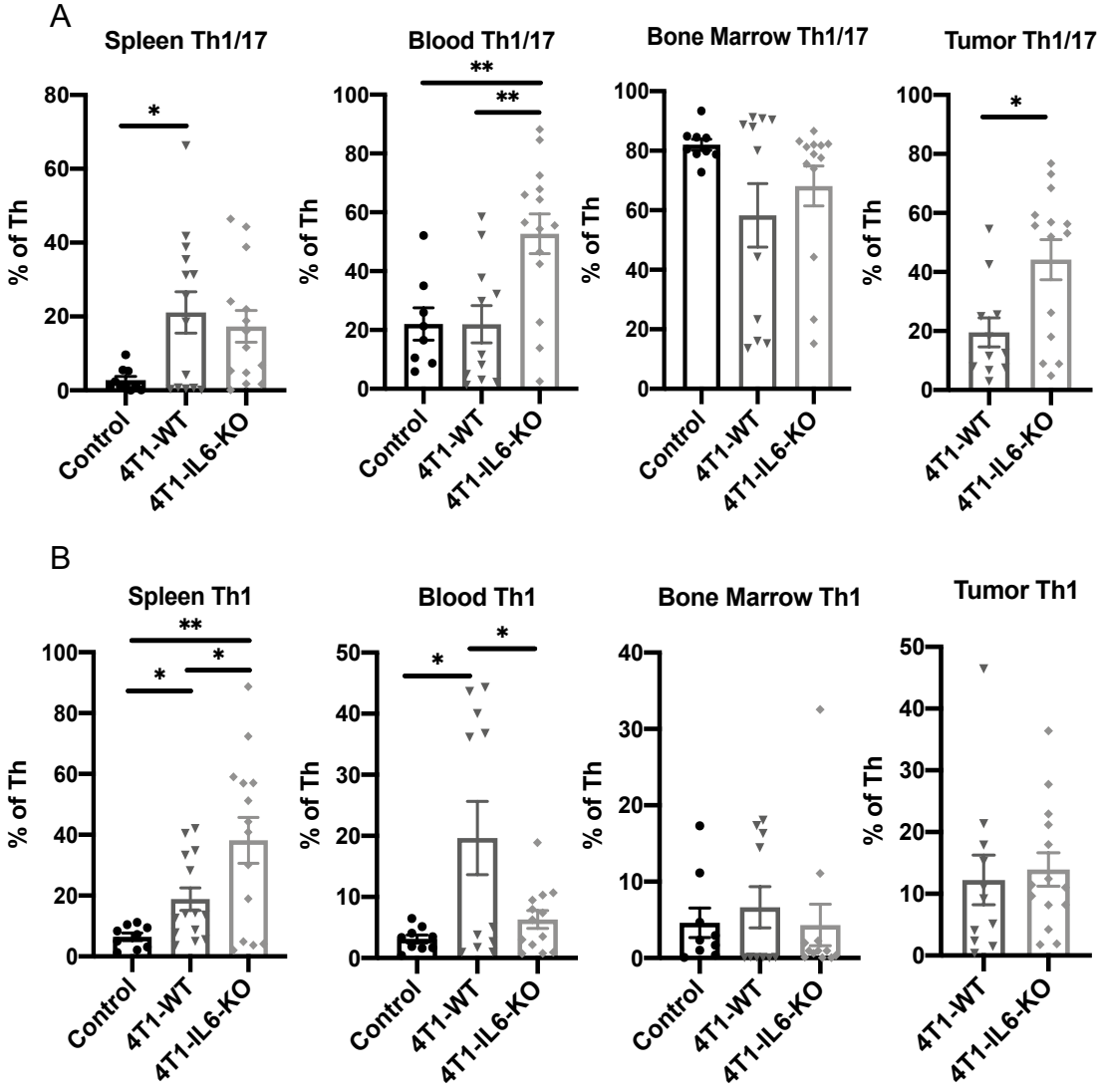


Figure 14: Depleting tumor IL-6 increases T_h1 and $T_h1/17$ cells. Recruitment at d 28 of (**A**) $T_h1/17$ and (**B**) T_h1 in response to 4T1-WT vs. 4T1-IL6-KO tumors. Data are represented as means ± SEM and n= 4-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001

Removing tumor cell-derived IL-6 led to a significant reduction of both M-MDSC and PMN-MDSC in all peripheral organs compared to 4T1-WT tumors (Figure 15A, C), further supporting the idea that IL-6 promotes MDSC expansion and that blocking systemic IL-6 signaling could prove beneficial to cancer patients. However, only tumor infiltrating M-MDSC were significantly reduced in 4T1-IL6-KO tumors (Figure 15B), whereas PMN-MDSC remained unchanged (Figure 15D).

