THE ROLE OF THE IL-17/IL-17R AXIS IN BREAST TUMOR GROWTH AND METASTASIS

By

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ABSTRACT

Clinical trials and animal experiments have revealed that chronic inflammation may contribute to all steps of tumor development from initiation, all the way to metastatic progression, predisposing for the development of many types of cancer including breast cancer. Interleukin-17A (IL-17A), the hallmark cytokine of T helper-17 (Th17) cell subset, has an important role in mediating chronic inflammation as a pro-inflammatory cytokine. IL-17A-producing cells are detected in various cancer samples; however, existing information on the role of the IL-17/IL-17R axis in cancer remains paradoxical. In this study, an adenoviral delivery system was used to over-express IL-17A (AdIL-17A) or IL-17RA antagonist (AdIL-17RA:Fc) in 4T1 murine mammary carcinoma cells. These cells were used to investigate the specific role of IL-17A in breast cancer using a murine model.

Our study demonstrates that IL-17A, promotes breast tumor growth via reinforcement of myeloid-derived suppressor cells (MDSCs) that dampen the immunosurveillance response, and increased lung metastasis via chemokine-mediated attraction of tumor cells into lungs. However, the pro-tumor effect of IL-17A could be reversed into an anti-tumor one when the tumor was resected. This reversal could be due to surgery-mediated reduction of MDSCs following tumor resection and IL-17A-mediated activation of CD4 (Th1) and CD8 (CTLs) cells, which would favor an anti-tumor response. This study provides novel insights into the role of IL-17/IL-17R axis in breast tumor development and has major implications for targeting IL-17A in the treatment of tumors.

LIST OF ABBREVIATIONS USED

ACK	Ammonium-chloride-potassium buffer
µL	Microliter
µm	Micrometer
6-TG	6-thioguanine
7AAD	7-amino-actinomycin D
Ab	Antibody
Ad-	Adenovirus
Addl	Adenovirus vector with E1/E3 deletion
Ad-IL-17A	Adenovirus vector expressing IL-17A
Ad-IL-17RA:Fc	Adenovirus vector expressing soluble IL-17RA subunits fused to IgG Fc
Ag	Antigen
APC	Antigen presenting cell
BRCA1/2 genes	Breast cancer type 1 early onset/2 early onset
BS	Bovine Serum
BSA	Bovine serum albumin
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DOC	Sodium Deoxycholate
E1/E3	Adenovirus early replication genes 1/3
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead transcription factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hanks' buffered salt solution
HEK293	Human embryonic kidney 293
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HER2/neu	Human epidermal growth factor receptor 2/neuro
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma

Ig	Immunoglobulin
IL	Interleukin
IMC	Immature myeloid cell
iNOS	Inducible nitric oxide synthase
IWK	Isaak Walton Killam Hospital for Children
Ly6-	Lymphocyte antigen 6-
mAb	Monoclonal antibody
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cell
MEM F11	Minimal essential medium
MHC	Major-histocompatibility complex
mL	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger Ribonucleic acid
myc	Myelocytomatosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T
NO	Nitric oxide
OD	Optical density
OVA	Ovalbumin
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cells
PDL-1	Programmed death-ligand 1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PFU	Plaque forming unit
PGE_2	Prostaglandin 2
PMA	Phorbol 12-Myristate 13-Acetate
PR	Progesterone Receptor
RB1	Retinoblastoma 1
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RPMI 1640	Roswell Park Memorial Institute 1640
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
STAT3	Signal transducer and activator of transcription 3
TAA	Tumor associated antigen
TAMs	Tumor-associated macrophages
TCR	T cells receptor
TDLN	Tumor draining lymph node
TGF-β	Tumor-growth factor beta
Th	T-helper
TIL	Tumor infiltrating leukocytes
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
T_{regs}	T regulatory cells
TSA	Tumor specific antigen
VEGF	Vascular endothelial growth factor

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والحمدُ لله رَبّ العالمين أولاً وآخراً

Chapter 1. INTRODUCTION

1.1. Breast Cancer

1.1.1. Epidemiology and risk factors

Lesions of the breast, which are much more common in females than in males, usually take the form of tumors (1). While the majority of these tumors are benign, breast cancer (characterized by the malignant form of these tumors) is, nonetheless, the second most common cancer found in women worldwide (after skin cancer). Moreover, it is the second biggest cause of cancer mortality, after lung cancer (1). Geographically, breast cancer is more prevalent in wealthy, developed countries such as Canada, USA, and those in Western Europe (2). Based on 2007 estimates by the Canadian Cancer Society, 1 in 9 women is expected to develop breast cancer during her lifetime, and 1 in 29 will die from it; 2013 alone was expected to have 23,855 new cases and 5000 deaths (3).

There have been great advances in breast cancer diagnosis and treatment, yet the overall etiology remains poorly understood. Nonetheless, some people are more likely to develop breast cancer depending on their genetics, environment and lifestyle (1, 4). The Canadian Cancer Society (2014) identifies the following risk factors for breast cancer:

- **Gender**: Females are about 100 times more likely than males to develop breast cancer, a difference possibly related to males' relative lower amounts of estrogen and progesterone hormones, which may play roles in breast cancer (5).
- **Age**: The risk of developing breast cancer increases with age: about two out of three diagnosed breast cancer patients are over the age of 55 (5).
- **Family history (genetics)**: About 10-15 % of women with breast cancer have a family member diagnosed with the disease, suggesting a role for genetics. Indeed,

having one first-degree relative with breast cancer doubles the risk of having the disease, and having two such relatives triples the risk (5, 6). Inherited mutations in *BRCA1* and *BRCA2* tumor suppressor genes are the most common cause of hereditary breast and ovarian cancer (5). Less commonly, mutations in genes such as *p53* and retinoblastoma 1 (*RB1*) have been associated with breast cancers, although these are commonly found in sporadic cases (see section 1.1.2) (7).

- **Menstrual history:** Women who have had a longer history of menstrual periods (early menarche or late menopause) are of slightly higher risk than others (5).
- **Pregnancy:** Nulliparous (a woman who has never given birth to a viable infant) and late age pregnancy increases the risk of breast cancer (6).
- **Environmental factors**: Numerous environmental factors, including ingredients in cosmetics, chemicals in plastics, and pesticides, have been implicated in breast cancer, although the extent of such connections is not yet clear (8).
- **Smoking**: Several recent studies have suggested a link between long-term smoking and breast cancer development, especially in women who start smoking at early ages (5).
- **Inflammation**: Although inflammation is natural response in the host against infections and tissue damage, several reports have suggested a link between breast cancer and chronic inflammation (see section 1.2.3).

In addition to these well-established risk factors, obesity, alcohol consumption, oral contraceptives, and hormonal replacement therapy likely play roles in breast cancer (9).

1.1.2. Breast cancer biology

The breast is mainly composed of two types of tissues, the glandular tissues (composed of the lobules and the ducts) and the supporting tissues (composed of connective tissues and fat). Normal breast growth, development and lactation are regulated by hormones and growth factors (10). These hormones include estrogens (estrone, estradiol, and estriol), progesterone, growth hormone, prolactin, and oxytocin. Estradiol and progesterone act on epithelial cells indirectly by inducing growth factors that induce or inhibit epithelial proliferation, either in an autocrine or in a paracrine manner (11). Mitogenic growth factors include fibroblast growth factors (FGF), epidermal growth factor (EGF), and insulin and insulin-like growth factors (IGF-1 and IGF-2), which induce normal breast development (7, 12). In opposition, inhibitory growth factors such as TGF-β prevent the breast epithelial development (13). The interaction of hormones and growth factors with their specific receptors triggers intracellular signals that result in the activation or inhibition of various genes (14). The cellular component of breast tissues divides frequently, making the development of cancer more likely in the breast compared to other more slowly dividing tissues. Especially at puberty, breast tissue grows rapidly as it develops under the influence of the previously mentioned hormones and growth factors. Breast tissue does not become fully mature until a woman has given birth and produced milk for the first time, hence, nulliparous women, who have immature breast cells, are more likely to develop breast cancers than women who give birth and breast-feed their babies (15). Cancers usually develop through complex multistep stages that include initiation, promotion, progression and metastasis.

Initiation of the tumor usually begins in normal cells that acquire at least four to five mutations, which create the potential for neoplastic transformation (16). DNA mutations may happen within genes that prevent tumor initiation (tumor suppressor genes) or within genes responsible for neoplastic transformations (oncogenes). **Tumor suppressor genes** play a critical role in preventing the onset of cancer, and losing the normal function of these genes contributes greatly to the promotion of malignancy. *BRCA1* and *BRCA2* genes act as tumor suppressor genes under normal conditions, mediating DNA repair, and disruptions of these genes are found in 80-90% of hereditary breast cancer cases (14, 17). Other tumor suppressor genes include the *RB1* and *p53*, each of which produces proteins that function as negative regulators of cell growth, division, and apoptosis (18, 19). Mutations in these tumor suppressor genes lead to the production of abnormal proteins that, unable to perform their primary functions, cannot prevent DNA-damaged cells from proliferating (18). In contrast, genes that induce cancer development are referred to as **oncogenes**; in healthy organisms, these genes function as growth regulators. Activation of oncogenes leads to the production of growth factors or growth factor receptors, some of which can be activated without a cognate ligand (14). Several oncogenes have been characterized in human cancers, but relatively few oncogenes have been connected to breast cancer. One well-studied example is the human epidermal growth factor receptor 2/neuro (*HER-2/neu* or *erb*B-2) gene, which encodes a transmembrane growth factor receptor for EGF. This gene is overamplified and upregulated in 20%-30% of invasive breast cancers (20). The overexpression of EGF receptor leads to the constitutive activation of it without the existence of its cognate ligand, leading to continuous intracellular signaling and, as a consequence, uncontrolled cell growth (14). Other oncogenes

overexpressed in breast cancer include members of the myelocytomatosis (*myc*) family, which encode transcriptional regulator proteins involved in cellular proliferation, differentiation, and apoptosis (21, 22). These genes are amplified in 15%-20% of breast tumors (22). The overexpression of c-*myc* and *HER-2* genes induces the initiation of both benign and malignant tumors in transgenic mice (23).

Tumor promotion is a cell proliferation stage during which mutagenic cells exhibit uncontrolled growth and form primary tumors. The promotion phase is usually subclinical, and can be partially reversed by the immunosurveillance response (see section 1.2.2.1). The mechanisms required for tumor promotion are complex and include tumor cell proliferation, enhanced survival, and increased angiogenesis (24). The majority of the promoting agents are also mutagens, and can therefore induce more neoplastic transformation. In breast cancer, a significant proportion of tumor cells express receptors for the hormones and the growth factors that are responsible for cancer development and retain some degree of dependence on these hormones or growth factors (14). The expression of these receptors may be altered from normal levels (increased or decreased), potentially causing aberrations in signalling pathways and gene activation or inhibition (14). Aberrations in these hormone and growth factor signalling pathways are linked to abnormal proliferation that augments tumor development (14). Other factors that can promote breast tumors are inflammatory mediators (see section 1.2.3.1).

Tumor progression is an extension of the promotion that involves increased tumor growth and invasiveness (increased malignancy). During tumor progression, more cells become mutated, leading to increased heterogeneity of the cell population (25). In this stage, the immunosubversion response (see section 1.2.2.2) becomes more predominant

and tumors grow to clinically detectable sizes that are mostly irreversible (25). Agents that induce tumor progression are similar to the initiators and promoters including hormones, growth factors and inflammatory cytokines (see section 1.2.3.1).

Metastasis is the main cause of death in patients with breast cancer, and is therefore considered the worst prognosis. Metastasis requires complex biological processes to enable primary tumor cells to spread to other organs and cause secondary tumors (26). Cancer cells must evade the immunosurveillance system (see section 1.2.2.1) and migrate from the primary tumor site to other organs or tissues through the blood stream or the lymphatic system, where they can colonize and develop to form a secondary cancer (26). Typically, the spread of metastases follow organ-specific patterns that vary depending on the cancer type (**Table 1**) (27). The organ-specific targeting of metastasis has been viewed as a non-random process since 1889, when the English surgeon Stephen Paget published the "seed and soil" hypothesis, in which specific tumor cells (the seed) have affinity for certain organs (the suitable soil) (28, 29). Although the mechanism by which organs are susceptible to harbor metastasis is poorly understood, some possibilities have been suggested. For example, cancer cells can be arrested in the microvasculature depending on their size or the involvement of specific adhesion molecules and coagulation factors (fibrinogen, fibrin, thrombin, and tissue factor) (26, 30). Another possible mechanism is the chemotactic homing of metastatic cells to organs under the influence of specific chemokines and their cognate receptors. Subpopulations of cancer cells in the primary tumor show heterogeneity with respect to the potential of metastasis (31, 32). The heterogeneity comes from a wide variety of molecules expressed on the cell surface, which can be important in directing these cells to specific organs through receptor-ligand

interactions (33). Furthermore, vascular endothelium is also heterogeneous in the context of surface receptors to certain ligands in healthy and pathological conditions (34). In the literature, it has been reported that chemokines like CXCR4 and CXCR2 play a critical role in tumor growth and metastasis using a 4T1 mammary carcinoma model (35, 36). Identification of new chemokines and chemokine receptors that play a significant role in the metastatic process is essential for developing therapeutic strategies to prevent or treat end-stage breast cancer patients.

Once the metastatic cancer cell survives the dissemination phase and localises to a suitable 'organ' it will colonize to form micro-metastasis, which can remain latent for a period before progressing to form macro-metastasis (37).

1.1.3. Breast cancer classifications

Breast cancer is considered a heterogeneous disease in terms of histology, treatment response, metastatic dissemination, patient outcomes, and most recently, molecular aberrations (38). Histologically, the majority of breast cancers are derived from the epithelium and are called carcinomas, which may arise from either the ducts (ductal carcinoma which account about 85% of breast cancers) or the lobules (lobular carcinoma) (39). Less commonly, some breast cancers are derived from the connective tissues, and are called sarcomas (39).

Traditionally, clinicians tend to describe breast cancers as either non-invasive (*in situ*, no metastasis) or invasive (with metastasis) (39). Advances in genetic research like DNA microarrays have provided a new tool to classify breast cancers depending on gene expression in tumor cells compared to normal cells (40 - 42). The use of immunohistochemistry (IHC) and the genomic profiling have led to the establishment of

seven subtypes: luminal A, luminal B, basal-like, HER2-enriched, normal breast-like, claudin-low, and molecular apocrine (see **Table 2**) (40). These subtypes differ from each other in the levels of estrogen receptor (ER), progesterone receptor (PR), HER2/ErbB2, and Ki67 protein.

These molecular differences among the subtypes lead to different prognoses: luminal A has the best prognosis, owing to its higher level of ER expression, while the basal-like subtype has the worst prognosis (43, 44). Classifying breast cancer based on gene profile not only helps in determining the prognosis of the breast cancer but also vital to determining the appropriate treatment (42, 45).

1.1.4. Breast cancer staging

Clinically, the stages of breast cancer are defined according to the TNM (tumour, node, metastasis) system developed by Pierre Denoix in 1942 (46) and revised by the American Joint Committee on Cancer (AJCC) (**Table 3**). The size and the spread of the breast cancer are helpful in stage determination, which can also clarify the patient's prognosis (47). This classification is best thought of as a snapshot of the cancer's status, taken at the time of diagnosis (47), providing a general indication of prognosis and treatment. Stages 0-II and early stages of III can be treated and cured with high potential, while the late stages of III and stage IV are advanced stages with poor prognoses (47).

1.2. Immune System

1.2.1. Overview

The immune system is responsible for protection against infections and alterations in healthy organisms. This protection system utilizes a network of cells, tissues, and factors that work together to form a line of defense against diseases (1, 48). The key role of the

immune system is to distinguish between the body's normal structures (self) and foreign (non-self) or abnormal ones (such as cancer cells). Altered cells can be detected by the immune system, which recruits different types of immune cells and molecules to eliminate them from the host. In vertebrates, the immune response is divided into two types, the innate and adaptive arms that provide protection for the host in a collaborative and interactive manner. Soluble and cell-surface elements are involved in the complex interactions of the innate and adaptive immune responses. Cytokines, one of the soluble elements, are a group of low-molecular-weight glycoproteins that work as messengers between immune cells to regulate innate and adaptive immune responses (49). Cell-surface molecules, such as major-histocompatibility complex (MHC) molecules, provide critical interactions between innate and adaptive immune cells. MHC molecules are encoded by the human leukocyte antigen (HLA) genes in humans and the H-2 complex genes in mice (50). MHC molecules are subdivided into three classes: MHC-I, MHC-II, and MHC-III; the MHC-I and MHC-II classes are vital to the immune response. MHC-I is a glycoprotein expressed on the surface of almost all nucleated cells that presents antigens to $CD8^+$ cells. MHC-II is a glycoprotein primarily expressed on antigen-presenting cells (APCs), which include macrophages, dendritic cells, and B cells; its major function is to present antigens to $CD4^+$ cells. Finally, MHC-III is a region located between MHC-I and MHC-II loci. It contains genes for several complement factors and pro-inflammatory cytokines (51). Immune system malfunctions can lead to a variety of problems, including the development of cancer.

1.2.1.1. Innate immunity

The innate immune response is the first line of host defense, recognizing and destroying pathogens and cancerous cells (1, 48, 49). The innate arm of the immune system consists of physical barriers as well as cellular and soluble components. The physical barriers of innate immunity can be mechanical (like skin, saliva, tears and gastric acid), biological (like commensal flora), or chemical (like antimicrobial peptides (52, 53). Innate immunity is mainly mediated through the functional activities of different types of cells, such as neutrophils, macrophages, dendritic cells (DCs), and natural killer cells (NK) and various soluble factors including cytokines, chemokines, the complement system, enzymes, and antimicrobial agents (54).

1.2.1.1.1. Neutrophils

Neutrophils are the most abundant leukocytes in human blood (50-70%), whereas they only represent 10-20% of leukocytes in mice (55). These cells play a significant role in innate immunity infiltrating sites of inflammation earlier than other cells. These cells are particularly prominent in bacterial infections, trauma, and cancer (49). The typical function of neutrophils is phagocytosis, wherein bacteria are digested and killed within the phagosome. Furthermore, neutrophils also release the components of their granules containing proteins that have anti-microbial effects; like myeloperoxidase, NADPH oxidase, and lysosomal proteases (56 - 58).

Under certain pathological conditions, such as infection or cancer, neutrophils response to inflammation and release a neutrophil-derived DNA webs called neutrophil extracellular traps (NETs) that can trap and kill pathogens; they also may play a role in trapping metastatic cancer cells (59). Neutrophils are a significant fraction of the

inflammatory cells in the tumor microenvironment (60, 61). Although neutrophils can exhibit cytotoxic effects against cancer (see section 1.2.2) (62), an increasing number of studies have found that tumor-infiltrating neutrophils are associated with a poor prognosis in several types of cancer, including renal carcinoma, melanoma, hepatocellular carcinoma, gastric adenocarcinoma, and colorectal cancer (60, 63).

1.2.1.1.2. Macrophages

Macrophages differentiate from monocytes after they leave the bloodstream and pass into the tissues. Like neutrophils, macrophages are phagocytic cells that specialize in the removal of dead cells, cellular debris, antigens coated with antibodies, viruses, and bacteria (49). Although macrophages are capable of capturing antigens and presenting them on the surface via MHC-II to CD4 T cells, they are weaker APC compared to DCs (64). Rather, macrophages appear to influence host defense through their activation status. Macrophages have two phenotypes, M1 and M2. The M1-macrophages or the classically activated macrophages reciprocally activate Th1 cells and have a pro-inflammatory role; this macrophage phenotype has the ability to kill pathogens in addition to its tumoricidal effects (65 - 67). These cells are MHC-II^{hi}, CD86^{+}, CD80^{+}, and express TLR2, TLR4 and Th1 cell-attracting chemokines CXCL9 and CXCL10 (68 - 70). In contrast, the M2-macrophages or the alternatively activated macrophages reciprocally activate Th2 cells and have an anti-inflammatory role; these cells are involved in tissue healing, through inhibiting inflammatory response and inducing angiogenesis, and they exhibit poor tumoricidal capability (71). The M2-macrophages are MHC-II^{+}, CD206^{hi}, CD163^{+} and *Arg-1* in the mouse; these cells also produce T$_{regs}$-attracting chemokines CCL17, CCL22 and CCL24 (68 - 70). Macrophages play a significant and complex role in cancer that

depends on the M1/M2 phenotype. The macrophages that are found within tumor masses are also referred to as tumor-associated macrophages (TAM), (see section 1.2.2.2.3).

1.2.1.1.3. Dendritic cells (DCs)

DCs are also phagocytic cells and highly specialized APCs that play a critical role in T helper cell activation and differentiation. DCs are a heterogeneous population of cells that capture and process foreign bodies, and present them via MHC-1 and MHC-II molecules to naïve T cells at lymphoid organs; thus, DCs act as a bridge between innate and adaptive immunity (72). DCs can be classified into two major subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Unlike cDCs, pDCs have lower levels of phagocytosis and do not efficiently present antigens via MHC-II (73). DCs play a significant anti-tumor role in cancer, but this function can be compromised or altered over the course of tumor progression by immunosubversion responses (see section 1.2.2) (74).

1.2.1.1.4. NK cells

These cells are responsible for killing of virally infected and tumor cells. NK cells distinguish self from non-self via a developed detection system composed of activating and inhibiting receptors. The activating receptors activate NK cells if they detect danger signals, from stressed cells that might overproduce self-molecules or other danger signals (49, 75). These receptors, such as NKG2D, can recognize the stress-inducible molecules like MHC-I-related chain A and B (MICA/ MICB), and UL-16 binding protein (ULBP) (76), as well as NKp46, which recognizes viral hemagglutinin (77). Whereas, the inhibitory receptors inhibit NK cells when they detect MHC-I molecules on healthy cells (self); however, the down-regulation of these molecules results in the loss of inhibitory signal, thus activating NK cells (49, 75). In the context of cancer, NK cells actively monitor the

host for abnormal cells that exhibit stress proteins or low MHC-I levels and destroy them before they develop into cancer (78). Upon activation, NK cells release their cytotoxic granules (perforin, various granzymes) and upregulate FasL interaction, which lead to the death of targeted cells through apoptosis (49, 78).

1.2.1.1.5. Natural killer T cells (NKT)

NKT cells have characteristics of both NK cells and T cells. Like NK cells, NKT cells express NK cell markers (e.g. CD16, CD56, and CD161) and produce granzymes. Similar to T cells, NKT cells have a T cell receptor (TCR) with a unique rearrangement that interacts with non-polymorphic CD1d molecules but not with MHC-I or MHC-II molecules. Unlike T cells, NKT cells can be activated by glycolipid antigens presented on CD1d molecules. Most NKT cells express an invariant TCR that can recognize the antigen α-galactosylceramide (α-GalCer); such cells are called type I NKT cells. A smaller proportion of NKT cells, known as type II NKT cells, express a wide range of TCRs that recognize CD1d but not α-GalCer (79). These cells fill the gap between the innate and the adaptive immune system, and are able to play both effector and regulatory roles in infectious and autoimmune diseases as well as cancer, depending on their ability to release different cytokines (e.g., IL-2, IFN-γ, TNF-α, and IL-4) (80). The dual functional role of NKT cells is relevant to cancer development: type 1 NKT cells are mostly protective and have an anti-tumor effect, while type 2 NKT cells primarily have an immune inhibitory activity (80).

1.2.1.2. Adaptive immunity

The adaptive immune system, or specific immunity, is the second line of defense to a primary invasion. This response is facilitated by antigen-specific T cells and B cells,

and usually it takes four to seven days to develop (1, 81). T and B-lymphocytes are the major players of the adaptive immune system, which possess specificity, diversity of repertoire, and memory (48, 49). The specificity of lymphocytes arises from their ability to distinguish between two peptides that differ in as little as one amino acid, and they can recognize the change in the 3D conformational structure (49, 82). This is achieved via rearrangement of several gene segments that encode the antigen receptors during development in the thymus (T cells) and bone marrow (B cells) (48, 49). Lymphocytes are kept from recognizing self-antigens through a rigid selection process that keeps only the nonself-recognizing cells and kills the self-recognizing cells (48, 49). In contrast to other cells in the immune system, lymphocytes have the ability to exhibit immunological memory, allowing for rapid response to secondary exposure of the antigen, and conferring life-long protection against some infectious agents (49). As detailed below, T lymphocytes can be subdivided into two phenotypes depending on function and cell surface markers.

1.2.1.2.1. T-lymphocytes

T cells are responsible for cell-mediated immunity, and can be distinguished from B cells and NK cells by the T-cell receptors (TCR) on their surface; most T cells, express α and β TCR chains, whereas γδ T cells utilize γ and δ TCR chains. T cell precursors originate from bone marrow and migrate to the thymus, where they mature and differentiate into different subtypes (49). There are two well-defined phenotypes of T cells: T helper (Th) cells and cytotoxic T cells (CTLs); they can be distinguished from each other by the surface expression of CD4 and CD8, respectively.

$CD4^+$ T cells become activated when they encounter an antigen presented by MHC-II molecules on the surface of an APC. Activated $CD4^+$ cells can differentiate into T helper

1 (Th1), T helper 2 (Th2), T helper 17 (Th17), or regulatory T cells (T_{regs}) based on their profile of cytokine secretion (83) (see **Table 4**). Th1 cells are effector $CD4^+$ cells that differentiate under the influence of IFN-γ and IL-12, with the involvement of the T box transcription factor (T-bet). These cells mainly develop following infections with intracellular bacteria and viruses (84, 67). Th1 cytokine IFN-γ activates macrophages leading to M1 phenotype and is required for IgG2a isotype switching (67). Th2 cells differentiate from naïve $CD4^+$ T cells under the influence of IL-4, with the involvement of the GATA3 transcription factor. These cells predominate in response to infestations by gastrointestinal nematodes (84, 67). The effects of Th2 cytokines such as IL-4, distinct from Th1, are responsible for IgG1 isotype switching, eosinophil activation, and inhibition of several macrophage functions like phagocytosis, leading to M2 phenotype (67). Th17 cells polarize under the influence of TGF-β, IL-6, IL-21, IL-1β, and IL-23, the last of which stabilizes the Th17 phenotype in order for it to become an effector cell (85). Th17 differentiation requires the activation of the transcription factor ROR-γt, which is signal transducer and activator of transcription 3 (STAT3)-dependent (86, 85). Th17 cells and their cytokines play an important role in cancer, autoimmunity, allergy and host defense against extracellular bacteria, as well as some fungi (87). T_{regs} are $CD4^+CD25^+$ T cells, which develop either in the thymus (natural T_{regs}) or in the periphery from naïve $CD4^+$ cells (induced T_{regs}), polarize under the influence of TGF-β and IL-2, with the involvement of the forkhead transcription factor FOXP3. These cells are critical to the maintenance of immune homeostasis and regulating effector T cell responses (84).

Overall, the role of $CD4^+$ T cells in cancer is highly dependent on their phenotypes: Th1 cells promote an anti-tumor effect while Th2 cells and T_{regs} support pro-tumor effects, and the role of Th17 cells remains controversial (see 1.2.2) (88).

$CD8^+$ T cells become activated upon recognition of an antigen presented by MHC-I molecules and, similarly to $CD4^+$ T cells, they develop into effector cytotoxic T cells or memory cells with distinct subsets. $CD8^+$ T cells are defined by expression of the same characteristic cytokines of T helper cells and are referred to as T cytotoxic 1 (Tc1), T cytotoxic 2 (Tc2), and T cytotoxic 17 (Tc17) (89, 90). These $CD8^+$ T cell subsets also differ from each other in their cytotoxic response, as Tc1 cells destroy their target cells through perforin/granzyme and/or Fas/FasL mediated pathways, Tc2 cells primarily use perforin/granzyme, while Tc17 cells show less cytotoxic activities as they have low levels of granzyme B, perforin and FasL (89, 91). Although the role of $CD8^+$ T cells in tumor immunity is well established, the prognosis of infiltrating $CD8^+$ T cells within several types of cancers is still controversial, which could be due to the heterogeneity of $CD8^+$ T cells (92, 93). Faghih *et al*, have shown an increase in Tc2 and Tc17 subsets and a decrease in the Tc1 subset within tumor draining lymph nodes (TDLNs) of advanced-stage breast cancer patients, suggesting a role for Tc2 and Tc17 in promoting tumor progression (89).

1.2.1.2.2. B-lymphocytes

B cells are responsible for humoral immunity, and their surface B-cell receptors are immunoglobulins (antibodies). B cell development, defined by CD19, CD45R (B220), and surface IgM, starts in the liver before birth, and in bone marrow in adults (94, 95). Naive B cells expressing both IgM and IgD leave the marrow and migrate to the secondary lymphoid tissues where they settle in the follicular region. They can undergo somatic

hypermutaion and class switching to IgA, IgG, or IgE if they encounter an antigen (94). The B cells in the mucosal associated lymphoid tissues are more likely to produce IgA, whereas the B cells in the lymph nodes and spleen are more likely to produce IgG (95). Once a B cell encounters a specific antigen matching its membrane-bound antibody, it acts as an APC that engulfs, processes, and presents peptides of the antigen on its surface to be recognized by T helper cells (49, 50). T helper cells induce the activation of B cells, which undergo clonal expansion and develop into effector B cells (plasma cells) and memory B cells (49). Plasma cells are able to produce large amounts of antibodies that bind to specific antigens, which can be neutralized either by the Ab itself, or with the help of other mechanisms such as the complement or effector cells that can bind to the Fc portion of the antibody (96). In the context of cancer, several studies have shown controversial results regarding the role of B cells in tumor progression. For example, some studies have shown that B cells inhibit T cell-mediated regression when compared with B cell-deficient mice (97, 98). In contrast, other studies have shown positive roles for B cells on T cell responses to cancer cells (99, 100). This controversy was attributed to the status of the B cells (activated or not), as T cell responses have been shown to be inhibited by resting B cells and enhanced by activated B cells (101). The role of B cells in tumor progression is also recognized through the production of several antibodies against tumor antigens in human cancer patients (94).

1.2.2. Tumor immunity

Healthy organisms have multiple cell-intrinsic and cell-extrinsic barriers that protect against cancer development. The cell-intrinsic factors are controlled via different genes that are responsible for tumor suppression (see section 1.1.2), while the cell-extrinsic

factors are usually the innate and the adaptive immune system. Dysfunction in any of these barriers can trigger tumorigenesis (102). From an immunological perspective, cancer results from altered self-cells, which might have some products or debris that can serve as tumor antigens. Tumor antigens can be classified into **tumor-specific antigens (TSA)**, which often unique to an individual tumor, and **tumor-associated antigens (TAA)**, which can be found in both tumor cells and normal cells (103). The anti-tumor adaptive immune response can be triggered upon capture and recognition of TSA and TAA. Although certain immune responses can be host-protective, other immune processes, such as chronic inflammation, can promote the initiation and/or progression of cancer (104, 105). Cancerous cells that form clinically diagnosed cancers must have succeed in escaping anti-tumor responses (**immunosurveillance response**) (102), either through selection of resistant variants **(immunoediting)** or through subverting the immune system to support pro-tumor response (**immunosubversion response**) (102, 106, 107).

Cancer immunoediting typically occurs in a milieu called the tumor microenvironment, which is composed mainly of tumor cells, immune cells, blood vessels, connective tissues, and different soluble factors (108). The immune cells in the tumor microenvironment, of both innate and adaptive types, are the key players in cancer immunoediting. These cells can be classified into two main classes: the immunosurveillance cells (anti-tumor) and the immunosubversion cells (pro-tumor) (109).

1.2.2.1. Immunosurveillance

One of the major functions of the immune system is to detect and destroy cancer cells via recruitment of both the innate and adaptive arms of immunity. The immune system often succeeds in eliminating the transformed cells through the immunosurveillance

response, first hypothesized in the 1950s by Burnet and Thomas (106, 110, 111). Immunosurveillance is responsible for eliminating the majority of the altered cells and the cancerous precursors before they develop into clinically diagnosed tumors. This response has been identified through observation of certain malignancies that occur in animals or humans with disorders in certain parts of the immune system, but are not found in healthy organisms (112). Furthermore, there is strong evidence that the accumulation of lymphocytes in the tumor microenvironment is conducive to a good prognosis for patients with several types of cancer, including breast, melanoma, bladder, colon, prostate, ovary, and rectal cancers (113 - 119). The immunosurveillance response against tumor cells begins when tumors start to invade surrounding tissues, inducing inflammatory signals and recruiting innate immunity cells like NK cells, NKT cells, macrophages, and DCs (102, 120). NK cells, perhaps the best-characterized effector cells in innate immunosurveillance of cancer, are able to kill cancerous cells without sensitization, as shown in some *in vitro* experiments (121). Moreover, many transplanted tumors are rejected by NK-cell-dependent mechanisms through direct interaction and perforin-mediated killing (122 - 125). Recognition of transformed cells by innate immunity leads to the production of IFN-γ from immunosurveillance cells such as NK cells (126). The role of IFN-γ in tumor immunosurveillance is important; it has an anti-proliferative effect on tumor cells through the induction of apoptotic death in these cells (127). Furthermore, IFN-γ also induces the production of CXCL9 and CXCL10 by TIL, leading to the recruitment of NK cells, which express CXCR3 (128). The antigens produced by tumors (TSA and TAA), which are released as the debris due to tumor cell lysis, are ingested by DCs. In the draining lymph nodes, the antigen-loaded DCs induce activation and differentiation of

$CD4^+$ Th1 cells, which in turn facilitate the activation of $CD8^+$ CTLs (129 - 131). Tumor-specific Th1 cells and CTLs are among the most potent anti-tumor cells; they home back to the tumor microenvironment where they recognize tumor-specific antigens presented on MHC molecules and exert their effects (see section 1.2.1.2) (132).

Neutrophils, as part of the innate immune system, make up a significant portion of the inflammatory cells in the tumor microenvironment. Several studies suggest a pro-tumor effect of tumor-associated neutrophils (TANs) especially at late stage tumors compared to early stages where they exhibit anti-tumor cytotoxicity (133). Moreover, TAN can induce chemoattraction of T_{regs} through the secretion of CCL-17, thus enhancing tumor growth (134), In contrast, TGF-β blockade can enhance the recruitment and activation of neutrophils that mediate anti-tumor responses (62). Furthermore, neutrophils can modify the inflammatory and immune responses by interacting with other innate immune cells (135, 136).