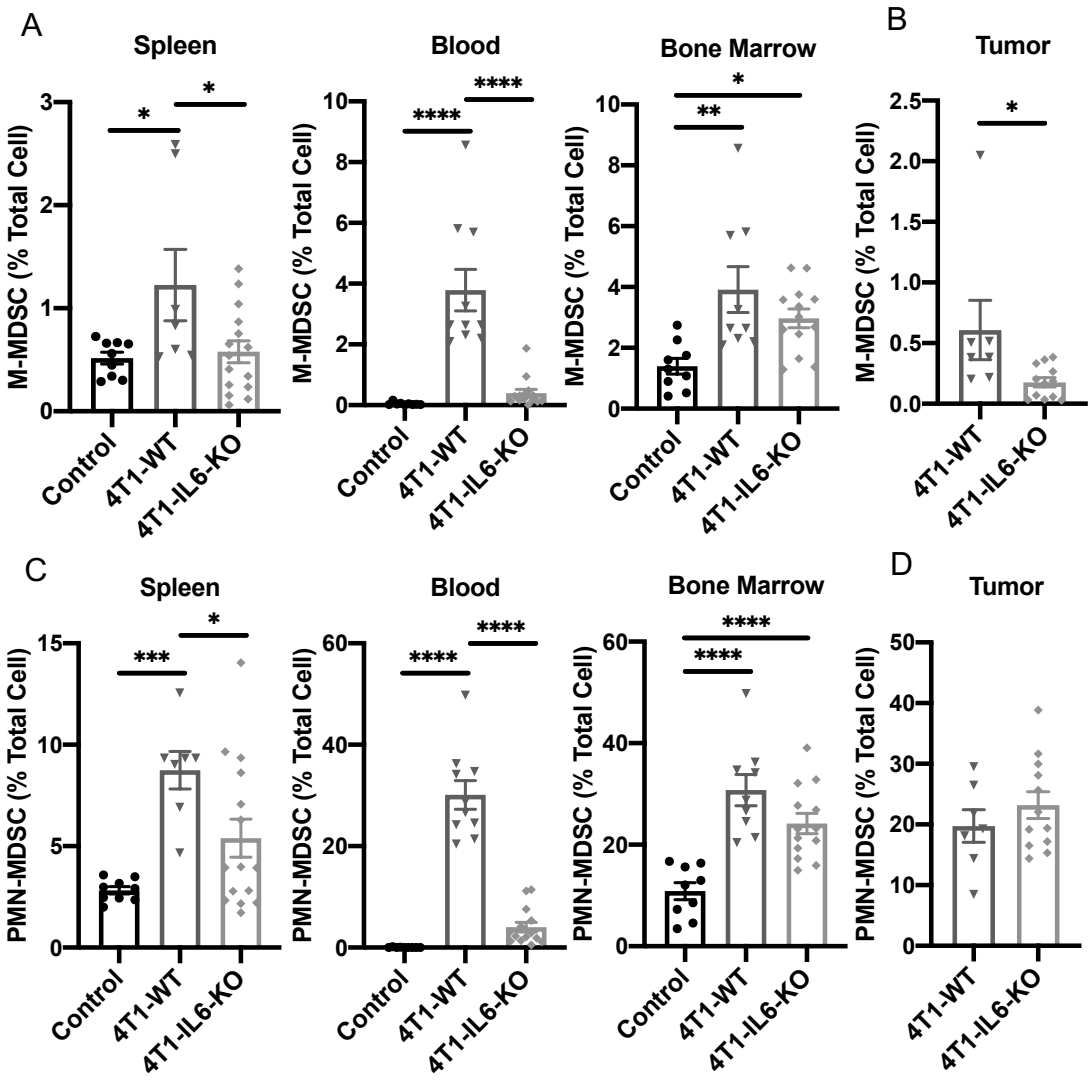


Figure 15: Tumor IL-6 depletion reduces both M-MDSCs and PMN-MDSCs. (**A-B**) M-MDSCs and (**C-D**) PMN-MDSCs in healthy controls, 4T1-WT and 4T1-IL6-KO in periphery and tumors of tumor bearing mice. 4T1-IL6-KO a 4T1 specific IL-6 knockout. Data are represented as means ± SEM and n= 4-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001.

CHAPTER V

DISSCUSSION

4T1 Tumor Time-Course Studies

Implications of Type 3 Polarization in Breast Cancer

Our results show that Type 3 T_h are dynamically recruited over time in response to mammary carcinoma. T_h17 were recruited initially in spleen, blood, bone marrow, and tumor and diminished at later time points. The opposite was seen of T_h22, which were recruited to the tumor at later timepoints. Due to the dynamic nature of type 3 T_h in response to mammary carcinoma, our findings indicate that the efficacy of clinical checkpoint inhibitors could be substantially diminished by enhancing type 3 immunity. It remains to be seen whether checkpoint therapy is able to enhance a pro-tumor T_h2, T_h17 or T_h22 response as opposed to increasing anti-tumor T_h1 or cytotoxic T cells, further experiments are necessary to answer this question. In theory, if type 3 T_h are amplified, it is possible that a negative immune response promoting tumor growth may persist with little amplification of type 1 immunity (68, 168). Indeed, clonal expansion followed by the polarization of type of immunity is possible following checkpoint inhibitor treatment, however, it remains to be seen what antigen these T cells recognize. It may be that they do not recognize tumor specific neoantigen displayed on MHC are merely bystanders.

Checkpoint inhibitor-stimulated expansion of T_h22 may be detrimental to the patient due to the polarization away from Type 1 immunity, T_h22 may also facilitate

metastasis in high grade breast cancer via IL-22 (81, 97). Thus, amplifying a T_h22 population in breast tumors may increase metastatic potential. The 4T1 model is representative of a high-grade mammary tumor (160, 161), which our data demonstrates robust T_h22 recruitment as time progresses. Future studies using this, and similar models should be carried out to elucidate the potential effects of T_h22, and by extension IL-22, on tumor and metastatic burden.

Are T Helper Cell 17 and T Helper Cell 22 Different Type 3 Subtypes?

Historically there has been debate as to whether T_h17 and T_h22 should be classified as distinct T_h subsets (31). Our data suggest that T_h17 and T_h22 are inversely correlated in the spleen but directly correlated in the context of a metastatic tumor (Figure 8 G and I). The spleen is a secondary lymphoid structure where T_h subset polarization occurs. Based on the negative correlation we observed between T_h17 and T_h22 in the spleen, we suggest that T_h17 and T_h22 are unique functional subsets due to the ability to be maintained in an opposing nature. A positive correlation of T_h17 and T_h22 suggests the potential repolarization of T_h17. Whether the reprogramming of T_h17 to a T_h22 phenotype is possible remains unclear and the present data underscore the need for additional focus on IL-22 in promoting metastasis (169). Moreover, IL-22 is known to promote wound healing in the skin and gastrointestinal tract, suggesting that this cytokine may enhance a breast tumor microenvironment and provide aid for the growth of malignant cells (33, 170).