1.2.2.2. Immunosubversion

Even though nascent tumors encounter effective immunosurveillance, clinical tumors can still develop. In some cases, the immunosurveillance response fails to stop cancer development completely because tumor cells are not sufficiently immunogenic to be targeted, the tumor mutates to lose antigens, or, alternatively, a suppressive microenvironment develops (102, 137). To date, different mechanisms, intrinsic and extrinsic, have been suggested to escape or evade the immune system (immunosubversion). It has been shown that 40-90% of human tumors exhibit low MHC-I expression on the cell surface compared to normal cells (138), making them less likely to be recognized by CTLs (102, 139). Tumor cells can also become resistant to the immune response and

chemotherapy through evasion of apoptosis (140, 141), accomplished through the expression of anti-apoptotic proteins like Bcl-2, Bcl-X_{L}, or $FLIP_{LS}$, which enhance cell survival (141 - 143). In addition, some cancer cells gain the ability to synthesize growth factors, and proliferate in response to them through positive-feedback loops (autocrine response) (102). For example, in breast cancer, several cell lines (e.g., MCF-7, MDA-MB-231, ZR-75-1, and Hs578T) are able to produce insulin-like growth factor-I (IGF-I) and respond to it (144). Although IGF-I plays a role in normal mammary gland development, it also regulates survival, proliferation, and metastasis in breast cancer (145). Increased proliferation may also occur through overexpression of growth factor receptors, resulting in hyper-responsiveness of cancer cells to normal amounts of growth factor (102). For example, the epidermal-growth-factor receptor is upregulated in breast and stomach carcinomas (see section 1.1.2) (102, 14). Correspondingly, several cytokines have been reported in different cancers to act in an autocrine manner and induce tumor progression. For example, IL-4 and IL-10 are found at high levels in thyroid carcinoma and acute myeloid leukemia (AML) and can skew immunity from the anti-tumor Th1 response to the pro-tumor Th2 response (146 - 148). In prostate cancer, IL-6 overproduction can induce tumor progression through the activation of STAT3, which blocks both the production and sensing of inflammatory signals by multiple immune cells leading to reduced interactions between innate and adaptive immune responses, as well as its role in promoting MDSC expansion (149 - 151).

The extrinsic factors that help the tumor to evade immunity are mainly composed of immunosuppressor cells and their products, such as MDSCs, TAMs, and T_{regs}. These cells are recruited to the tumor microenvironment under the influence of several

chemokines and their corresponding receptors. For example, CCL2-CCR2, CCL1-CXCR2, and IL-1-IL-1R are important for MDSC infiltration (152, 153); likewise, CX3CL1-CX3CR1 is important for TAM infiltration, and CCR4 and CCR7 are critical for T_{reg} infiltration (154, 155). These chemokines and their receptors are induced during the inflammatory response in the tumor microenvironment under the influence of several pro-inflammatory molecules, like IL-1β, TNF-α, IL-17A and prostaglandin2 (PGE2) (152).

1.2.2.2.1. Myeloid-derived cells suppressor cells (MDSCs)

MDSCs are a heterogeneous population of immature cells of the myeloid lineage that exhibit potent suppression of the immune response and expand in patients with cancer (156), autoimmunity (157), inflammation (158), and infection (159). MDSCs often accumulate in cancer patients and experimental animals in response to pro-inflammatory mediators, and they are considered a serious obstacle to immunotherapy (156). These cells have been known for more than three decades under different names, like natural suppressor cells, immature myeloid cells, and suppressor macrophages (160 - 164). The current terminology has been used since 2007, mainly because it accurately reflects the origin and biologic function of these cells (165, 166). In healthy individuals, hematopoietic stem cells differentiate into common myeloid progenitor and then into immature myeloid cells, which migrate and differentiate in the peripheral lymphoid organs to produce monocytes/macrophages, DCs, or granulocytes. However, under pathological conditions, the maturation of immature myeloid cells is partially altered, such that they accumulate as heterogeneous populations of MDSCs (monocytic-MDSCs and granulocytic-MDSCs) (167). Different growth factors and cytokines, many of which are associated with chronic inflammation, have been associated with the expansion of MDSCs. These include GM-

CSF (168), G-CSF (169), IL-17A (170), VEGF (171), PGE2 (172), IL-1β (173), IL-6 (151), and SCF (174). The transcription factor STAT3 plays an important role in MDSC expansion and survival, via upregulation of multiple proteins required for myeloid cell differentiation, in addition to its role in inducing proliferation and preventing apoptosis (175, 176). Moreover, STAT6 and STAT1 are important for the activation of MDSCs, which leads to the upregulation of arginase 1, inducible nitric oxide synthase (iNOS), and TGF-β (167). The STATs are activated by several factors released by tumor cells and activated T cells, such as IL-13, TGF-β, IL-4, and IFN-γ (167).

MDSCs are characterized differently between mice and humans. In mice, MDSCs are broadly characterized by the co-expression of both Gr-1 and CD11b surface markers (177). CD11b is an alpha component of the α_M integrin that can be found normally on myeloid cells (granulocytes, monocytes and macrophages, DCs) and on some lymphocytes (B and T cells) (167). Gr1 is a myeloid differentiation antigen, which is found normally on immature myeloid cells in bone marrow and granulocytes in the periphery. Gr-1 is detected by the RB6-8C5 monoclonal antibody, which recognizes the epitope shared by two lymphocyte antigen 6 complexes (Ly6C and Ly6G), which are commonly used for the classification of two MDSC subsets in tumor-bearing mice: $CD11b^{+}Ly6C^{low}Ly6G^{+}$ with a granulocytic morphology (G-MDSCs) and $CD11b^{+}Ly6C^{high}Ly6G^{-}$ with a monocytic phenotype (M-MDSCs) (178-180). Yuon *et al*, have shown that a large proportion of M-MDSCs in tumor-bearing mice can differentiate into G-MDSCs through silencing of *Rb1* gene (181). Moreover, Corzo *et al*, have shown that, under hypoxic conditions, MDSCs can dramatically differentiate into TAMs (182).

In humans, identifying MDSCs in cancer patients is more challenging due to the lack of the Gr-1 marker, the difficulty in collecting samples (164), and the existence of different MDSC subsets depending on tumor type (183). Nevertheless, several studies in cancer patients have identified G-MDSCs ($CD11b^{+}CD14^{-}CD33^{+}CD15^{+}$) (184) and M-MDSCs ($CD11b^{+}CD14^{+}CD33^{+}HLA\text{-}DR^{low/-}$) (185). Nonetheless, in light of the above difficulties, the only way to define a MDSC population is to assess their suppressive function.

The pro-tumor role of mouse MDSCs arises from their potent suppression of several immune cell types, including M1 macrophages, NK, DCs and T cells (167). *In vivo* and *in vitro* experiments suggest direct and indirect mechanisms by which MDSCs suppress immune cells (167). The direct effects of MDSCs require cell-cell contact, which indicate that they function either through cell-surface receptors or through the secretion of short-lived soluble mediators (167). Some of the factors that MDSCs produce are immune-suppressive, such as the products of arginase 1, iNOS, and reactive oxygen species (ROS), (167). Three other mechanisms for MDSCs suppression activity have been suggested. First, the expression of ADAM metallopeptidase domain 17, leading to the cleavage of L-selectin (CD62L) on T cells, thus impair the migration of naïve T cells ($CD4^{+}$ and $CD8^{+}$ T cells) (186). Second mechanism is shared with $FoxP3^{+}$ T_{regs}, through depleting the cysteine, which is an essential amino acid for T cell activation, leading to impaired T cell activation and function (187). Third mechanism is through the interaction between Tim-3 on IFN-γ-producing cells and its ligand, galectin-9, on MDSCs; this interaction leads to the inhibition of Th1-cell response by triggering cell death (188). In contrast, the indirect effects can be

via induction of other suppressor cells, such as M2 macrophages and T_{regs} through secretion of IL-10 and TGF-β (189, 190, 88).

Arginase 1, an enzyme that converts L-arginine to urea and L-ornithine, is highly upregulated under the influence of IL-4, IL-13, TGF-β, and GM-CSF (180, 191). Overproduction of arginase 1 leads to the depletion of L-arginine, which in turn causes disruption of CD3ς mRNA stability, thus impairing T cell proliferation and maturation (191 - 193). **NOS2**, which utilizes L-arginine to produce nitric oxide (NO), is produced under the influence of Th1 cytokines like IFN-γ, IL-2 and TNF (194). NO is able to disrupt T cell activation through a number of mechanisms, including the inhibition of Janus Kinase 3, activation of STAT5, and inhibition of MHC-II expression, as well as the induction of T cell apoptosis (194 - 196). **ROS**, including H_2O_2, O_2^-, and OH^-, are also characteristic of MDSCs in cancer patients (197). Several cytokines and growth factors can increase ROS production by MDSCs, including IL-3, IL-6, and GM-CSF (198). The receptors for these cytokines signal through Janus kinase 2 and STAT3, which activate NADPH oxidase leading to increased production of ROS within myeloid cells (167); increased production of ROS inhibits the differentiation of myeloid cells, playing a role in the accumulation of MDSCs (167, 199). ROS, especially H_2O_2, produced during antigen-specific interactions between MDSCs and T cells, suppress IFN-γ production and inhibit CD3ς expression on T cells, thus disrupting T cell responses (199, 200).

Recent studies have shown that the MDSC subpopulations, G-MDSCs and M-MDSCs, employ different mechanisms to suppress immune responses. For example, G-MDSCs have higher STAT3 and NADPH activity, leading to higher ROS levels and low NO (156, 167). In contrast, M-MDSCs exhibit higher STAT1 activity that increases NOS2,

leading to higher NO levels but low ROS (156, 167). Both subtypes exhibit comparable levels of arginase 1 (180). The variable activity of these transcription factors and effector molecules between MDSC subsets is correlated with different suppressive potency between the two subsets, and is higher in M-MDSCs compared to G-MDSCs (201).

Although NK cells are potent anti-tumor cells, their cytotoxicity is inhibited in the presence of MDSCs. MDSCs can induce anergy of NK cells through membrane-bound TGF-β1 as well as blocking IFN-γ and perforin production (202, 203, 156). Moreover, the crosstalk between MDSCs and macrophages leads to the polarization of macrophages into the pro-tumor M2 phenotype (204). MDSCs also support tumors indirectly via the induction of T_{regs}, which can suppress anti-tumor immunity. The expansion of T_{regs} by MDSCs is induced via IL-10 and is TGF-β dependent (186); it can also be induced via arginase, in which case it is TGF-β independent (190).

1.2.2.2.2. Regulatory T cells (T_{regs})

Tumors are usually infiltrated with various numbers of $CD4^+$ and $CD8^+$ T cells. T_{reg} cells, a subset of $CD4^+$ cells characterized by the markers CD25 and FOXP3, are often expanded in tumors relative to their frequency in the periphery (108). T_{regs} cells favor tumor outgrowth because they are able to suppress the anti-tumor immune responses, and therefore represent one of the obstacles to an effective anti-tumor response, and contribute to the failure of immunotherapy (205). Several mechanisms have been defined through which T_{regs} can inhibit anti-tumor response. Tregs can physically inhibit the interactions between DCs and conventional T cells. In addition, Tregs express inhibitory receptors such as cytotoxic T lymphocyte-associated antigen-4, which can compete for co-stimulatory molecules, such as CD80 and CD86, on DCs leading to impaired T cell activation and

proliferation (137, 206). Furthermore, Tregs can secrete perforin and granzyme B, which can induce apoptosis and cytolysis in both DCs and conventional T cells (206). In addition, the overproduction of IL-10 and TGF-β from T_{regs} within tumor induces the tumor growth through suppressing effector $CD8^+$ cells response (207).

1.2.2.2.3. Tumor associated macrophages (TAMs)

In response to several factors in the tumor microenvironment, like IL-4, IL-13 and IL-10, TAMs can skew toward the suppressive M2-like phenotype (137). TAMs induce angiogenesis via the release of VEGF and endothelin-2 (208), apoptosis resistance by attenuating the activation and cleavage of caspase-dependent apoptotic signaling (209), and tumor cell invasion and metastasis by releasing matrix metalloproteinases-2 and -9, which degrade the extracellular matrix and the basement membrane (210, 211). Furthermore, TAMs are poor APCs and produce immunosuppressive factors including IL-10 (212), PGE_2 and TGF-β (213). Other pro-tumor effects of TAMs include the inductions of T cell apoptosis via interaction of PDL-1 on TAMs with PD-1 on T cells, and their production of CCL22, which, together with IL-10, can recruit T_{regs} and maintain their activity (137, 214, 215).

1.2.3. Inflammation and Tumorigenesis

1.2.3.1. Overview

Inflammation is one of the most vital orchestrators of host defenses directed towards eliminating infections and damaged cells, as well as healing injured tissues. In general, inflammation can be classified as acute or chronic, depending on the duration of the process. Acute inflammation is a short-term response that is accompanied by intense local or systemic symptoms. In contrast, chronic inflammation is a long-term response that

is typically accompanied by continuous immune activation that can cause tissue remodeling due to destruction and healing processes. In the context of cancer, inflammation can be classified into several types that differ from each other in cause, outcome, mechanisms, and severity (152). These types include chronic inflammation, tumor-associated inflammation, and therapy-inducing inflammation.

Chronic inflammation, which can be caused by persistent infections, prolonged exposure to environmental irritants, and autoimmunity, has been suggested to precede tumorigenesis (216 - 219). Chronic inflammation may promote tumorigenesis through various mechanisms, such as the induction of oncogenic mutation, genomic instability, which leads to neoplastic transformation (152, 216, 219). However, not all chronic inflammation diseases increase the risk of cancer development, such as psoriasis and rheumatoid arthritis, which could be due to the lower exposure to dietary and environmental carcinogens within joints or on the skin (152, 218).

The inflammation that develops during tumor progression is called **tumor-associated inflammation**. This type of inflammation promotes tumor through enhancing angiogenesis, tumor progression, and metastasis, augmenting genomic instability, and inducing immunosubversion (see section 1.2.2.2) (152).

Therapy-induced inflammation refers to the inflammation that follows some cancer treatments, which is not fully understood. This type of inflammation may enhance antigen presentation, leading to tumor regression (152, 220). In contrast, some therapies, such as radiation, might cause cancer necrosis that leads to increased inflammation, thus, promotes tumor progression in a way similar to the tumor-associated inflammation (152, 221, 222). In the other hand, therapy-induced inflammation can increase the

presentation of tumor antigens and stimulates an antitumor immune response that improves the therapeutic outcome (152).

Inflammation plays an instrumental role in promoting all steps of cancer development. This role is highly dependent on the soluble factors that exist in the tumor microenvironment, the most relevant of which are cytokines, chemokines, and growth factors (regardless of their cellular source) (223). Some molecules, such as reactive oxygen and nitrogen compounds, which can be released under the influence of pro-inflammatory cytokines, damage DNA and RNA leading to genomic mutations and instability (224). If these mutations occur within tumor suppressor genes or oncogenes, the result might be neoplastic transformation and initiation of tumorigenesis (224). However, neoplastic transformation is usually irreversible and can be transferred to daughter cells; transformed cells are susceptible to becoming tumors unless they are eradicated by the immunosurveillance response (see section 1.2.2.1) (225). Many pro-inflammatory molecules, such as IL-6 (226), IL-1β (227), IL-17A (228), and IL-23 (229), have been shown to play an important promoter role in different types of cancers. These inflammatory cytokines can promote tumor growth through the activation of STAT3, NF-κB, and AP-1, which in turn control apoptosis, proliferation, angiogenesis, invasiveness, and motility, as well as the expression of chemokine genes (230). Angiogenesis is one of the tumor-associated inflammation characteristics that serve as tumor promoter. Hypoxia is the primary trigger for angiogenesis in the tumor microenvironment (231), acting as a danger signal that can be received by TAMs, MDSCs, T_{regs} and other cells (232). Subsequently, these cells are able to induce angiogenesis via activation of STAT3, NF-κB, and AP-1, which in turn induce several angiogenic factors (e.g., VEGF, HIF-1α, IL-8, and CXCL-1)

(233, 234). The significance of angiogenesis in cancer development arises from the recruitment of several inflammatory cell types, which can be attracted into the tumor microenvironment, to be polarized into immunosuppressor phenotypes (see section 1.2.2.2), thus, enhancing tumor progression. Furthermore, inflammation can also induce the metastatic process through different mechanisms. Besides its role in inducing angiogenesis that is required for metastasis spread in circulation, inflammation can also enhance the release of metastatic cancer cells via increasing vascular permeability (230). Moreover, inflammation induces chemotaxis of cancer cells to specific organs by stimulating expression of different chemokines and their receptors on both metastatic cells and targeted organs, in addition to preparing the adequate milieu for metastasis to grow and survive immunosurveillance response (235, 236). These chemokines (receptors and ligands) that can be induced on tumor cells or organs are variable among tumor types and constantly updated with new discoveries (237).

1.2.3.2. IL-17/IL17R family

IL-17 cytokine family is composed mainly of six pro-inflammatory cytokines (IL-17A-IL17F), while their corresponding receptor family is composed of five receptor subunits (IL-17RA-IL-17RE). The best-characterized cytokines in this family are IL-17A and IL-17F, both of which can be found as homodimers or heterodimers (238). Regardless of the form, they signal through the same receptor, typically composed of at least two IL-17RA subunits and one IL-17RC subunit (239). IL-17RA is a commonly shared receptor subunit that can form heterodimers with IL-17RC, IL-17RE and IL-17RD, and it is highly expressed in hematopoietic tissues (240). IL-17RC is a required subunit for IL-17A and IL-17F signaling, through pairing with IL-17RA; unlike IL-17RA, the expression of IL-

17RC is low in hematopoietic tissues but high in non-immune cells of the liver, kidney, prostate, and joints (241).

IL-17A and IL-17F are the hallmark cytokines of Th17, which is a subset of $CD4^+$ cells (see section 1.2.1.2.1); they can also be secreted by other cells, such as γδ T-cells, NKT cells, $CD8^+$ T cells, and lymphoid tissue inducer cells (242). The effect of IL-17A is mainly characterized by its pro-inflammatory role, which has a potent effect on the stromal cells of various tissues (87). Downstream signaling through IL-17R initiates activation of NF-κB and MAPK, leading to the production of a wide array of inflammatory cytokines and chemokines (242). These products induce inflammation through recruitment of different leukocytes, especially neutrophils and macrophages, thus forming a bridge between innate and adaptive immunity (242, 87).

Th17 cells represent a minor population in human peripheral blood under normal conditions and exhibit minor changes in the case of cancer (243). Nonetheless, the increased frequency of IL-17A-producing cells at the tumor site suggests a significant role for this cytokine in cancer development (244). Many of the soluble factors in the tumor microenvironment, like IL-6, PGE2, TGF-β, IL-21, IL-1β, and TNF, favor Th17 differentiation over other Th subtypes, and some of these cytokines are induced by IL-17A itself, resulting in a positive feedback loop (245, 246). Although IL-17A-producing cells are detected in multiple cancers, including breast cancer in humans and mouse models, the role of IL-17A in the tumor microenvironment is controversial (247 - 249). The majority of functions attributed to IL-17A in the tumor microenvironment are thought to support tumor progression. This pro-tumor effect is due to the ability of IL-17A to induce the production of pro-inflammatory cytokines, chemokines, and growth factors that favor

tumor progression (242, 85). In contrast, some studies have suggested that IL-17A can exhibit an anti-tumor effect; IL-17A has shown to be able to inhibit the growth of some tumors, like plasmacytoma and mastocytoma, through activation of CTLs (250). Moreover, IL-17A induces DC progenitor maturation by increasing CD11c, MHC-II, and costimulatory molecule expression, which can induces T cell priming and activation (251).

1.2.3.3. Chemokine Receptor CCR4 and Its Ligands CCL22/CCL17

One of the impacts of cancer-related inflammation is the induction of various chemokines and their receptors (see section 1.2.3). It is becoming increasingly clear that, during dissemination, tumor cells use mechanisms similar to immune cells, trafficking via chemokine receptors. Recent studies have linked several chemokines receptors, CXCR4, CCR10, CCR4 and CCR7, with breast cancer metastasis (155, 252), as well as CCR6, CXCR5, CCR7, and CX3CR1 with lung and pancreatic cancers (155, 253). Olkhanud *et al* (155) have shown using the 4T1 mammary carcinoma model that the chemokine receptor CCR4 and its ligands, CCL17 and CCL22, are required in the metastatic process, with the involvement of T_{regs}.

CCR4, a seven-transmembrane G-protein-coupled receptor, is the specific receptor for macrophage-derived chemokine MDC/CCL22 and thymus activation-regulated chemokine TARC/CCL17 (254). Expression of CCR4 has been described on various types of cells, but is most prevalent on T_{regs}, memory T cells, and Th2 cells (155, 255). However, CCR4 is also upregulated on several types of cancer cells, including breast cancer, B-cell chronic lymphocytic leukemia, and adult T-cell leukemia/lymphoma. The chemokines CCL17 and CCL22 are induced in some tumors, drawing $CCR4^+$ T_{regs} to the tumor site, where they promote tumor growth and suppress immunity (256, 257). CCR4 ligands are

also remotely induced by the primary tumors in specific organs where breast cancer cells tend to metastasize (155). Owing to the importance of this chemokine receptor, phase-I clinical trials using an anti-CCR4 mAb have been initiated for treatment of patients with CCR4-positive neoplasms (256). However, the main inducers of CCR4 and CCL17/CCL22 have not yet been fully identified.

1.3. Research Tools

1.3.1. Mouse model of mammary carcinoma

Several murine models have been utilized in breast cancer research, enabling scientists to study important aspects of breast cancer, including angiogenesis, metastasis, tumor-host interactions, and potential treatments (258). In mice, tumor progression is highly dependent on the strain; for example, BALB/c and DBA2F are more permissive for tumor development than C57BL mice (259). There are numerous methods for studying breast cancer using animal models, the most common being grafting or transplantation of transformed cells or whole tissues into the host (259). Grafting and transplantation methods depend on several factors: the donor of the cell line or tissue (human or animal), the host (syngeneic or non-syngeneic), and grafting site (orthotopic, ectopic, or systemic) (259).

Each model has its own advantages and disadvantages that must be evaluated and assessed in the context of the research objectives. Syngeneic transplantation models, such as the 4T1 model in BALB/c mice, are one of the most commonly used models for studying metastasis biology (260, 261). The advantages of syngeneic models include the lack of immunologic host-versus-graft reaction as well as the ability to evaluate interactions between the intact immune system and cancer, (261). Although these models can mimic human breast cancer, the lack of human elements in them limits the range of clinical

application. In contrast, xenograft transplantation models (like MDA-MB-231 cells in nude mice) are usually used to study the growth and metastasis of human breast cancer cells within immunocompromised mice (262). Although these models use human elements, they cannot be used to understand tumor initiation because the grafts are already transformed, and the immune system, which is important in tumor development, has been impaired (263). Although there is no model that can perfectly reflect human breast cancer, genetic profiling has enabled the selection of murine mammary cell lines that can exhibit molecular similarity to certain human breast cancer subtypes (264).

4T1 mammary carcinoma: The murine 4T1 model is commonly used to study the spontaneous metastatic process in breast cancer. The 4T1 cell line is syngeneic to BALB/c mice, and can be injected orthotopically into the mammary fat pad in order to establish a primary tumor (265, 266). This model is suitable for studying the late stages of breast cancer because 4T1 cells can grow locally and metastasize to lung, brain, bone, and liver within one to two weeks of establishment (265). The main route for spread of metastasis in this model is the blood stream, with a minor role for the lymphatic system (265, 266). The 4T1 cells are known to be resistant to 6-thioguanine (6-TG) (266). This characteristic of 4T1 cells has been used to identify specific organ metastasis through culturing targeted organs in medium supplemented with 6-TG so that only 4T1 cells will grow and form colonies that can be counted (267). Another advantage of using the 4T1 cells is that they can induce a leukemoid reaction in tumor-bearing BALB/c mice through induction of MDSCs (268). MDSC levels increase in the peripheral blood (PB), spleen, and the bone marrow of the tumor-bearing mice (268), simulating what happens in some human cancers. Thus, 4T1 is an excellent model for the study of MDSCs in breast cancer.

1.3.2. Adenovirus

Adenovirus is a linear double-stranded DNA virus of approximately 34-43 kb contained within a non-enveloped icosahedral capsid. Adenoviruses are classified into different types depending not only on the species but also on the serotype within each species. Human adenoviruses are divided into 51 serotypes classified into six subgroups (A-F; B has B1 and B2) depending on their genome sequence and their ability to agglutinate red blood cells (269). The DNA genome is composed of eight transcription units classified into five early units (E1A, E1B, E2, E3, E4, and E5), two intermediate, and one late (L, divided into L1-L5) (see **Figure A 6**) (269). E1 is essential for adenovirus replication, and deletion can therefore be used to attenuate the virus replication (270). E3 is non-essential for replication but regulates the host immune response to viral infection (270). The most commonly used first generation vectors contain E1and E3 deletions (271). These vectors are unable to replicate in normal cells, but they can be propagated in cells transfected with E1 and E3 genes, (e.g., HEK293 cells) (272). Deletion of the E1 and E3 sequences creates an empty space that allows for the addition of different genes to a maximum length of 8 kb; this addition can be utilized for gene delivery to the transduced cells, allowing for the production of excess amounts of specific proteins. The first generation of these adenovirus vectors are used in experiments requiring transient gene expression, because they still have undeleted adenoviral genes that are able to induce immunity against transduced cells. A new generation of these vectors, helper-dependent virus vector, has all the viral genes deleted but only contains *cis*-acting sequences that are required for DNA replication and virus packaging (273). The extensive deletion allows the vectors to avoid immune responses, hence favoring long-term transgene expression.

However, the propagation steps of these fully deleted vectors are more complicated and require a helper virus (274).

The human serotype (5) adenovirus is the most commonly used adenovirus vector for a number of reasons (269):

- These adenoviruses are stable and able to produce high titers in tissue culture.
- The genetic structure is well characterized, allowing for the deletion of some genes and insertion of others.
- It is easy to purify and manipulate.
- It is able to infect a wide range of mammalian cells from different species.
- Adenovirus vector infected cells are able to produce transgene products efficiently.

Upon infection of targeted cells, adenoviral vectors allow for transmission of their genes to the host nucleus but do not integrate them into the host genome, thus the transgenes are only transiently expressed without passing into daughter cells (275).

1.4. Objectives and Hypothesis

Given that inflammation is a key player in tumor progression, including breast cancer, therapies targeting specific inflammatory mediators with adverse effects is of vital importance for the future development of cancer treatments. The increased levels of IL-17A and IL-17A-producing cells within the tumor microenvironment of different types of cancer suggest an important role for this cytokine in tumor progression. Since both pro-tumor and anti-tumor effects of IL-17A are reported, the primary objective of this study was to define the role of IL17A production within the tumor microenvironment of a 4T1 tumor, which mimics human breast cancer. The secondary objective of this study was to

define the role of IL-17A or a decoy receptor antagonist as a potential biotherapy preceding surgery. The overall role of IL-17A as a pro- or anti-tumor mediator was assessed by examining the host immunological response, as well as tumor growth and lung metastasis. We hypothesized that IL-17A plays a significant role in promoting tumor growth and lung metastasis through the induction of chronic inflammation at the tumor site.

Chapter 2. Materials and Methods

2.1. Mice

Mice were housed under pathogen-free conditions in a ventilated barrier rack within the IWK *in vivo* facility, and experiments were undertaken according to guidelines of the Canadian Council for Animal Care guidelines. BALB/c female mice, aged 6-8 weeks, were purchased from Charles River Laboratories (Senneville, QC) and allowed to acclimate for 48 hrs. BALB/c DO11.10 transgenic mice, aged 6-8 weeks, were bred at the IWK *in vivo* facility. Food and water were supplied *ad libitum*.

2.2. Cell Lines

2.2.1. HEK293 cells

Human embryonic kidney HEK293 cells expressing the adenovirus E1 and E3 genes were grown and maintained in complete F11 medium (**Table 11**). To start a cell line, a frozen aliquot was thawed in a 37°C water bath, mixed immediately in 10 mL of complete F11, and centrifuged (300xg, for 10 min, at 4°C). After the supernatant was discarded, the cells were resuspended in T75 flask containing 15 mL of fresh medium and maintained in an atmosphere of 5% CO_2 at 37°C. HEK293 cells were passaged every 4-5 days once they became 80% confluent. To passage HEK293 cells, the culture medium was discarded and the cells were washed with 10 mL of PBS. To detach the cells, 2 mL of citrate saline solution was added to the flask for 5 min, and then 10 mL of complete F11 was added to the suspension and mixed very well. Two mL of the suspension mix was kept in the flask (or transfer to another) and supplemented with extra fresh medium (final volume is 15 mL/flask). HEK293 cells were discarded prior to passage 40.

2.2.2. 4T1 mammary carcinoma

The 4T1 mouse mammary carcinoma was obtained from Dr. Tim Lee (Dalhousie University). 4T1 cells were grown in complete DMEM (**Table 11**). The cell line was initiated, passaged and maintained in the same way as the HEK293 cells, with the exception that 2 mL of TrypLE^{TM} solution was used to detach 4T1 cells instead of citrate saline (see section 2.2.1). The 4T1 cells were passaged every 1-2 days once they became 80% confluent, and were discarded prior to passage 40.

2.2.3. Endothelial bEnd.3 cells

The mouse brain endothelial cell line bEnd.3 was maintained in the same way as the 4T1 cells by using complete DMEM as culture medium and TrypLE^{TM} solution to detach cells between passages (see section 2.2.2). The bEnd.3 cells were passaged once they became 80% confluent, and were discarded prior to passage 30.

2.3. Adenovirus Vectors

2.3.1. Vectors propagation, storage and purification

HEK293 cells were grown in 150 mm cell culture dishes until they became confluent. When confluence was reached, the culture medium was discarded and the monolayer was overlaid with 1-2 mL of adenovirus-containing supernatant in order to transduce cells. Following incubation for 20 min at RT, 20 mL of fresh medium was added to each dish. Transduced HEK293 cells were incubated at 37°C for 2-4 days until 80-90% of the HEK293 cells detached from the dish indicating the cytopathic effect of viral transduction. The cells were completely detached from the plate using a cell scraper, transferred into 50 mL tube, and centrifuged (850xg, for 10 min, at 4°C). The supernatant was stored at -80°C for further viral amplification; the cell pellets were resuspended in 10

mL of 0.1 M Tris-HCl (pH 8.0) and frozen at -80°C until purification. To purify the adenovirus from the infected HEK293 cells, these cells were subjected to three freeze-thaw cycles (freeze at -80°C, thaw at 37°C) and then centrifuged (850xg, for 10 min, at 4°C). The supernatant was discarded, while the pellet was resuspended in 10 mL of 0.1 M Tris-HCl (pH 8.0) containing 0.5% Sodium Deoxycholate and mixed slowly for 30 min at RT over a moving rocket platform. The pellet was digested with DNase I (final concentration 50 µg/mL) in $MgCl_2$ (final concentration 0.02 M) at 37°C for 45 min. The tube was inverted once every 10 min, and then centrifuged (2095xg, for 20 min, at 4°C). The supernatant was collected and added on top of a gradient composed of three densities of CsCl (1.25 top, 1.35 middle, and 1.5 bottom) (**Table 11**); which was then centrifuged for 1 hr, 35000 rpm, at 10°C (using 14x89 mm centrifuge tubes and SW41 rotor in a Beckman Coulter Optima L-100XP ultracentrifuge). From the bands that formed in the CsCl gradient following centrifugation, the lowest band, where the adenovirus was situated, was collected in the smallest volume possible and transferred into a 13x51 mm centrifuge tube; CsCl (d=1.35) was added on top to bring the final volume to 4.8 mL. Tubes were then centrifuged 35000 rpm for 16 hrs, at 4°C (using a SW55 rotor). The resulting adenovirus band was collected in the smallest volume possible and underwent 2-4 cycles of dialysis using 10 mM Tris-HCl pH 8 over two days. The dialysed virus was collected and diluted in 10% glycerol solution, then aliquoted and stored at -80°C.

2.3.2. Adenovirus vector titration

HEK293 cells were cultured in 60mm culture dishes until they became confluent. Serial dilutions of the purified adenovirus were prepared in PBS^{++} (the virus binds and infects cells more efficiently in the presence of Ca^{2+} and Mg^{2+}) ranging from 1:10 to $1:10^{11}$.

The media were removed from 293 cultures, and the cells were infected drop by drop with 200 μL of specific viral dilution and then incubated at 37°C for 20 min. A mixture of 5 mL of 2x complete F11 medium and 5 mL of 10g/mL agarose solution (maintained in liquid status by incubating it at 44°C) was added to the HEK293 cells until the agarose-containing medium solidified at RT. The plates were incubated for 8-12 days at 37°C, until viral plaques became visible. The viral plaques were counted and the titer of each viral preparation was calculated using the following formula:

Titer **(**pfu/mL) = number of plaques x 1/0.2 x dilution factor.

2.3.3. Transduction of 4T1 cells with adenovirus vectors

4T1 cells were cultured in 60 mm tissue culture dishes in a density of $1x10^6$ cells per dish and incubated at 37°C until they became adherent. The supernatant was carefully removed and the cells were overlaid with 400 μL of PBS^{++} containing the desired adenovirus vector at a multiplicity of infection (MOI) of 200 (i.e., each cell should get 200 pfu of the adenovirus). After 20 min at RT, 10 mL of complete RPMI was added to each plate, and plates were incubated for 12-24 hrs at 37°C, for use in *in vivo* or *in vitro* experiments.

Plates were divided into four groups depending on the type of adenovirus vector used. In the first group (IL-17A), the 4T1 cells were transduced with AdIL-17A in order to make cells capable of overproducing mouse recombinant IL-17A. In the second group (IL-17RA:Fc), the 4T1 cells were transduced with AdIL-17RA:Fc in order to enable cells to produce soluble IL-17RA receptor subunit; in mice, the use of IL-17RA:Fc can block IL-17A, IL-17F, and IL17A/F (242). In the third group (Addl), the 4T1 cells were transduced with empty adenovirus vector Ad170-3 for use as a negative control. In the fourth group

(PBS), cells were overlaid with PBS^{++} alone, without the addition of any virus. The four groups were investigated *in vitro* for efficiency of transduction after 24 hrs by PCR (see section 2.12.1), the expression of chemokine receptor CCR4 on 4T1 cells surface after 48 hrs by flow cytometry (see section 2.10.3), and the production of cytokines after 48 hrs by Luminex® (see section 2.11).

2.4. Experimental Models

2.4.1. Primary tumor with adenovirus-transduction model

This transduction model is outlined in **Figure 1**. BALB/c mice were divided into four groups (IL-17A, IL-17RA:Fc, Addl, and PBS) based on the type of 4T1 cells injected (see section 2.3.3). At day 0 of the experiment, the culture medium of 4T1 cells was discarded. The cells were washed with 2 mL of PBS then incubated for 5 min with 500 µL of TrypLE to detach the cells. The reaction was stopped using 1 mL of complete DMEM; the cells were washed twice using basic DMEM without FBS and the cell number was determined. The cells were splitted into 1.5 mL tubes; each tube contains $1x10^6$ cells in a volume of 50 µL of basic DMEM without FBS and preserved on ice until injection. Prior to injection, the tube contents were mixed gently. Using a 1 mL BD™ syringe with a 27 G x 1/2" needle the cells were aspirated and injected subcutaneously in the fourth mammary fat pad of each mouse. Peripheral blood samples were collected at different time points (days 0, 4, 7, and 14) for the assessment of immune profiles and serum cytokines via flow cytometry and Luminex®, respectively (see sections 2.6.1 and 2.11). Seventeen days after tumor inoculation, mice were sacrificed using an overdose of CO_2. Tumor, lung, TDLN, and spleen were collected in 5% Bovine Serum (BS) RPMI, preserved on ice, and then processed accordingly (see section 2.6).