Potential Impacts of Myeloid Derived Suppressor Cells on Checkpoint Inhibitor Therapy

The timing of checkpoint administration may be important as MDSC presence varies over time. Here, following tumor injection, PMN-MDSC and M-MDSC are induced early in the bone marrow but are not significantly elevated in blood and spleen until days 21 and 28 respectively. Early expansion followed by the reduction of MDSC in the bone marrow suggests splenic takeover of the maintenance of MDSC (Figure 9A and B). Early expansion of MDSC in the bone marrow, compared to other tissues is logical because MDSC are of the myeloid lineage, and myelopoiesis occurs in the bone marrow. It is, however, interesting that this increase in MDSC production occurs day 7 before a palpable tumor is detected suggesting early remodeling of immune tissue. A similar phenomenon is seen in the spleen at day 7, spleen mass is significantly lower than non-tumor bearing controls which may suggest immune remodeling of the spleen prior to an enlarged mass (Figure 6C). Understanding the pattern of MDSC recruitment may serve as an additional parameter to determine the stage of tumor progression (43).

When evaluating the relationship between MDSC recruitment and T_h17 it was found that there was a positive correlation in the bone marrow but a negative correlation in the blood (Figure 10J, N, K, and O). It may be possible that MDSC are capable of promoting T_h17 responses before achieving full suppressive capacity. These data may suggest that when MDSC are first generated in the bone marrow they may not have acquired suppressive capacity. Thus, are able to promote T_h17. While this is a stimulatory response, in the context of a metastatic tumor this is still negative as it would deplete the

necessary type 1 immune components necessary to mitigate tumor progression. Future studies evaluating the suppressive capacity of MDSC in the bone marrow over time following 4T1 mammary tumor injection are needed to support this claim. Still, this finding is in line with research in rheumatoid arthritis where MDSC promote T_h17 responses but are not able to suppress effector T_h to halt an autoimmune reaction (9). Further, there were no significant correlations between MDSC and T_h22 in the bone marrow and blood. This is more evidence suggesting that T_h17 and T_h22 should be regarded as different cell types, which may have unique functional relevance in the context of breast cancer.

Polarization to Type 1 Immunity

In addition to T_h17, T_h22, and MDSC recruitment in 4T1 mammary carcinoma, we assessed the recruitment of $T_h1/17$ as a potential indicator of polarization to anti-tumor type 1 immunity. The T_h17 phenotype has been shown to be more plastic than the T_h1 phenotype (112). Due to this, it is thought that $T_h1/17$ are transiting away from T_h17 toward the T_h1 phenotype (112) as opposed to the reverse. Our data support that previous work. We see that there is a significant positive correlation between $T_h1/17$ and T_h17 in the tumor across all time points. This suggests that when there are more T_h17 present there is also a greater chance of detecting $T_h1/17$. Further, there was no correlation observed between $T_h1/17$ and T_h17 in spleen, blood, or bone marrow indicating that the tumor microenvironment might play a unique role in the re-polarizing immune response. In the spleen, blood, and bone marrow, when comparing $T_h1/17$ accumulation to T_h1 we see a significant negative correlation. This event suggests once again that $T_h1/17$ likely occur when T_h1 polarization is low, indicating the potential to transition from type 3 to a

type 1 immune response. Additionally, if $T_h1/17$ are transiting towards T_h1 eventually you would expect the number of T_h1 to increase. It is possible that a complete repolarization of $T_h1/17$ is dependent on tumor specific milieu of cytokines and should be evaluated further. Understanding the conditions in which $T_h1/17$ are likely to be amplified over T_h17 following checkpoint therapy may prove to be therapeutically beneficial.

Despite $T_h1/17$ generation, which indicates that repolarization of T_h17 into T_h1 may be possible it is still possible polarization away from type 1 to type 3 immunity. When evaluating the recruitment of type 1 and type 3 T_h we saw significant negative correlations between both T_h1 and T_h17, and T_h1 and T_h22 in the spleen and tumor. This may indicate that there is potential for polarization away from type 1 towards a type 3 T_h phenotype in mammary carcinoma given the proper stimulation of cytokines from the tumor microenvironment. We did not examine which population of T_h cells (T_h1 or T_h17) were actively undergoing clonal expansion, however, T_h17 is to be a more plastic T_h phenotype as it is easily repolarized by the target tissue, such as a tumor (112). Due to this, we speculate that $T_h1/17$ generation likely originates from a T_h17, not a T_h1.

Tumor Interleukin 6 Studies

Following our time course study using 4T1-WT tumor bearing mice, we focused on the effects of tumor-derived IL-6. IL-6 was targeted in this study for two reasons: 1) it acts as a broad pro-tumor cytokine, and 2) the presence of already clinically approved tocilizumab to target IL-6 signaling in rheumatoid arthritis (142). IL-6 promotes tumorigenesis by facilitating immune suppression, T_h polarization away from type 1 immunity and promoting angiogenesis (171). Further, IL-6 facilitates MDSC

development and type 3 T_h polarization (30, 139). We used CRISPR to knock out IL-6 in the 4T1 cell line (4T1-IL6-KO) and compared T_h17, T_h22, $T_h1/17$, and MDSC responses to changes in IL-6. When assessing T_h17 and T_h22 in the 4T1 tumor IL-6 knockout we found that IL-6 acts differently on T_h17 and T_h22. In the tumor, T_h17 are expanded whereas T_h22 are significantly reduced when the tumor source of IL-6 is removed. IL-6 is a key cytokine in fate determinate of both T_h17 and T_h22 lineage (31), but it has been thought that TGF-β1 is the major player in determining T_h17 or T_h22 polarization. TGF-β1 presence drives T_h17 polarization while inhibiting T_h22 formation, while IL-6 is necessary but not sufficient to discriminate between these lineages. Our data suggest that there is an IL-6 concentration-dependent mechanism of T_h22 or T_h17 skewing, which should further be evaluated as there is currently little information on differential expression of IL-6R or IL-6R function on these cell types.

Additionally, the reduction of tumor resident T_h22 may be therapeutically beneficial due to the link between IL-22 growth and metastasis in other types of cancer (80). It remains unclear whether an increase of T_h17 in this context would lead to pro or anti-tumor immunity. Specifically, this is because T_h17 are a plastic subset of T_h cell and their phenotype may be easily manipulated by the cytokine profile of the tumor microenvironment, thus they may remain pro-tumor T_h17 or repolarize into tumor promoting T_{reg} or tumor mitigating T_h1 (108, 110, 112).

Moreover, a significant increase in $T_h1/17$ was observed alongside T_h17 increase in the 4T1-IL6-KO tumor compared to 4T1-WT. Further supporting the notion that these cells are transitioning from T_h17 to T_h1 phenotype. $T_h1/17$ were significantly elevated in the blood of 4T1-IL6-KO tumor bearing animals compared to 4T1-WT. The increase of

anti-tumor IFN-γ positive $T_h1/17$ in the absence of tumor IL-6 suggests that IL-6 is a logical therapeutic target to accompany checkpoint immunotherapy.

Additionally, tumor localized IL-6 should be investigated as a therapeutic target in this setting, because we observed a significant increase in total T_h cells present in 4T1-IL6-KO tumors as well as blood compared to 4T1-WT tumor-bearing animals. In addition, we observed a statistically significant reduction in M-MDSC across all tissues including tumor and a significant reduction in PMN-MDSC in all peripheral organs. Indeed, this was our initial prediction and impetus for the IL-6 portion of the study. Reducing MDSC accumulation in the periphery as well as in the tumor has been shown to bolster T cell-mediated immunotherapy (172). Additionally, the effects of IL-6 on extramedullary hematopoiesis (EMH) should be considered. 4T1 tumors are known to initiate extramedullary myeloid hematopoiesis in the spleen and liver of tumor bearing mice (137). Despite the decrease of MDSC in the spleen, we saw spleen masses were significantly increased in 4T1-IL6-KO compared to 4T1-WT tumor bearing mice (Figure 11C). This may suggest that splenic EMH is increased in the absence of tumor IL-6 but allowing for the maturation of myeloid cells and potentially extramedullary lymphoid cell hematopoiesis. Additionally, in our 4T1-WT time course study, we observed MDSC expansion at d 7 in bone marrow accompanied by a significant decrease in splenic mass. In subsequent days MDSC in the bone marrow decreased while spleen size and MDSC accumulation in the spleen increased. This suggests that there is a delay in splenic EMH and the ability for splenic takeover of MDSC production over bone marrow derived MDSC. This raises further questions about when and how MDSC acquire suppressive capacity.

In our model knocking out IL-6 in 4T1 cells did not significantly reduce total IL-6 in the tumor (Figure 4C), and we suspect this is due to infiltrating immune and stromal cell and systemic ability to produce IL-6 is intact, despite this we observed a significant impact on the local and systemic immune environment. Thus, we propose that a reduction in IL-6 from the tumor may be sufficient to alter the immune landscape. Although we saw positive anti-tumor alterations in immune response when eliminating tumor IL-6, it is important to further investigate whether the local source of IL-6 is important for this reduction or whether these systemic responses occur in a concentration dependent manner.

Conclusion and Summary

In sum, this study demonstrated that there is a dynamic pro-tumor type 3 immune response in reaction to 4T1 mammary carcinoma (Figure 16). These data demonstrate the ability for breast tumors to polarize away from an anti-tumor type 1 immunity toward a type 3 response, highlighting that it might be possible for checkpoint therapy to amplify the wrong immune response. This has not been adequately explored, warranting future research into the impact of immune polarization on checkpoint efficacy. Furthermore, the effects of tumor-derived IL-6 on the tumor and peripheral immune landscapes were explored. It was found that knocking out tumor IL-6 significantly increased tumor infiltrating T_h cells, significantly increased IFN-γ producing $T_h1/17$, and reduced MDSC recruitment (Figure 16). These results demonstrate that IL-6 should be considered a therapeutic target in select circumstances where aberrant type 3 immune skewing is involved, which may better facilitate an initial, appropriate anti-tumor immune response. Indeed, IL-6 may serve as a therapeutic target for the above reasons but IL-6 is necessary

to initiate an immune response. Lack of IL-6 has been shown to lower lymphopoiesis and poor infiltration of immune cells into sentinel lymph nodes. Due to these collective observations, the concentration of IL-6 will likely be important if implementing anti-IL-6 therapy (173).