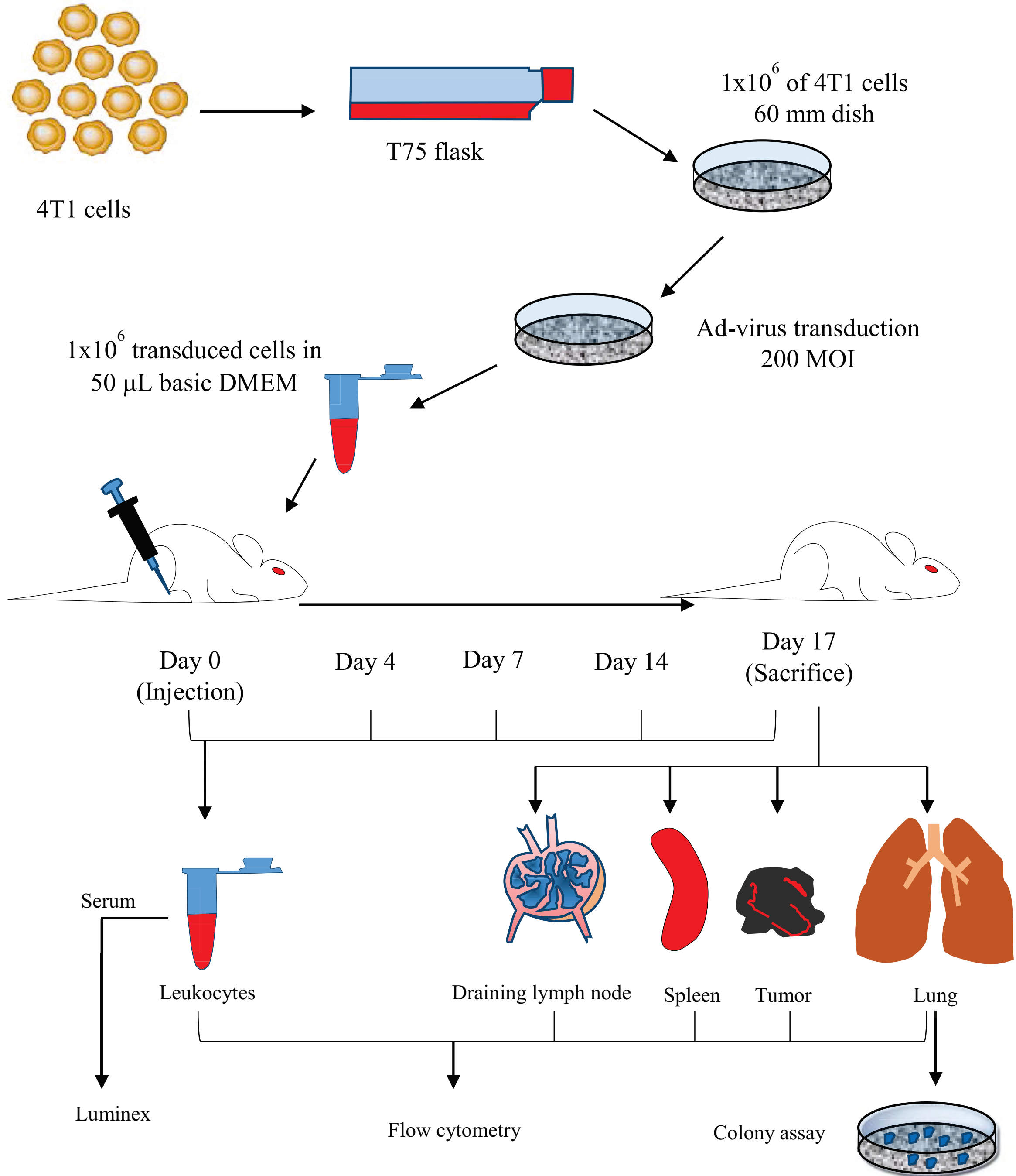


Figure 1: Schematic diagram of the model of primary tumor with adenovirus-transduction.

Schematic diagram shows the *in vivo* model using adenovirus-transduced 4T1 cells. Mice are injected with $1x10^6$ of different adenovirus-transduced 4T1 cells. The immune profile is monitored at several time points (D0-D4-D7-D14). At day 17, mice are sacrificed and spleen, tumor, lung, and TDLN are collected and studied.

2.4.2. Primary tumor with adenovirus-injection model

This model is outlined in **Figure 2**. At day 0 of the experiment, BALB/c mice were injected subcutaneously in the fourth mammary fat pad with $2x10^5$ of 4T1 cells, suspended in 50 μL of basic DMEM. Seven days after inoculation, 50 μL of PBS^{++} containing $5x10^8$ pfu of Ad-IL-17A, Ad-IL-17RA:Fc, Addl, or 50 μL of PBS^{++} alone were prepared and preserved on ice. Mice were divided into four groups and injected (intra-tumor) with one of the treatments (using a 1 mL BD™ syringe with 23 G x 1" needle). Twelve days after tumor injection, the primary tumor and the TDLN were surgically removed (see section 2.5). Mice were sacrificed at day 28 post tumor injection (or 16 days post tumor resection) using an overdose of CO_2. PB samples were collected at different time points (days 0, 6, 11, 18 and 24) for assessment of immune profiles (see sections 2.6.1).

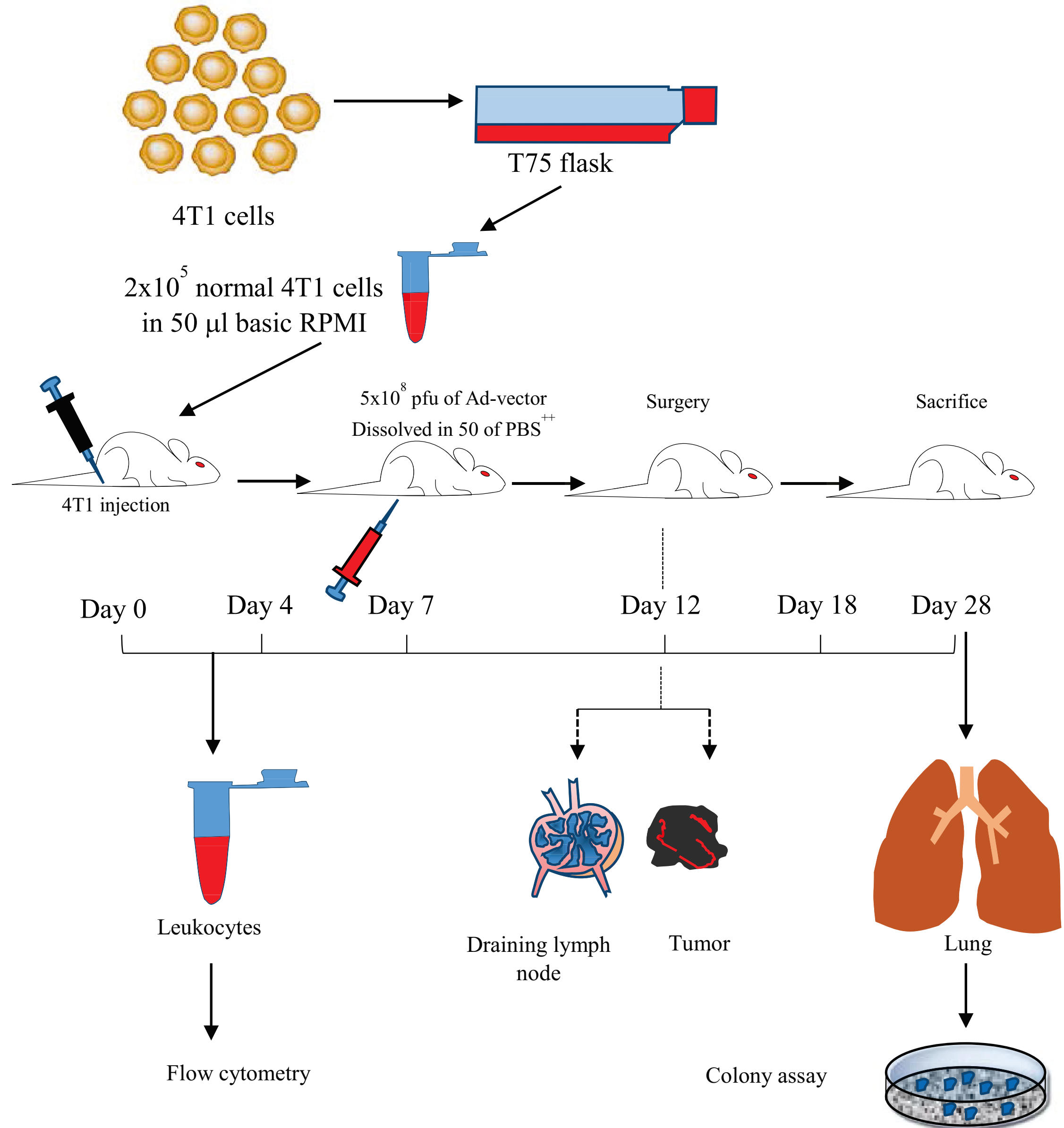


Figure 2: Schematic diagram of the model of primary tumor with adenovirus-injection

Schematic diagram shows the *in vivo* model of intratumoral injection of different Ad vectors prior to the surgery. Mice are injected with $2x10^5$ cells of 4T1 cells. After 1 week, mice are injected intratumoral with $5x10^8$ pfu of Ad-vectors or just PBS. Five days later, mice undergo surgery where the tumor and TDLN are removed. The immune profile is monitored at several time points (D0-D4-D7-D12-D18-D24). At day 28, mice are sacrificed, and the lungs are collected and analyzed for metastasis.

2.5. Surgical Removal of Primary Tumor and Draining Lymph Node

To remove the primary tumor, mice were anesthetized and maintained with vaporized isoflurane. The surgical site was disinfected using 70% alcohol and then followed with iodine tincture (2%) using a cotton tip applicator. Following a small incision around the tumor, complete resection of the tumor was performed using a curved scissor, with care not to damage the peritoneum and the abdominal organs. The draining lymph node was identified and carefully removed without damaging the surrounding tissues. Care was taken to minimize blood loss using sterile gauze before closing the wound. The skin and the subcutaneous tissues were closed as a single layer using monofilament 18" blue nylon sterile sutures (Davis + Geck Montreal, QC, CA). The mice were injected with 50 μL of Ketorolac Tromethamine (30 mg/mL) (an NSAID pain killer) and monitored until they woke up. Their water source was supplemented with 2 mg/mL of neomycin sulfate as an antibiotic for one week after surgery. Sutures were removed 5-7 days after surgery, when the wound was healed completely. Recurrence of the primary tumor at site of surgery was observed in 9 mice out of 56 (16%) from three experiments and was independent of the treatment group (2 in the IL-17A group, 2 in the IL-17RA:Fc group, 1 in the Addl group, and three in the PBS group). These mice were excluded from our results, as they exhibited higher rate of metastasis, likely due to imperfectly removal of primary tumor.

2.6. Isolation of Immune Cells from Organs of Tumor-Bearing Mice

2.6.1. White blood cells (WBCs)

Blood was collected from the tail (50 μL per mouse) into a 75 μL micro-hematocrit capillary tube containing 25 μL of 0.1 M EDTA. After collection, samples were diluted in a 1:3 ratio with PBS and centrifuged (300xg, for 10 min, at 4°C). The PBS-diluted plasma

samples (about 70-80 µL) were extracted and stored at -80°C. The red blood cells (RBCs) within the pellet were lysed by mixing samples with 2 mL of Ammonium-Chloride-Potassium buffer. Following 5-7 min of incubation, the reaction was stopped using 6 mL of 5%BS RPMI. The cells were then washed with 5%BS RPMI, centrifuged (300xg, for 10 min, at 4°C), and then resuspended in 1 mL of complete RPMI. Total cell number was determined, and leukocytes were suspended at a predetermined cellularity for use in either suppression assays or immune profiling by flow cytometry analysis.

2.6.2. Spleen

The spleen was excised aseptically, collected on ice in 5% BS RPMI, and then minced into fine pieces into a petri dish containing 4 mL of 5% BS RPMI using frosted slides. The minced organ was collected into a 15 mL tube and centrifuged (300xg, for 10 min, at 4°C). The supernatant was discarded and RBCs were lysed using 4 mL of ACK buffer per spleen for 5-7 min, stopped with 20 mL of 5%BS RPMI. The cells were filtered through a 70-µm cell strainer to remove any cellular debris, washed twice using 5%BS RPMI and centrifuged (300xg, for 10 min, at 4°C). The cells were counted and resuspended in complete RPMI at a predetermined cellularity, to be used for either suppression assays or immune profiling by flow cytometry analysis.

2.6.3. Tumor

At the time points indicated for each experimental model (see section 2.4), tumors were resected from mice and weighed. Each tumor was minced in 2.5 mL of HBSS buffer using sharp scissors then supplemented with an additional 2.5 mL of HBSS containing collagenase II enzyme (Bioshop, Burlington, ON) (final concentration 150 µg/mL) to digest connective tissues by incubating the sample for 20 min at 37°C. The mixture was

filtered through a 70-µm cell strainer and the cell pellets were washed twice using 5%BS RPMI. Cells were counted and resuspended at a predetermined cellularity to be used for immune profiling by flow cytometry analysis.

2.6.4. Lung

Lungs were removed after sacrifice, swirled in HBSS to remove remaining blood. Each lung was minced in 2.5 mL of HBSS using sharp scissors, and then supplemented with additional 2.5 mL of HBSS containing 1 mg/mL collagenase IV and 10 units of elastase. After the cocktail was mixed for 75 min at 4°C on a rotating wheel, the digested lungs were filtered through 70-µm cell strainers and washed twice with complete RPMI. Cells were counted and re-established at a predetermined cellularity for use in immune profiling by flow cytometry analysis and metastasis colony assay (see section 2.9).

2.6.5. Lymph node

The inguinal lymph node, which drains the mammary gland, was removed aseptically into 1mL of HBSS. Lymphocytes were isolated by mashing the lymph node tissue with frosted slides, and resuspended in complete RPMI. Cells were filtered through a 70-µm cell strainer, counted, and resuspended in a predetermined cellularity to be used for immune profiling by flow cytometry analysis.

2.7. Isolation of Serum Samples

Blood was collected from the tail (50 µL of whole blood per mouse) using a 75 µL micro-hematocrit capillary tube containing 25 µL of HBSS. After collection, samples were supplemented with another 25 µL of HBSS (total volume 50 µL of blood and 50 µL of HBSS). The samples were centrifuged (21,000xg, for 20 min, at 4°C). The PBS-diluted

serum samples (about 70-80 µL) were extracted and stored at -80°C for cytokine measurements.

2.8. Cell Counting

An aliquot of cells was diluted (1:1 – 1:10) with 0.1% trypan blue stain and 10 µL of the resulting cell-stain mixture was loaded onto a hemacytometer. Viable and dead cells (stained blue) were counted, and the total cell number was determined from the equation below. The concentration of cells was adjusted depending on the requirement of each experiment.

$$Total\ cells\ (Cell/ml) = \frac{\text{Total cells counted} \times \text{dilution factor} \times 10^4}{number\ of\ squares}$$

2.9. Colony Assay to Measure Lung Metastases

To recover 4T1 cells that metastasized to the lungs, a combination of mechanical and enzymatic digestion was performed in order to release cells from lung connective tissues (see section 2.6.4). The single-cell suspension was resuspended in complete DMEM supplemented with 60 µM of 6-TG and seeded into 100 mm tissue culture dishes. Plates were incubated at 37°C for 10-14 days, until colonies were visible. After the medium was discarded from the dishes, the colonies were fixed with 5 mL of methanol for five min and then washed with distilled water. After that, the fixed colonies were stained by adding 5 mL of 0.03% methylene blue stain, and blue colonies were counted using ImageJ software. Data were expressed as the total number of metastatic colonies per lung.

In some experiments, we investigated levels of CCR4 on 4T1 lung metastasis. Similar to the previous steps, the lungs were cultured for 10-14 days in complete DMEM supplemented with 60 µM of 6-TG. A pen was used to mark the location of colonies on the plate. ImageJ software was used to enumerate colonies after scanning the plates at a

high resolution using EPSON Expression 1680 scanner. The metastatic cells were detached following the counting, and used for CCR4 staining (as described in section 2.10.3).

2.10. Flow Cytometry

Flow cytometry was used to analyze various types of cells, including tumor cells and immune cells in tumors, blood, lung, spleen and lymph nodes. Cells were counted and aliquoted (0.5-1x10^6 cells into each assay tube), and then washed using flow cytometry wash buffer (PBS supplemented with 1% BS). To define cellular phenotypes or study the expression of chemokine receptors, cells were stained with specific extracellular fluorescence-conjugated mAbs and isotype controls (see section 2.10.1). To detect cytokine production, lymphocytes were stained with intracellular fluorescence-conjugated mAbs (see section 2.10.2). Multicolor assays were controlled and compensated using single-stained cells as positive controls and unstained cells as negative controls.

2.10.1. Extracellular staining

Extracellular staining identifies different cell types within a heterogeneous population, based on surface antigens. Cells were washed with flow cytometry wash buffer; to avoid non-specific Fc-mediated binding, each pellet was blocked using 20 µL of wash buffer containing 10% heat-inactivated normal rat serum, incubated for 20 min at 4°C, and then washed with wash buffer. Pellets were resuspended in 50 µL of wash buffer containing a cocktail of fluorescent conjugated antibodies that bind specific surface markers at appropriate dilutions and colors (**Table 7**). Cells were incubated with antibodies for 20 min at 4°C, and washed three times with the wash buffer. Cells were fixed with 200 µL/tube of 1% formalin fixation buffer and transferred into flow cytometry mini-tubes (1 mL). Data

were acquired on a Becton Dickinson FACSCalibur and analyzed using FCS Express 4 Flow research edition (De Novo, Los Angeles, CA).