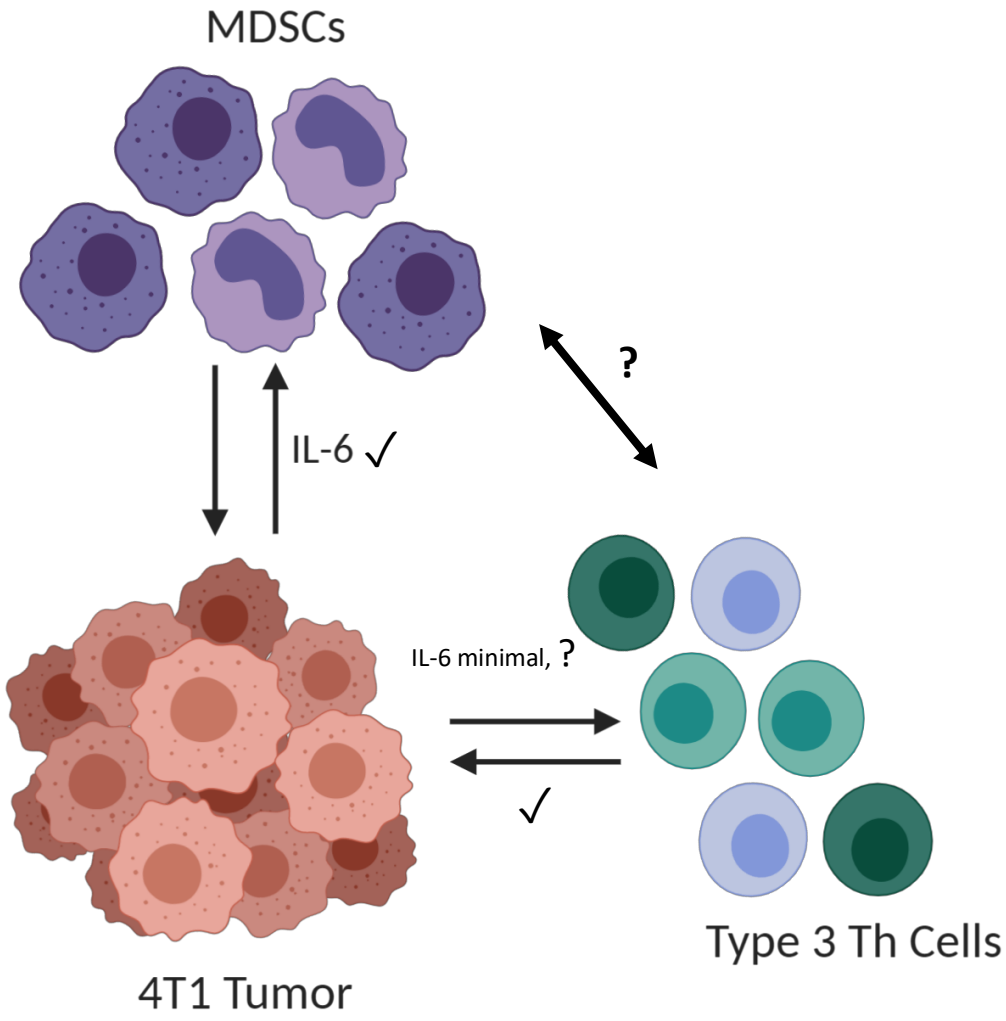


Figure 16: Summary of interactions between 4T1 tumor cells, type 3 Th, and MDSCs; that are mediated by interleukin IL-6. In this study, we demonstrated that both T_h17 and T_h22 are dynamically recruited in the 4T1 tumor model of mammary carcinoma. Tumoral IL-6 plays a role in the formation of MDSC as removal lead to a systemic reduction in MDSCs. Tumor derived IL-6 was important to type 3 T_h locally to the tumor but this effect was minimal, and possibly aids in repolarization of Th immunity. Additionally, total T_h were expanded in the tumor when IL-6 was knocked out of the 4T1 cells. The connection between MDSC and type 3 Th has yet to be determined in mammary carcinoma.

Future Studies and Questions

Myeloid Derived Suppressor Cell and Type 3 Immunity

Our data suggest that in the context of 4T1 mammary carcinoma there is not a relationship between MDSC recruitment and type 3 T_h polarization. I make this assumption because type 3 T_h and MDSC recruitment do not appear in lockstep with one another. Further studies evaluating whether or not this conclusion is accurate should be done. Additionally, a study should be done to reveal when MDSC achieve suppressive capacity, both in a time course setting and at the level of tissue specific MDSC. A study like this may reveal why we observed a positive correlation between T_h17 and MDSC in the bone marrow of tumor-bearing mice; MDSC may be able to promote T_h17 polarization before they achieve suppressive capacity. MDSC have been shown to produce high levels of TGF-β1 (9), which in turn may drive a T_h17 phenotype over a T_h22 phenotype. This may be why we saw a positive correlation between presumably pre-suppressive MDSC and T_h17 in the bone marrow and not between MDSC and T_h22. Additionally, it would be interesting to assess the link between MDSC, T_h17 and mast cells in a mammary carcinoma setting. The rationale here being TGF-β1 high–producing MDSC have been shown to promote mast cell activation. Mast cells upon TGF-β1 challenge have been shown to secrete high levels of IL-6 (26), which has the potential to promote both MDSC and T_h17 (9).

T Helper Cell 17 and Mammary Carcinoma

We found that T_h17 are recruited at early time points in response to 4T1-WT tumors and then are reduced over time. I think in the future it would be interesting to

evaluate why we see this reduction of T_h17. Is it possible that T_h17 are initially polarized but following migration to the tumor they are driven into a T_{reg} phenotype? Additionally, is it possible to capitalize on this early T_h17 response and completely repolarize T_h17 to tumor fighting T_h1 help?

Interleukin 6 and Checkpoint Immunotherapy

In this study, the main immunological benefits found by removing tumor IL-6 were the increase of tumor infiltrating T_h and the reduction of MDSC. Lack of TIL and the recruitment of MDSC are thought to be major contributors to the failure of immunotherapy treatment in solid tumors. I propose that adding an IL-6 neutralizing antibody may increase the efficacy of checkpoint inhibitor therapy function. This combination has not yet been evaluated and should be considered for future studies. Additionally, it would be interesting to look at the polarization of T_h immunity under the influence of checkpoint therapy as well as analyze the interactions between activated T_h and their antigen in a mammary carcinoma setting.

Study Limitations

Throughout the course of this study, we acknowledge the following limitations. In our flow cytometric panel, we did not include markers that allowed us to identify T_h2, cytotoxic T cells or T_{reg}. This meant that we were unable to evaluate changes to these cell populations. Most importantly, however, we are unable to say for certain whether the T_h populations we evaluated fall into a T_{reg} or effector T_h category. Despite this, I argue that polarization of Type of immunity meaning the alteration in the tumor immune microenvironment and thus cytokine profile released by total T_h may shape the outcome to immunotherapy treatment.

Additionally, this research was only conducted using one model of mouse mammary carcinoma. Due to this, we do not know if similar results will be observed in other models of solid tumors, such as the B16 melanoma in C57/BL6 mouse. Another limitation arises when evaluating the 4T1-IL6-KO line. 4T1-IL6-KO were derived from two single knockout 4T1 clones. This presents a unique situation where there is not an exactly matched 4T1-WT control. Presumably, before using CRISPR to knock out IL-6 I could have started 4T1-WT colonies from a signal cell and used this to knockout IL-6 and saved some as controls. Indeed, this was an option, but we decided not to proceed, because each colony would be cultured separately for a number of passages, which invariably introduces clonal divergence.

Lastly, I would like to state that I myself did not perform a functional suppression assay on MDSC in our model of 4T1 mammary carcinoma. However, this phenomenon has been reported extensively in the literature (10). Additionally, Jacob Garritson, a member of our laboratory performed MDSC T cell suppression assays from this model, and he observed that MDSC were indeed suppressive at the day 28 time point (work currently in peer review).

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND
USE COMMITTEE APPROVAL

College of Natural and Health Sciences, School of Biological Sciences, Ross Hall, University of Northern Colorado, Greeley, CO 80639

Dear Review Committee,

This letter is intended to confirm that the research being conducted by my graduate student, Ms. Viva Rase, is covered under a current and ongoing IACUC protocol. All of our procedures have been pre-approved under IACUC protocol numbers: 1906CE-RH-RM-22, (previously 1511CE-RH-RM-18) and 1702C-NP-M-20. These protocols were created by, and are maintained by, our research collaborators Dr. Reid Hayward in the Sports and Exercise Sciences Program and Dr. Nicholas Pullen School of Biological Science. Dr. Hayward and his graduate students have played an important role in the completion of these studies as outlined in the Thesis acknowledgements. Please feel free to contact me or Dr. Hayward or Dr. Pullen if there are any specific questions regarding the IACUC protocol and our ongoing research collaboration.

Sincerely,

James M. Haughian

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APPENDIX B

ANIMAL VITAL STATISTICS

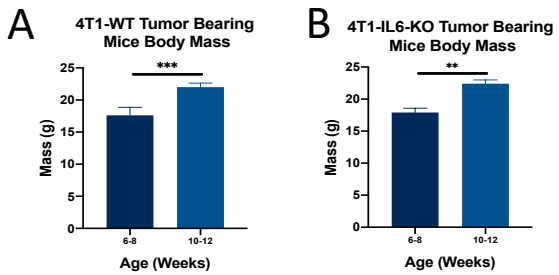

Figure 17: Female mouse body mass before injection and at day 28 post injection. Mass of (**A**) 4T1-WT and (**B**) 4T1-IL6-KO tumor bearing mice before injection and at day 28 post injection. Mice received tumors at 6-8 weeks old and were euthanized around 10-12 weeks old. This weight gain is expected since the average weight of a healthy 6-8-week-old female mouse is about 18g and of a 10-12-week-old female mouse is on average 21g. Data are represented as means ± SEM and n= 4-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001.

APPENDIX C

CLUSTER OF DIFFERENTIATION 40^+ T HELPER CELLS IN 4T1 MAMMARY CARCINOMA

Background

Classically, CD40 is thought of as a costimulatory molecule expressed on professional APC such as dendritic cells and B cells, and its ligand CD40L is expressed on activated $T_{h.}$ CD40 is not classically thought of as expressed of T_h cells. In dendritic cells, the CD40/CD40L signaling axis is essential for dendritic cell survival and differentiation. Arguably, more importantly, $CD40L^+$ effector T_{fh} interact with CD40 on B cells to facilitate immunoglobin class switching. When CD40L is rendered functionally impaired, T_{fh} help of B cell class switching is not achieved and hyper IgM syndrome is observed (174). Hyper IgM is characterized by increased serum IgM and insufficient levels of IgG, IgA, and IgE, thus and chronic risk of pathogenic infection (175).

Despite the notion that CD40 is classically restrictively expressed on APC while CD40L is largely expressed on T cells and not CD40, the Wagner lab at Anschutz Medical Campus in Denver have recently classified a subset of pathologically active T cells that express CD40, which they have entitled T_h40. The Wagner lab has identified T_h40 or $CD40^+$ T_h in experimental autoimmune encephalomyelitis and in Type 1 diabetes. In both of these autoimmune diseases, their results indicate that $CD40^+$ T_h are pathologically active and drive these autoreactive immune responses (176–179). After seeing this work presented at the AAI in 2019, naturally we were curious if this

particularly autoreactive T_h40 subset was present in our 4T1 model of mammary carcinoma. If so, it may be of therapeutic benefit to enrich an autoreactive $CD40^+$ T_h in order to improve immunotherapeutic efficacy in breast cancer. To our knowledge, this is the first study to evaluate $CD40^+$ T_h recruitment in mammary carcinoma.

Research Questions

Q1 Are $CD40^+$ T_h cells recruited in 4T1-WT Tumor bearing mice?

Q2 Are $CD40^+$ T_h cells affected by tumor IL-6?

Methods

The same methodology was utilized as in the thesis proper, however, with the addition of a CD40 (BioLegend 124631) antibody to the T_h cell flow cytometric panel. In short, a time course study was performed with 4T1-WT tumors evaluating $CD40^+$ T_h recruitment in spleen, blood, bone marrow and tumor at days 0, 7, 14, 21, and 28 post injection time point. Following this a day 28 time point was used to evaluate the effects of tumor IL-6. This was accomplished through CRISPR/Cas9 knockout of IL-6 generating 4T1-IL6-KO cells. $CD40^+$ T_h were evaluated in 4T1-WT and 4T1-IL6-KO in spleen, blood, bone marrow and tumor. All data are presented as mean ±SEM and statistical tests used $\alpha = 0.05$. All multiple comparisons were calculated using one-way ANOVA with Turkey's multiple comparisons test. Single comparisons were calculated using Student's t-test.