2.10.2. Intracellular staining

Intracellular staining identifies cells based on markers inside the cells. Cells were washed, resuspended in wash buffer at a concentration of $2x10^6$ cells/mL, and triplicate 100 μL samples were seeded in separate wells on a 96-wells tissue culture plate. 100 μL of complete RPMI supplemented with 1 ng/mL of PMA, 1x Brefeldin A, and 1 μg/mL of Ionomycin was added to each well (final volume/well is 200 μL) and incubated for 4-5 hrs at 37°C. After that, 2 μL of 1 mM EDTA was added to each well and incubated for 5-10 min at RT. Cells were then transferred to a V-bottom 96 well plate and washed with wash buffer. For blocking, each well was supplemented with 20 μL of wash buffer containing 10% rat serum, incubated for 20 min at 4°C, and washed. Extracellular staining was performed (see section 2.10.1) without the fixation step. After extracellular staining and wash, 100 μL of intracellular fixation buffer was added to each well, followed by incubation in the dark for 20 min at RT. Without washing, 100 μL of 1x permeabilization buffer was added to the mixture, which was centrifuged for 10 min (750xg, 4°C). Washing was repeated again using 200 μL of permeabilization buffer. Pellets were then resuspended in 50 μL of permeabilization buffer containing a cocktail of fluorescent conjugated antibodies against specific intracellular cytokines (IFN-γ, IL-17A, IL-4) at appropriate dilutions and colors. Following incubation for 20 min at 4°C, cells were washed using 100 μL of 1x permeabilization buffer, washed three times with wash buffer, fixed with 200 μL/tube of fixation buffer, and transferred into flow cytometry mini-tubes. Data were

acquired on a Becton Dickinson FACSAria and analyzed using FCS Express 4 Flow research edition (De Novo, Los Angeles, CA).

2.10.3. CCR4 staining

Chemokine receptor CCR4 levels were tested in 4T1 cells cultured *in vitro* for 48 hrs after adenovirus-transduction (see section 2.3.3) and in 4T1 lung metastasis cells *ex vivo* (see section 2.9) by flow cytometry using a fluorescent anti-mouse CCR4 mAb. 4T1 cells from both *in vitro* and *ex vivo* experiments were processed using the same procedure, as follows. The culture medium in each plate was discarded and the cells were washed with 5 mL of PBS to remove any trace of the medium. The cells were incubated for 5 min with 500 μL of TrypLE to detach the cells. The reaction was stopped using 1 mL of flow cytometry wash buffer. Each sample was divided into two assay tubes containing 0.5-1x10^6 cells each, and then washed twice using 200 μL/tube of flow cytometry wash buffer. One tube of each pair was resuspended in 50 μL of wash buffer containing a PE-conjugated CCR4 antibody at appropriate dilutions; the second tube was resuspended in 50 μL of wash buffer containing a PE-conjugated isotype control antibody (**Table 7**). The tubes were incubated for 20 min at 4°C and then washed three times with the wash buffer. Stained cells were fixed with 200 μL/tube of 1% formalin fixation buffer and transferred into flow cytometry mini-tubes. Data were acquired on a Becton Dickinson FACSCalibur and analyzed using FCS Express 4 Flow research edition (De Novo).

2.10.4. Cell sorting

Cell sorting by flow cytometry uses cell surface markers to retrieve populations of interest from heterogeneous mixtures of cells. In our research, cell sorting was used to isolate MDSC subpopulations from spleen tissue. Splenocytes were isolated (see

section 2.6.2) and stained with anti-Mouse CD11b-FITC, anti-Mouse Ly6G-PE, anti-Mouse Gr1-APC, and anti-Mouse Ly6C-PerCPCy5.5 (see section 2.10.1). Splenocytes were suspended in flow cytometry sort buffer (40-60x10^6 cells/mL). After gating on $Gr1^+/CD11b^+$ cells, splenocytes were sorted into two subpopulations ($Ly6C^+/Ly6G^-$, $Ly6C^{low}/Ly6G^+$) using a Becton Dickinson FACSAria machine and collected into cold sort buffer to be used as effector cells in the suppression assay (see section 2.13)

2.11. Cytokine analysis

2.11.1. ELISA

IL-17A production from adenovirus-transduced 4T1 cells was analyzed in 4T1 supernatant at 24 hrs intervals for three days using the Ready-Set-Go! IL-17A ELISA kit (eBioscience). The 96-well ELISA plates (Greiner bio one) were coated with 50 µL of 0.05 M $NaHCO_3$ (pH 9.6) containing IL-17A capture antibodies. After incubation overnight at 4°C, the plates were washed 5 times with 200 µL/well of PBST (1xPBS + 0.05% Tween), blocked with 1x Assay Diluent (eBioscience) for 2 hrs at RT, and washed five times with 200 µL of PBST. The cytokine standards (eBioscience) and samples were serially diluted into to the wells (50 µL/well) using 1x Assay Diluent and incubated overnight at 4°C. Following incubation, the plates were washed five times with 200 µL/well of PBST. The biotin-conjugated detecting antibody (eBioscience) was diluted in 1x Assay Diluent, and added at 50 µL/well for 2 hr incubation at RT. The plates were washed five times with 200 µL/well of PBST. Streptavidin-conjugated horseradish peroxide SAV-HRP (eBioscience) was diluted in 1x Assay Diluent, added at 50 µL/well, and incubated for 30 min at RT. Following seven washes with PBST (200 µL/well of PBST), 50 µL of 3,3',5,5'-Tetramethylbenzidine TMB (eBioscience) was added to each well and the plates were

stored at RT in the dark for 10-20 min. The reaction was quenched with 2 N H_2SO_4, and the optical density (OD) of the wells was read at 450 nm using a BioTek synergy reader (BioTek, Winooski, VT)

2.11.2. Luminex Multiplex Assay

Multiple cytokines were analyzed in serum and 4T1 cell culture supernatants. Serum samples were collected at three time points (D0, D7, and D12; see section 2.7), and supernatant samples were collected after 48 hrs post adenovirus-transduction (see section 2.3.3). The cytokine concentrations were determined using a ProcartaPlex® Multiplex Immunoassay (eBioscience) and analyzed using Bioplex® 200™ with BioPlex Manager Software (BIO-RAD; Mississauga, ON). The Luminex analyzer was calibrated before each experiment and validated once every month using a calibration and validation kit (BIO-RAD) in order to insure optimal performance. Experiments were performed only when analyzer calibration and validation were successful. The capture antibody bead mix (containing beads for 19 selected cytokines, see **Table 8**) was loaded into 96-well plate (180 µL/well) and washed three times with wash buffer (eBioscience) using magnetic plate washer. The standards used to calculate cytokine concentrations in the samples were prepared by reconstituting the lyophilized standard in 250 µL of DMEM or Universal Assay Buffer (eBioscience). A total of seven standards were prepared in a 4-fold dilution series. All standards and samples were loaded into a 96-well plate (50 µL/well for standards and culture supernatant; 25 µL/well for serum samples diluted with 25 µL/well of Universal Assay Buffer). Two wells were left blank and loaded with 50 µL of DMEM. The plate was sealed, covered, and incubated in the dark at RT on a shaker at 500 rpm for 160 min. Following three washes, the biotinylated detection antibody mix was added to each

well (25 µL/well) and incubated on a shaker at 500 rpm for 30 min. Following three additional washes, Streptavidin-PE was added to each well (50 µL/well) and incubated on a shaker at 500 rpm for 30 min. Finally, the plate was washed three times. Reading buffer (120 µL/well) was added to all wells and incubated on a shaker at 500 rpm for 5 min and then inserted into the Luminex instrument for reading. A separate standard curve was produced for each cytokine measured, with a lower limit of quantification for each cytokine (see **Table 8**). In each case, concentration was calculated based on the dilution factor of the sample.

2.12. Polymerase Chain Reaction (PCR)

2.12.1. PCR for adenovirus hexon DNA

2.12.1.1. Sample preparation

4T1 cells were cultured in 60 mm tissue culture dishes in complete DMEM and transduced for 24 hrs with three types of adenovirus vectors or PBS^{++} to serve as negative control (see section 2.3.3). The culture medium was removed completely and 750 µL of DNAzol (Life Technologies) was added to each plate and swirled well to lyse 4T1 cells. The lysate was processed for genomic DNA isolation.

2.12.1.2. DNA isolation

For each sample, the lysate was transferred into a 1.5 mL tube, 375 µL of 100% ethanol was added, and the tube was gently inverted 3-6 times in order to mix the contents without shearing the genomic DNA, which became visible as a cloudy precipitate. The precipitated genomic DNA was spooled onto a pipette tip and transferred to another tube containing 1 mL of 75% ethanol in order to be washed. The tube was gently inverted 3-6 times and stored vertically for 30-60 sec until the genomic DNA settled to the bottom of

the tube, after which the ethanol was removed by pipetting; this washing step was repeated three times. Following ethanol removal, the precipitated genomic DNA was left to dry in the open tube for up to 15 seconds prior to resuspension in in 8mM NAOH (0.1 mL/ sample). The genomic DNA was dissolved in NAOH by slowly passing the genomic DNA through a pipette tip and then pH was adjusted to 7.2 by adding 2.3 μL of 1M HEPES. The purity and concentration of DNA samples were assessed by measuring the absorbance at 260 nm and 280 nm using a Take3 plate (BioTek, VT) in a BioTek Synergy HT reader and analyzing the results using Gen5 software (BioTek, VT). A typical ratio of A260/A280 for purified genomic DNA showed that all samples was in the range of (1.8-2) indicating a high purified sample preparation (276). All templates were then diluted to a concentration of 10 ng/mL with 8mM NAOH. A reaction mixture of 20 μL total volume was prepared by mixing 2 μL of genomic DNA template (20 ng of DNA), 10 μL PCR Master Mix (Promega), 1 μL of 1μM forward primer (see **Table 5**), 1 μL of 1μM reverse primer (see **Table 5**), and 6 μL of nuclease-free H2O.

2.12.1.3. DNA amplification, gel electrophoresis, and UV visualization

PCR amplification was performed in 20 μL reaction volumes (see section 2.12.1.2). Reactions were carried out using an Eppendorf Mastercycler PCR machine under the following cycling conditions: reaction volume was initially heated for 3 min at 95°C, processed through 40 cycles of sequential temperatures of 95°C (30 sec), 68°C (40 sec), 72°C (40 sec) and finally incubated for 10 min at 72°C. Samples were stored at 4°C prior to electrophoresis at 100 V on 0.8% agarose gels containing ethidium bromide for 40 min. Following electrophoresis, the bands of amplified DNA were visualized using UV Transilluminator (BioDoc-It, CA, USA).

(Note: The work described in sections 2.12.1.1 to 2.12.1.3 was conducted by Mr. Chi Yan)

2.12.2. Quantification of CCL17/CCL22 mRNA using quantitative reverse transcription PCR (qRT-PCR)

The effect of IL-17A on the expression of CCL17 and CCL22 was assessed using *in vitro* and *in vivo* samples of bEnd.3 cells following stimulation with rIL-17A, and lungs of adenovirus-transduced 4T1 tumor-bearing mice, respectively. The expression of these two ligands was evaluated by quantifying the mRNA levels encoded by CCL17 and CCL22 genes using qRT-PCR.

2.12.2.1. Sample preparation

For *in vitro* samples, the endothelial cell line bEnd.3 cells were cultured in 60 mm tissue culture dishes containing complete DMEM and treated with 100 ng/mL recombinant mouse IL-17A (eBIoscience) for 24 hrs. The IL-17A-treated bEnd.3 cells were completely detached from the plate using a cell scraper, transferred into 15 mL tube, and centrifuged (850xg, for 10 min, at 4°C). The supernatant was discarded and the pellet was resuspended in Trizol (Life Technologies) (800 µL of Trizol/1x10^7 cells).

For *in vivo* samples, the lungs were excised from mice that had been injected with 1x10^6 of adenovirus-transduced 4T1 cells (see section 2.4.1) and sacrificed after 12 days of tumor inoculation. The lungs were mechanically dissociated over a 70 µm sterile metal mesh using a plunger from a 1 mL syringe to obtain a single cell suspension. The cells were washed then centrifuged (850xg, for 10 min, at 4°C). The supernatant was discarded and the pellet was resuspended in Trizol (Life Technologies) (800 µL of Trizol/1x10^7 cells).

2.12.2.2. RNA isolation

Following resuspension in Trizol, samples were processed for RNA isolation. Using a 1 mL syringe and 26G needles, each Trizol-suspended sample was aspirated up and down. After that, chloroform was added to each sample (0.2 mL chloroform/mL of Trizol), mixed vigorously by hand, allowed to stand for 3 min, and centrifuged (16,000xg, for 10 min, at 4°C). The aqueous phase (the top clear layer) was collected, with care taken not to collect the phenol and waste layers (the pink layers). The collected layer was added to a tube containing 350 μL of RLT buffer (Qiagen RNeasy Mini kit) then mixed with 700 μL of 100% ethanol. The mixture was loaded onto an RNeasy mini column (700 μL each time with spinning down at 16,000xg, for 25 sec) (Qiagen RNeasy Mini kit). The flow through was discarded and 500 μL of RW1 buffer (Qiagen RNeasy Mini kit) was added; after 1 min, elute was centrifuged (16,000xg, rpm for 25 sec). Next, 70 μL of RDD Buffer was added to 10 μL of DNase, loaded into the column and left to stand for 15 min. After that, 350 μL of RW1 buffer was added and let to stand for 1 min, followed by centrifugation (16,000xg, for 25 sec). The column was transferred to a new collection tube, followed by the addition of 500 μL of buffer RPE, mixing, and centrifugation (16,000xg, for 25 sec). Following that, 500 μL of RPE buffer was added, followed by mixing and centrifugation (16,000xg, for 2 min). The flow through was removed by centrifugation (16,000xg, for 25 sec) in order to fully remove the RPE buffer. The column was put in a clean collection tube for RNA collection, and 50 μL of RNase free dH_2O was added; after several min, the mixture was centrifuged (16,000xg, for 1 min). The elusion contains the RNA that was used to quantify CCL17/CCL22 expression using qRT-PCR.

2.12.2.3. CCL17/CCL22 quantification using qRT-PCR

Total RNA isolated from samples was used to generate cDNA using Superscript III Reverse Transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (qPCR) was performed in triplicate with 1 μL of cDNA using Quantifast SYBR Green (Qiagen, Toronto, ON, Canada). Data were collected on RG-6000 Rotor-Gene (Corbett Research, Sydney, Australia), and analysis was conducted using the 2−ΔΔCt relative quantification technique and expressed relative to the internal control mRNA level (277). High-stringency primer pairs were used for mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) as an internal control, mCCL17, and mCCL22 (see Table **6**).

2.13. Suppression Assays

Two types of suppression assays were done: one to evaluate the effect of increased number of MDSCs on T cell proliferation, and another one to evaluate the quality of different MDSCs subpopulations on T cell proliferation (See **Figure 3** and **Figure 4**).

Responder cells were splenocytes isolated from DO11.10 TCR transgenic mouse (see section 2.6.2). T cells of DO11.10 mice were stimulated with ovalbumin (OVA) peptide $_{323\text{-}339}$. Splenocytes were washed with pre-warmed PBS, resuspended at a concentration of $10x10^6$ cells/mL in warm PBS containing 2.5 μM of cell proliferation dye eFluor 670 (eBioscience), and incubated for 10 min in the dark at 37°C. Labelling of cells was terminated by adding 4-5 volumes of cold complete RPMI (containing ≥10% FBS), and the cells were incubated on ice for 5 min and then washed three times using complete RPMI. Labeled cells were resuspended in complete RPMI ($2x10^6$ cells/mL) and 100 μL of the suspension ($2x10^5$ cells) was co-cultured with 100 μL of the effector cell suspensions

(see sections 2.13.1 and 2.13.2) in a U-bottom 96 well plate (BD Labware, Franklin Lakes, NJ). Each well was supplemented with 50 µL of complete RPMI containing OVA II peptide (final concentration 10 µg/mL) in order to stimulate the responder cells. Positive and negative controls (responder cells alone with and without OVA stimulation, respectively) were added to each plate. After being cultured for 72 hrs at 37°C, the cells were harvested and stained with anti-mouse CD4-FITC. CD4 T cells that proliferate in response to OVA II peptide exhibit a reduction in eFlor 670 fluorescence intensity, which can be detected using flow cytometry. This method accurately determines the fraction of $CD4^+$ cells in each round of division cells. The suppression rate was measured with reference to the proliferation of T cells in the positive and negative controls.

2.13.1. Suppression assay of WBCs by blood volume

The procedure for this assay is outlined in **Figure 3**. The effector cells were the leukocytes isolated from the PB of a tumor-bearing mouse. Blood was obtained from the tail (50 µL) of each mouse and leukocytes were isolated following RBC lysis (see section 2.6.1). Effector leukocytes were resuspended at three dilutions containing 50%, 25%, or 12.5% of the total leukocytes derived from the 50 µL of blood. Each dilution was co-cultured with $2x10^5$ of eFluor-labeled responder cells and OVA II peptide in a final volume of 250 µL per well. The plates were cultured for 72 hrs at 37°C (see section 2.13) and T cell proliferation was evaluated by flow cytometry.