Results and Discussion

Cluster of Differentiation 40$^+$ T Helper Cell in 4T1 Wild Type Time Course

CD40$^+$ T_h were detected in the spleen, blood, and bone marrow of both healthy and tumor-bearing mice (Figure 18A). This was interesting because CD40$^+$ T_h have largely been ignored but are present in high numbers in a healthy mouse. CD40$^+$ T_h remained unchanged following tumor injection in the spleen and bone marrow but were significantly elevated at day 14 in blood compared to no tumor controls and day 28 tumor bearing controls (Figure 18A). CD40$^+$ T_h were found in the tumor; however, we saw that they decreased day 21 to day 28 in 4T1-WT tumor bearing mice. CD40$^+$ T_h are thought to be an auto-reactive T_h (178, 179), which means that T_h40 cells may have the potential to provide anti-tumor immunity to the host. Due to this, we evaluated the particular T_h subset of CD40$^+$ T_h over time (Figure 18C-J). We found that the majority of CD40$^+$ T_h seemed to be either a T_h1 phenotype or a T_h17 phenotype (Figure 17C-D and G-H). The phenotype of CD40$^+$ T_h may be important when evaluating how CD40$^+$ T_h may provide an autoreactive or anti-tumor phenotype. In autoimmunity autoreactive T_h are generally pathologically active T_h17, however, some are T_h1 (180). In the case of CD40$^+$ T_h the autoreactive phenotype is a mix between T_h1 and T_h17 suggesting these cells may be able to provide anti-tumor immunity (177). However, at this time we do not know the functional significance of CD40 expression on T_h cells. Additionally, CD40$^+$ T_h that are T_h17 decreased over time in the spleen and blood, which followed the trend of total T_h17 over time in 4T1-WT tumors. CD40$^+$ T_h that are T_h22 were significantly increased at day

28 in the spleen compared to no tumor bearing mice, which directly opposed the depletion of $CD40^+$ T_h that are T_h17.

Cluster of Differentiation 40^+ T Helper Cell and Tumoral Interleukin 6

In the 4T1-IL6-KO tumor bearing mice, $CD40^+$ T_h are significantly increased in the spleen blood and bone marrow compared to 4T1-WT tumor bearing mice (Figure 19A). Indeed, this may be a consequence of total T_h increase in 4T1-IL6-KO compared to 4T1-WT tumor bearing animals, however, if $CD40^+$ T_h are more auto-reactive this increase may be therapeutically beneficial. Additionally, we evaluated T_h subset that were $CD40^+$ T_h (Figure 19B-E).

Future Directions

The functional importance of CD40 expression on T_h has yet to be elucidated. In fact, the idea that CD40 may be expressed on T_h has largely been ignored with the exception of the Wagner Laboratory. Uncovering functional significance of CD40 expression on T_h would be very interesting given that T_h40 have been identified as more autoreactive than $CD40^-$ T_h cells.

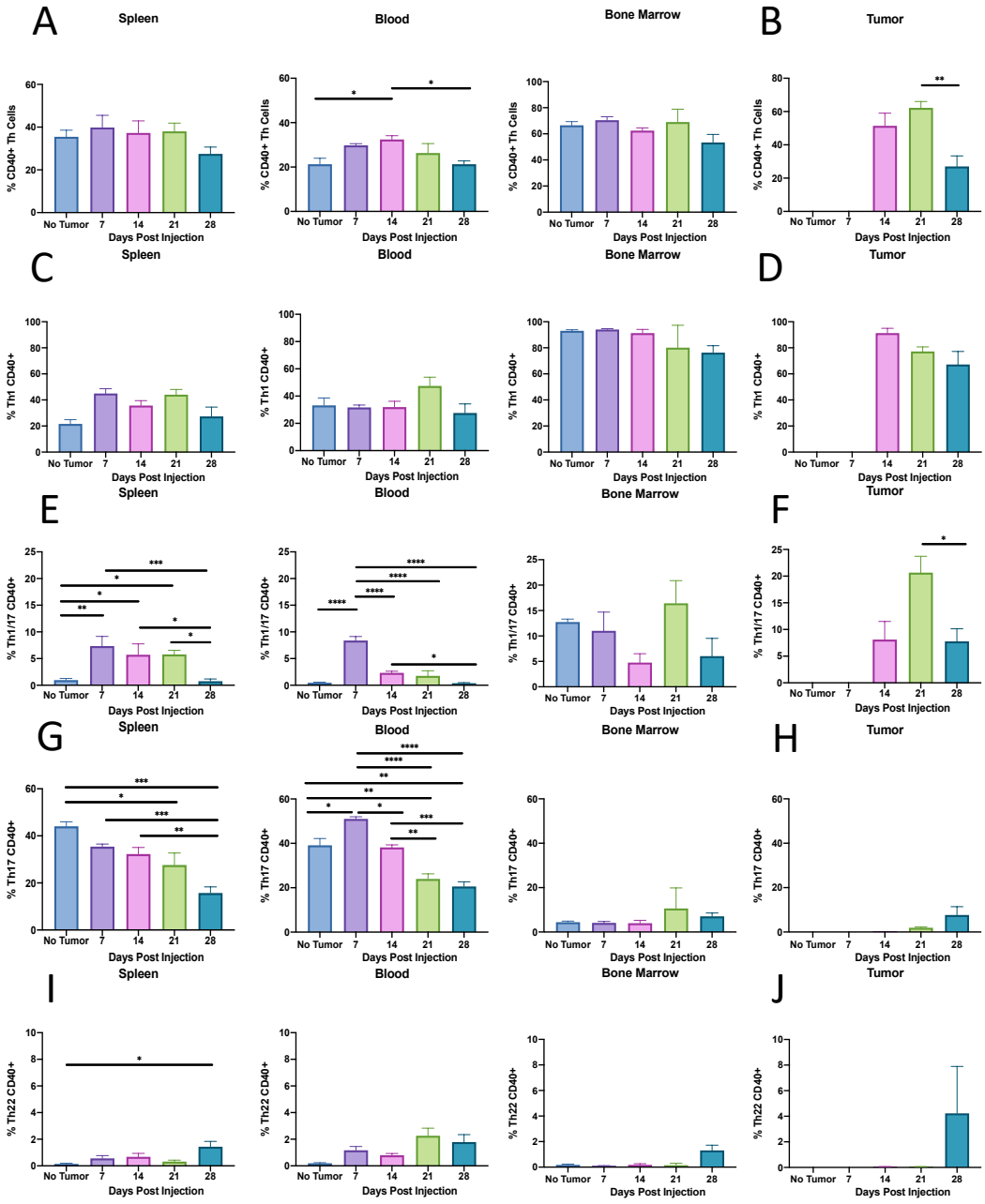

Figure 18: CD40$^+$ T_h expansion over time in 4T1-WT tumor bearing mice. Flow cytometric measurement of (**A**) total CD40$^+$ T_h in periphery (**B**) total CD40$^+$ T_h in tumor, (**C**) CD40$^+$ T_h1 in periphery (**D**) total CD40$^+$ T_h1 in tumor, (**E**) CD40$^+$ $T_h1/17$ in periphery (**F**) total CD40$^+$ $T_h1/17$in tumor, (**G**) CD40$^+$ T_h17 in periphery (**H**) total CD40$^+$ T_h17 in tumor, (**I**) CD40$^+$ T_h22 in periphery and (**J**) total CD40$^+$ T_h22 in tumor in response to 4T1-WT mammary carcinoma over time. Tumor free (no tumor) mice were used as control.1.0x10^4 4T1-WT cells were injected and T_h cell measured in individual mice days 7, 14, 21 and 28. Palpable tumors day 14. Statistical significance was measured using one-way ANOVA with Tukey's multiple comparisons. Data are represented as means ± SEM and n= 4-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001.

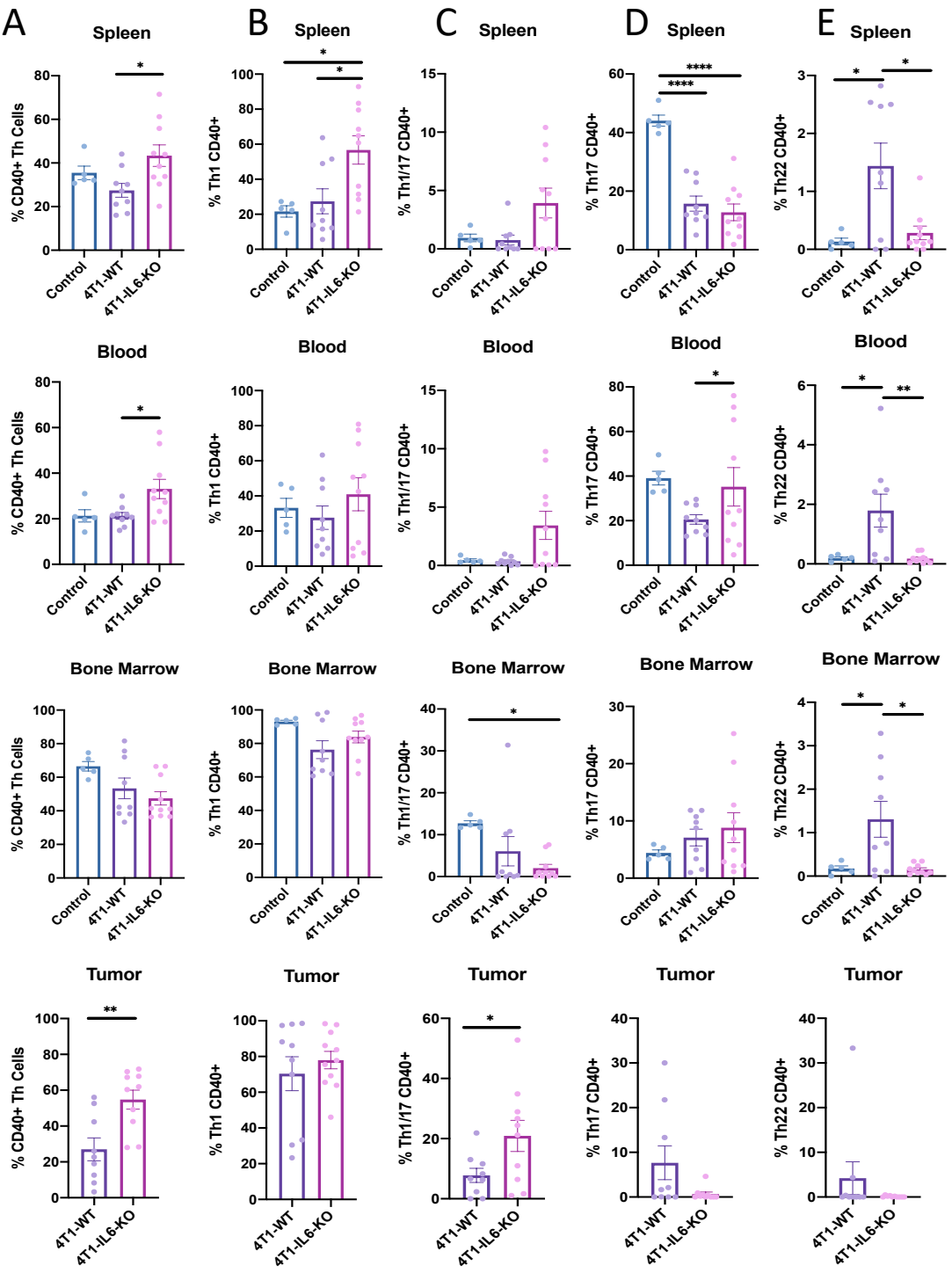

Figure 19: CD40$^+$ T$_h$ expansion in 4T1-WT vs. 4T1-IL6-KO tumor bearing Mice at day 28. Flow cytometric measurement of (**A**) total CD40$^+$ T$_{h,}$ (**B**) CD40$^+$ T$_h$1 in periphery, (**C**) CD40$^+$ T$_h$1/17 in periphery, (**D**) CD40$^+$ T$_h$17 in periphery, and (**E**) total CD40$^+$ T$_h$22 in tumor in response to 4T1-WT mammary carcinoma over time. Statistical significance was measured using one-way ANOVA with Tukey's multiple comparisons. Data are represented as means ± SEM and n= 4-11 mice. *p<0.05, **p<0.001, ***p<0.001 and ***p<0.0001.