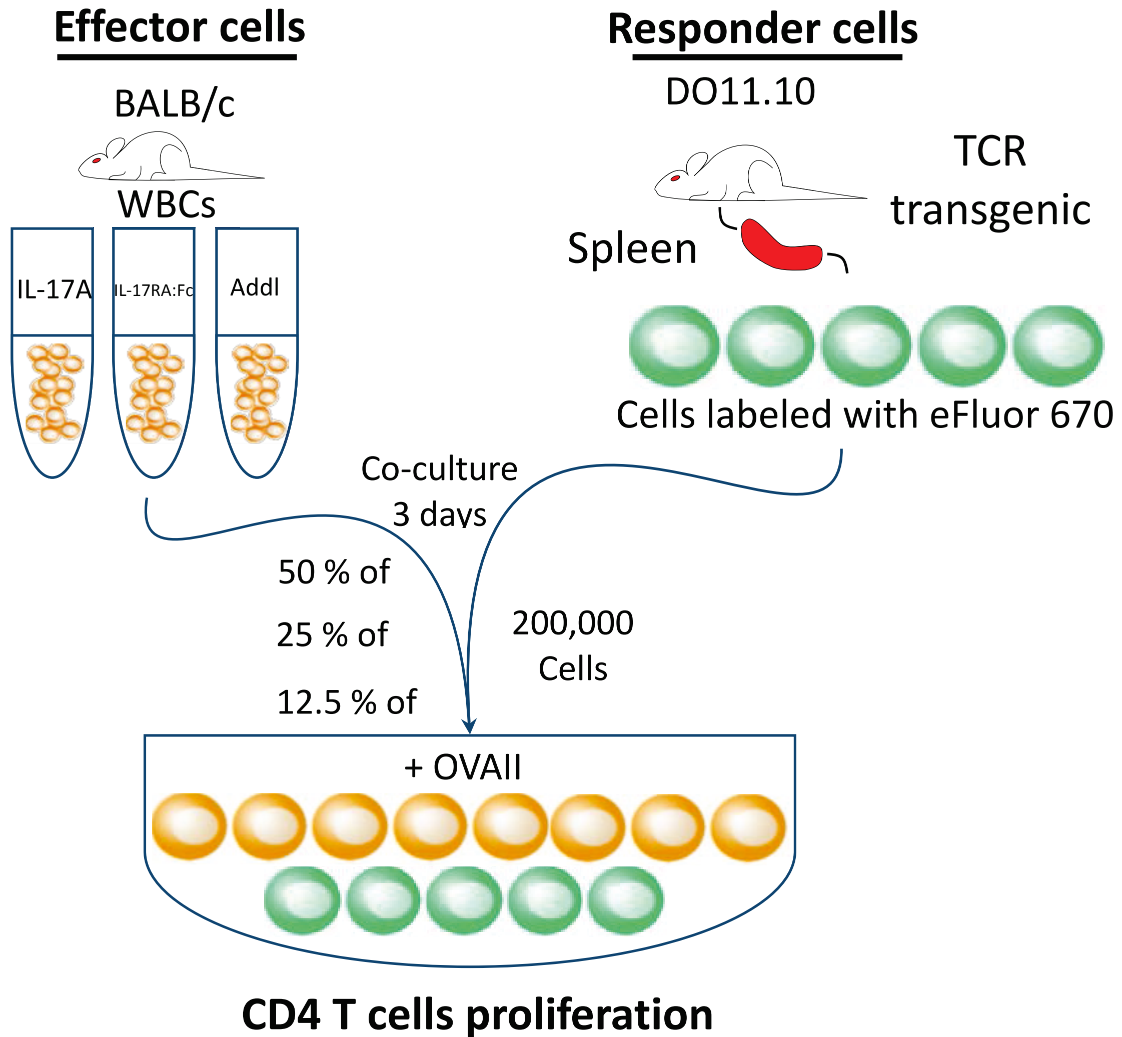


Figure 3: Schematic of suppression assay by blood volume

Leukocytes, isolated from BALB/c tumor-bearing mice, are co-cultured with $2x10^5$ of DO11.10 eFluor-labeled splenocytes in the presence of OVA peptide. After three days, T cell proliferation is measured based on eFluor-dilution using flow cytometry.

2.13.2. Suppression assay of MDSCs subpopulations by cell number

The procedure for this assay is outlined in **Figure 4**. MDSC subpopulations, G-MDSCs ($CD11b^{+}Ly6C^{low}Ly6G^{+}$) and M-MDSCs ($CD11b^{+}Ly6C^{high}Ly6G^{-}$) were used as effector cells, which were sorted out from the spleen of tumor-bearing mice (see section 2.10.4). Sorted MDSC subsets were washed twice with 5% BS RPMI and resuspended in complete RPMI at a concentration of $2x10^{6}$cell/mL. The effector leukocytes were resuspended into three dilutions containing $2x10^{5}$, $1x10^{5}$, and $5x10^{4}$ cells, and then resuspended into 100 μL of complete RPMI. These cells were co-cultured with $2x10^{5}$ eFluor-labeled responder cells; such that the ratio of effector cells to responder cells were 1:1, 1:2, and 1:4. The rest of the steps matched those of the suppression assay by blood volume (see section 2.13.1).

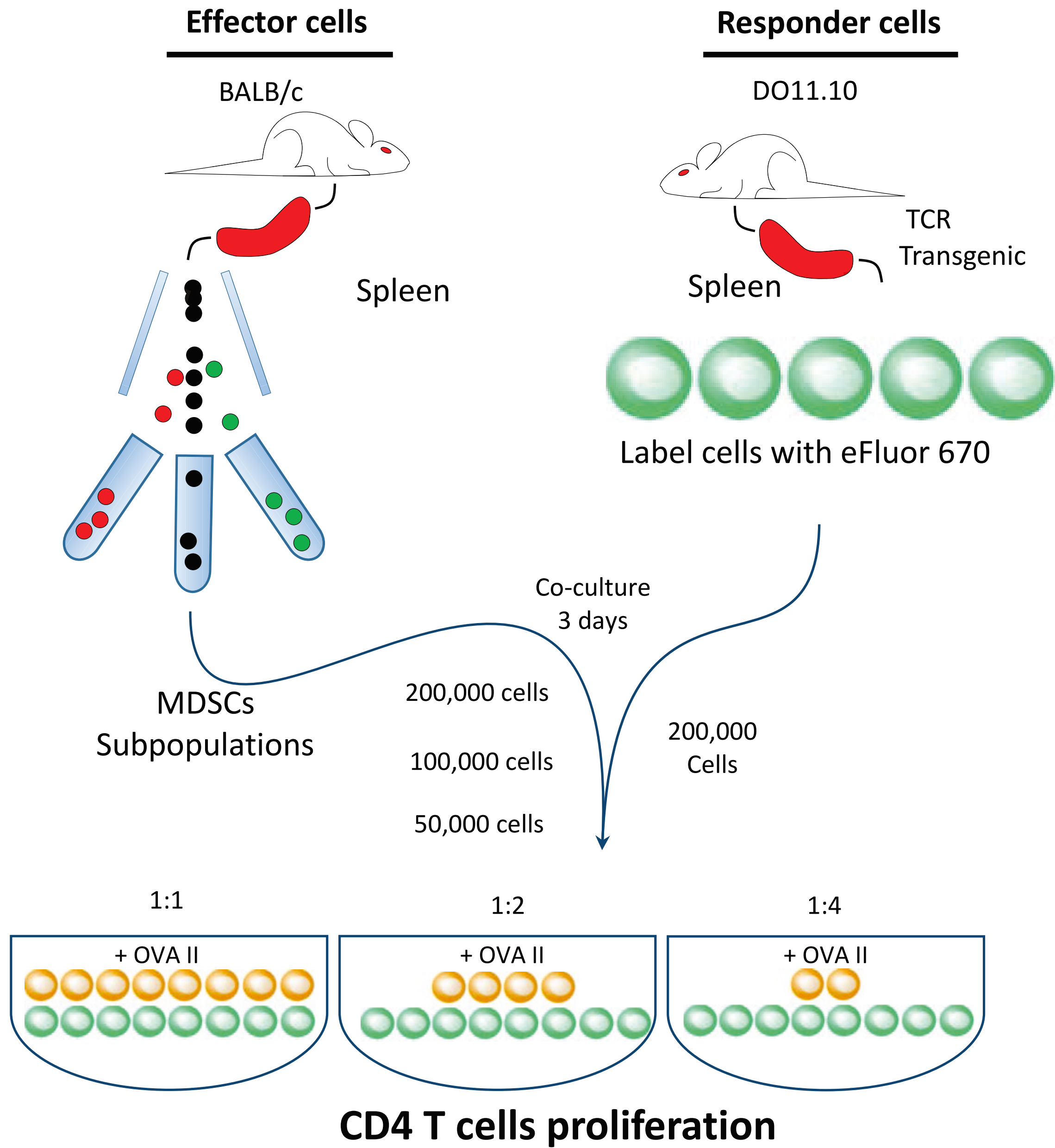


Figure 4: Schematic of suppression assay of MDSCs subpopulations.

MDSCs subpopulations are sorted-out from the spleen of a tumor-bearing mouse and co-cultured with 2x105 of DO11.10 eFluor-labeled splenocytes, at 1:1, 1:2, and 1:4 ratios in the presence of OVA peptide. After three days, T cell proliferation is measured based on eFluor-dilution using flow cytometry.

2.14. Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM) of pooled data sets, unless indicated otherwise. Data analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc.; La Jolla, CA, USA). One-way ANOVA was used to determine whether there were statistical differences among groups, with the Dunnett post-hoc test used to compare all groups to the control. Two-way ANOVA was used to compare the statistical differences among groups, with the Bonferroni post-hoc analysis to compare all groups to the control, as well as compare the changes in the control group at different time points versus the naïve. T-Test was used to determine whether there was statistical difference between two groups. The following symbols were used to denote statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 1: The common sites of metastases for different types of cancer

Tumour type	Metastasis targeted organs
Breast	Bone, lungs, liver and brain
Lung adenocarcinoma	Brain, bones, adrenal gland and liver
Colorectal	Liver and lungs
Prostate	Bones
Pancreatic	Liver and lungs
Skin melanoma	Lungs, brain, skin and liver

Adapted from *national cancer institute* (2013), where does cancer spread?

Table 2: Breast cancer classifications

	IHC markers	Proliferation cluster	Other markers	Outcome
Luminal A	ER+: 91–100% PR+: 70–74% HER2+: 8–11% Ki67: low	Low	*FOXA1* high	Good
Luminal B	ER+: 91–100% PR+: 41–53% HER2+: 15–24% Ki67: high	High	*FGFR1* and *ZIC3* amp	Intermediate or poor
Basal-like	ER+: 0–19% PR+: 6–13% HER2+: 9–13% Ki67: high	High	*RB1*:low/- *CDKN2A*: high *BRCA1*: low/- *FGFR2*: amp	Poor
HER2-enriched	ER+: 29–59% PR+: 25–30% HER2+: 66–71% Ki67: high	High	GRB7: high	Poor
Normal breast-like	ER+: 44–100% PR+: 22–63% HER2+: 0–13% Ki67: low/intermediate	Low / intermediate	--	Intermediate
Claudin-low	ER+: 12–33% PR+: 22–23% HER2+: 6–22% Ki67: intermediate	Intermediate / high	*CDH1*: low/- Claudins: low/-	Intermediate
Molecular apocrine	ER– PR– HER2 +/– Ki67: high	High	Androgen receptor: +	Poor

Adapted from *Lancet* (2011); 378: 1812–23

Table 3: Breast cancer staging

Stage		Tumor (T)	Node (N)	Metastasis (M)
0		Tis	N0	M0
I	A	T1	N0	M0
	B	T0	N1 mi	M0
		T1	N1 mi	M0
II	A	T0	N1	M0
		T1	N1	M0
		T2	N0	M0
	B	T2	N1	M0
		T3	N0	M0
III	A	T0	N2	M0
		T1	N2	M0
		T2	N2	M0
		T3	N1	M0
		T3	N2	M0
	B	T4	N0	M0
		T4	N1	M0
		T4	N2	M0
	C	Any T	N3	M0
IV		Any T	Any N	M1

Adapted from *NCCN Guidelines*™, version 2. (2011), staging breast cancer.

T (0= no primary tumor, 1= size $\leq$ 20 mm, 2= size > 20 mm but $\leq$ 50 mm, 3= size > 50 mm, T4= any size with spread to skin or chest wall)
N (0= no draining lymph node metastasis, N1 mi= micro-metastasis, N1= nodes are palpable but not histologically detectable, N2= nodes are not palpable but histologically detectable, N3= nodes are enlarged and histologically detectable)
M (0= no distant metastasis, 1= distant metastasis)

Table 4: Effector molecules produced by effector T cells

Cell type	Cytokines
Th1	IFN-γ, LT-α
Th2	IL-4, IL-5, IL-13
Th17	IL-17A, IL-17F, IL-22
T_{regs}	IL-10, TGF-β, IL-35

Adapted from *eBioscience* (2010), Th cell differentiation

Table 5: Primers of Hexon and GAPDH genes

Hexon-Forward	5’AACACCGCCTCCACGCTT-3’
Hexon-Reverse	5’CCAGTGATGGGGTTTCCTTAGTC-3’
GAPDH-Forward	5’CGATGCCCCCATGTTTGTGAT-3’
GAPDH-Reverse	5’GCAGGGATGATGTTCTG-3’

Table 6: Primers of HPRT, mCCL17 and mCCL22

HPRT-Forward	5′-TTGATTGTTGAAGATATAATTGACACT-3′
HPRT-Reverse	5′-TTCCAGTTTCACTAATGACACA-3′
mCCL17-Forward	5′-TGGTATAAGACCTCAGTGGAGTGTTC-3′
mCCL17-Reverse	5′-GCTTGCCCTGGACAGTCAGA-3′
mCCL22-Forward	5′-GAGTTCTTCTGGACCTCAAATCC-3′
mCCL22-Reverse	5′-TCTCGGTTCTTGACGGTTATCA-3′

Table 7: Antibodies used in this study

Antibodies			
Product	**Clone**	**Manufacturer**	**Country**
Anti-mouse Ly-6G (Gr-1) FITC	RB6-8C5	eBioscience	San Diego, CA, USA
Anti-mouse CD11b PE	M1/70	eBioscience	San Diego, CA, USA
Anti-mouse Ly-6C PerCP-Cy 5.5	HK1.4	eBioscience	San Diego, CA, USA
Anti-mouse CD11c APC	N418	eBioscience	San Diego, CA, USA
Anti-mouse CD4 FITC	RM4-5	eBioscience	San Diego, CA, USA
Anti-mouse B220 PE	RA3-6B2	eBioscience	San Diego, CA, USA
Anti-mouse CD19 PE	eBio1D3	eBioscience	San Diego, CA, USA
Anti-mouse CD3e PerCP-Cy 5.5	145-2C11	eBioscience	San Diego, CA, USA
Anti-mouse CD335 (NKp46) eFluor 660	29A1.4	eBioscience	San Diego, CA, USA
Anti-mouse CD194 (CCR4) PE	2G12	BioLegend	San Diego, CA, USA
Armenian Hamster IgG Isotype PE	HTK888	BioLegend	San Diego, CA, USA
Anti-mouse CD45 PE-Texas Red	30-F11	Invitrogen	Carlsbad, CA, USA
Anti-mouse Ly-6G PE	1A8	BD Bioscience	Mississauga, ON, CA

Table 8: Cytokines measured using ProcartaPlex® Multiplex Immunoassay and their Lower Limit Of Quantification (LLOQ)

Cytokine	LLOQ (pg/mL)
GM-CSF	2.32
IFN-γ	0.63
IL-1β	1.75
IL-12p70	2.395
IL-13	3.201
IL-18	32
IL-2	4.971
IL-4	1.37
IL-5	3.2
IL-6	6.4
TNF-α	3.39
IL-10	4.669
IL-17A	1.33
IL-22	12
IL-23	7.884
IL-27	2.397
IL-9	20
G-CSF	8.583
M-CSF	0.491

Table 9: Cell culture, supplements, and stimulations used in this study.

Cell culture / Supplements / Stimulation		
Product	**Manufacturer**	**Country**
Minimum Essential Medium F11 (MEM powder with Earle's salts and L-Glutamine and no sodium bicarbonate)	Invitrogen	Carlsbad, CA, USA
Dulbecco's modified Eagle's medium (DMEM)	Multicell, Wisent Inc.	St-Bruno QC, CA
Roswell Park Memorial Institute 1640 medium (RPMI)	Multicell, Wisent Inc.	St-Bruno QC, CA
Hanks' Buffered Salt Solution (HBSS)	Multicell, Wisent Inc.	St-Bruno QC, CA
1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Multicell, Wisent Inc.	St-Bruno QC, CA
200 mM L-glutamine	Multicell, Wisent Inc.	St-Bruno QC, CA
Fetal Bovine Serum (FBS)	Invitrogen	Carlsbad, CA, USA
Bovine Serum (BS)	Invitrogen	Carlsbad, CA, USA
TrypLE^{TM} Express	Invitrogen	Carlsbad, CA, USA
Trypan blue stain 0.4%	Invitrogen	Carlsbad, CA, USA
Brefeldin A	eBioscience	San Diego, CA, USA
Intracellular (IC) fixation buffer	eBioscience	San Diego, CA, USA
10X Permeabilization buffer	eBioscience	San Diego, CA, USA
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich Co	St. Louis, MO, USA
Collagenase II	Bioshop	Burlington, ON, CA
DNAse I	Invitrogen	Carlsbad, CA, USA
OVA p323-339 peptide (OVA II)	United BioSystems	Herndon, VA, USA
Collagenase IV	Worthington	Lakewood, NJ, USA
Isoflurane (USP 99.9%)	PPC	Richmond Hill, ON, CA
Ketorolac Tromethamine (30 mg/mL)	Sandoz	Italy
10% Formalin	Azer Scientific	Morgantown, PA, USA
Ionomycin	Sigma-Aldrich Co	St. Louis, MO, USA
0.03% Methylene blue stain	Molecular Research Center (MRC), Inc.	Cincinnati, OH, USA
Iodine tincture 2%	Galenova	Saint-Hyacinthe, QC, CA

Table 10: Chemicals used in this study.

Chemicals		
Product	**Manufacturer**	**Country**
NaCl	Bioshop	Burlington, ON, CA
KCl	Bioshop	Burlington, ON, CA
Na_2HPO_4	Bioshop	Burlington, ON, CA
KH_2PO_4	Bioshop	Burlington, ON, CA
NH_4Cl	Bioshop	Burlington, ON, CA
Trisodium Citrate	Bioshop	Burlington, ON, CA
CsCl	Bioshop	Burlington, ON, CA
Tris-HCl	Bioshop	Burlington, ON, CA
Magnesium chloride ($MgCl_2.6H_2O$)	Bioshop	Burlington, ON, CA
Calcium chloride ($CaCl_2.2H_2O$)	Fisher-Scientific	Fair Lawn, NJ, USA
Dimethyl Sulfoxide (DMSO)	Bioshop	Burlington, ON, CA
$KHCO_3$	Sigma-Aldrich Co	St. Louis, MO, USA
Sodium Deoxycholate (DOC)	Sigma-Aldrich Co	St. Louis, MO, USA

Table 11: Media, solutions, and reagents used in this study.

Media/Chemicals/Solutions/Reagents		
Name	**Composition**	**Application**
Complete DMEM (cDMEM)	DMEM	4T1 cells, Colony assay
	10% FBS (heat-inactivated)	
	100 IU/mL penicillin,	
	100 µg/mL streptomycin	
	10 mM HEPES	
	2 mM L-glutamine	
Complete RPMI (cRPMI)	RPMI	T cells, Suppression assay
	10% FBS (heat-inactivated)	
	100 IU/mL penicillin,	
	100 µg/mL streptomycin	
	10 mM HEPES	
	2 mM L-glutamine	
Complete F11	MEM F11	HEK293 cells
	10% FBS (heat-inactivated)	
	100 IU/mL penicillin,	
	100 µg/mL streptomycin	
	10 mM HEPES	
	2 mM L-glutamine	
2x MEM (F11) 1 L pH (7.0 - 7.2)	19 g MEM powder (2 bags)	Adenovirus titration
	4.4 g sodium bicarbonate	
	dH2O	
Agarose solution	1 g agarose powder	Adenovirus titration
	100 mL of dH_2O	
10x Phosphate Buffered Saline (PBS) 1 L (pH 7.4)	80 g NaCl	Stock
	2 g KCl	
	14.4 g Na_2HPO_4	
	2.4 g KH_2PO_4	
	dH_2O	
1x PBS^{++} 100 mL	10 mL of 10X PBS	Isotonic buffer
	1 mL of 1% Mg^{2+} solution	
	1 mL of 1% Ca^{2+} solution	
	88 mL of dH_2O	
Flow cytometry wash buffer	1x PBS	Flow cytometry
	1% BS	
Flow cytometry sorting buffer	1x PBS	Flow cytometry
	2% FBS	
	1mM EDTA	
	25mM HEPES	
Flow cytometry fixation buffer	1% formalin	Flow cytometry
	1x PBS	

<table>
<tr><th colspan="4">Media/Chemicals/Solutions/Reagents</th></tr>
<tr><th colspan="2">Name</th><th>Composition</th><th>Application</th></tr>
<tr><td colspan="2" rowspan="2">1% Mg^{2+} solution</td><td>1 g $MgCl_2.6H_2O$</td><td rowspan="2">4T1 transduction</td></tr>
<tr><td>100 mL dH_2O</td></tr>
<tr><td colspan="2" rowspan="2">1% Ca^{2+} solution</td><td>1 g $CaCl_2.2H_2O$</td><td rowspan="2">4T1 transduction</td></tr>
<tr><td>100 mL dH_2O</td></tr>
<tr><td colspan="2" rowspan="4">2X Ammonium-Chloride-Potassium buffer (ACK)
1 L pH 7.3</td><td>16.58 g NH_4Cl</td><td rowspan="4">RBCs lysis</td></tr>
<tr><td>2 g $KHCO_3$</td></tr>
<tr><td>0.04 g EDTA</td></tr>
<tr><td>dH2O</td></tr>
<tr><td colspan="2" rowspan="3">Citrate saline solution
1 L</td><td>10.06 g KCl</td><td rowspan="3">HEK293 cells</td></tr>
<tr><td>4.41 g trisodium citrate</td></tr>
<tr><td>dH_2O</td></tr>
<tr><td colspan="2" rowspan="2">1M Tris-HCl
100 mL pH 7.5</td><td>15.76 g Tris-HCl</td><td rowspan="10">Adenovirus</td></tr>
<tr><td>dH_2O</td></tr>
<tr><td colspan="2" rowspan="2">10% DOC</td><td>10 g DOC</td></tr>
<tr><td>100 mL dH_2O</td></tr>
<tr><td rowspan="6">CsCl</td><td rowspan="2">Density 1.5</td><td>90.8 g CsCl</td></tr>
<tr><td>109.9 g of 10 mM Tris Hcl pH 8.0</td></tr>
<tr><td rowspan="2">Density 1.35</td><td>70.4 g of CsCl</td></tr>
<tr><td>129.6 g of 10 mM Tris Hcl pH 8.0</td></tr>
<tr><td rowspan="2">Density 1.25</td><td>54.0 g of CsCl</td></tr>
<tr><td>146.0 g of 10 mM Tris Hcl pH 8.0</td></tr>
</table>

Chapter 3. Results

3.1. Primary Tumor with Adenovirus-transduction Model

3.1.1. *In vitro* characterization of 4T1 cells upon transduction with different adenovirus vectors

Three replication-defective adenovirus vectors were used to investigate the role of the IL-17/IL-17R axis in breast cancer: one encoding IL17A (AdIL-17A), one encoding the soluble IL-17RA subunit fused to IgG Fc (AdIL-17RA:Fc), and an empty adenovirus vector (Ad*dl*70-3 or Addl). The adenovirus vectors were transduced into 4T1 cells (see section 2.3.3). After 24 hrs of transduction, we used PCR to amplify the hexon gene, which encodes one of the major virus capsid proteins, to investigate the efficiency of adenovirus-transduction of 4T1 cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used as a control. The hexon gene was detected at comparable levels in all of the adenovirus-transduced 4T1 cells, indicating the transduction of all viral vectors was successful (**Figure A 1**). An IL-17A ELISA was used to test the ability of transduced 4T1 cells to produce the transgene product. The supernatants of 4T1 cells (transduced and non-transduced) were collected at 24, 48, and 72 hrs after transduction with Ad-vectors. IL-17A production was detected only in the AdIL-17A-transduced 4T1 cells, with cytokine production increasing at each time point ($P<0.001$; **Figure 5A**). We also evaluated the effect of adenovirus-transduction on 4T1 cell proliferation at 24, 48 and 72 hrs after transduction, and found no impact on cell proliferation among the four groups ($P>0.05$; **Figure 5B**). Moreover, cell viability was investigated after 48 hrs in culture, and there were no significant differences among groups in terms of the frequency of apoptotic and necrotic cells (pooled together), as assessed by Annexin V/7AAD staining using flow

cytometry (**Figure 5C**). These data indicate that transduction of 4T1 cells with Ad-vectors was successful and able to produce transgene products in transduced cells without any direct effect of the vectors or their products on the viability and the proliferation of the 4T1 cells *in vitro*.

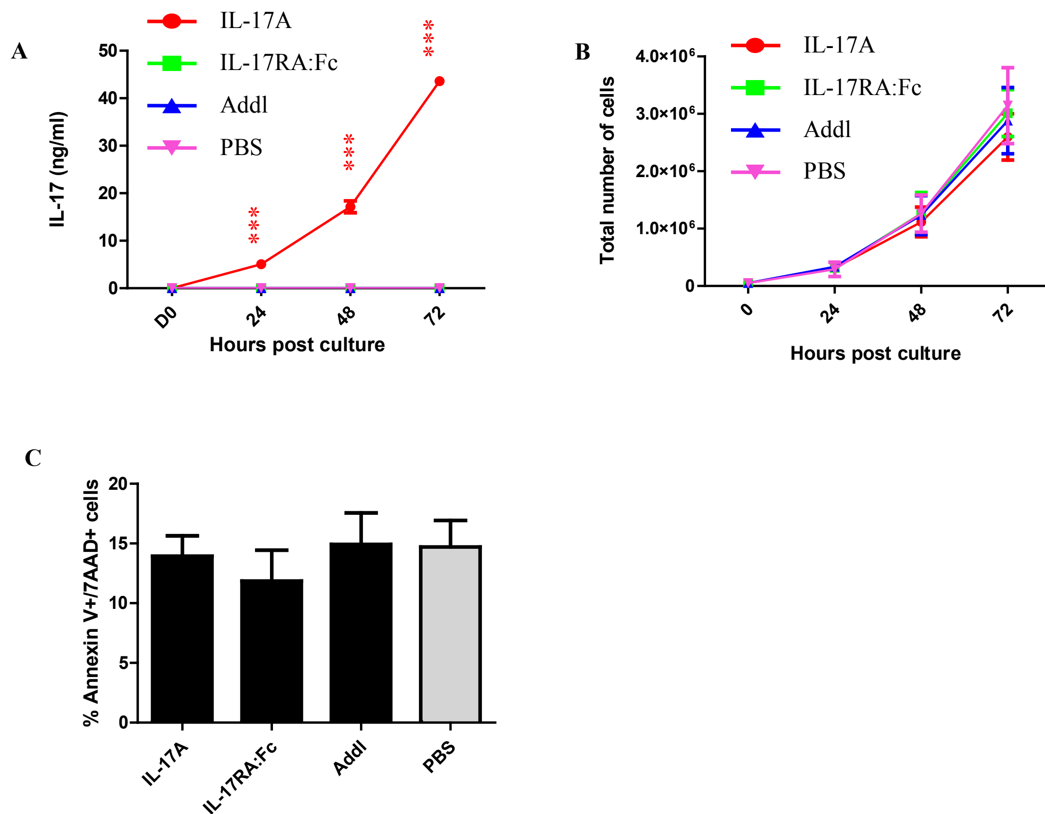


Figure 5: *In vitro* characterization of 4T1 cells upon transduction with different adenovirus constructs

4T1 cells were divided into four groups, each group was transduced with a different adenovirus vector (AdIL-17A, Ad-IL17RA:Fc, or Ad170) with MOI=200 and one group only was treated with PBS^{++} as a control. **(A)** Levels of IL-17A in adenovirus-transduced and non-transduced 4T1 cells at 24 hrs intervals and measured by ELISA (n=3 per group from single experiments). **(B)** *In vitro* growth curve of adenovirus-transduced and non-transduced 4T1 cells. Cell number was monitored by direct counting at 24 hrs intervals (n=3 per group pooled from 3 independent experiments). **(C)** 4T1 cells were stained for annexin V/7AAD after incubation for 48 hrs to detect apoptotic and necrotic cells (n=6 replicates per group pooled from 2 independent experiments). Data are presented as the mean ± SEM. ***P*<0.01; ****P*<0.001 versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.

3.1.2. AdIL-17A transduction in 4T1 cells induces production of G-CSF, GM-CSF, M-CSF, IL-6, and IL-10 *in vitro*

Cytokine production was measured in the culture supernatants of different adenovirus-transduced 4T1 cells in order to identify the effect of transduction on the secretion of other cytokines. Our results showed that there was a basic detectable secretion of IL-17A (approximately 10 pg/mL) in the AdIL-17R:Fc and the Addl groups, whereas the IL-17A level in the AdIL-17A group was 4000 fold higher than in the control group (**Figure A 2A**). In parallel with the production of IL-17A, there was an increased production of G-CSF (8 fold), GM-CSF (40 fold), M-CSF (7 fold), IL-6 (260 fold), IL-10 (5 fold), and TNF (6 fold) in the AdIL-17A-transduced 4T1 cells compared to the Addl group (**Figure A 2B-G**). Other cytokines, IL-13, IL-2, IL-22, IL23, IL-12p70, and IL18 were detectable in 4T1 culture medium but not different among the adenovirus-transduced groups (**Figure A 3**). These results indicate that IL-17A production from transduced 4T1 cells is able to stimulate these cells in an autocrine manner to produce other cytokines.

3.1.3. AdIL-17A transduction in 4T1 cells promotes mammary tumor growth and lung metastasis

Having assessed the behavior of adenovirus-transduced 4T1 cells *in vitro*, we wanted to investigate their behavior *in vivo*, in the context of primary and secondary tumors. Tumor growth was significantly reduced in the Addl group compared to the PBS group ($P<0.01$), suggesting an inhibitory effect of viral infection on 4T1 tumor growth. However, AdIL-17A transduction in 4T1 cells was able to restore the tumor growth to the level of the PBS group ($P<0.05$; **Figure 6A**). In comparison, tumor growth in the AdIL-17RA:Fc group and the Addl group were comparable. Thus, adenovirus-transduced 4T1 cells exhibited a different pattern of proliferation *in vivo* than they did *in vitro*, indicating that IL-17A has an indirect pro-tumor effect on primary tumor growth *in vivo*. However, blocking IL-17R via Ad vector delivery did not show obvious biological activity. Next, we investigated whether IL-17A production within primary tumor has any effect on lung metastasis. In our model, we observed a 4-5 fold increase in the number of lung metastasis in the AdIL-17A group compared with the Addl group ($P<0.01$; **Figure 6B**). Once again, AdIL-17R transduction showed no effect on the level of lung metastasis compared to the control Addl group. Collectively, our data suggests that production of IL-17A at the tumor site significantly induces tumor growth and lung metastasis *in vivo.*

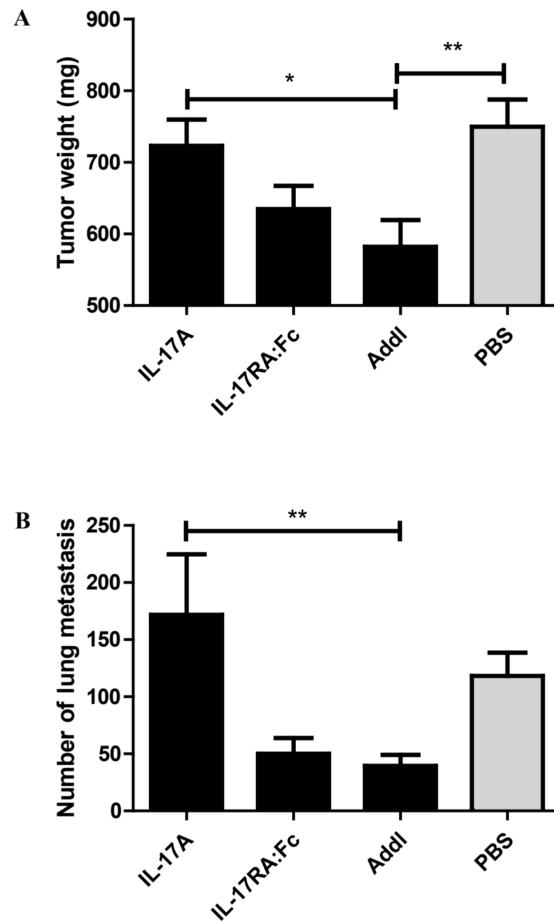


Figure 6: IL-17A promotes 4T1 tumor growth and lung metastasis

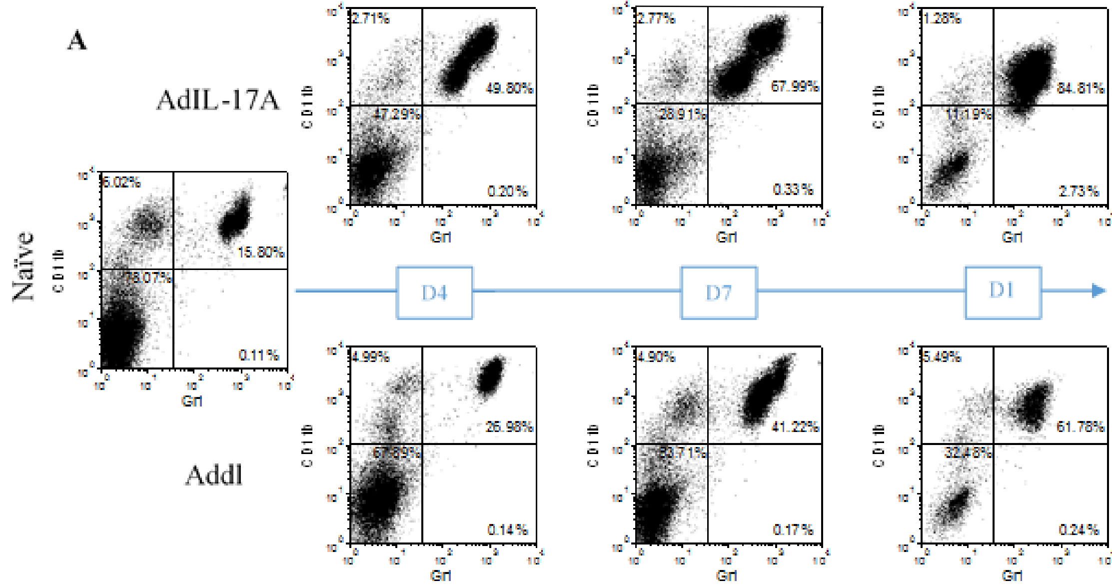
Mice were injected with $1x10^6$ 4T1 cells (transduced or non-transduced) into the fourth mammary pad and mice were sacrificed after 17 days. **(A)** Tumor weight was determined at 17 days post tumor injection (n=25 per group pooled from 5 independent experiments). **(B)** Lung metastasis was assessed at 17 days post tumor injection by culturing lung-derived single cell suspension in 6-TG-suplemented complete RPMI for 10-14 days (n=20 per group pooled from 4 independent experiments). Data are presented as the mean ± SEM. *P<0.05; **P<0.01 versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.

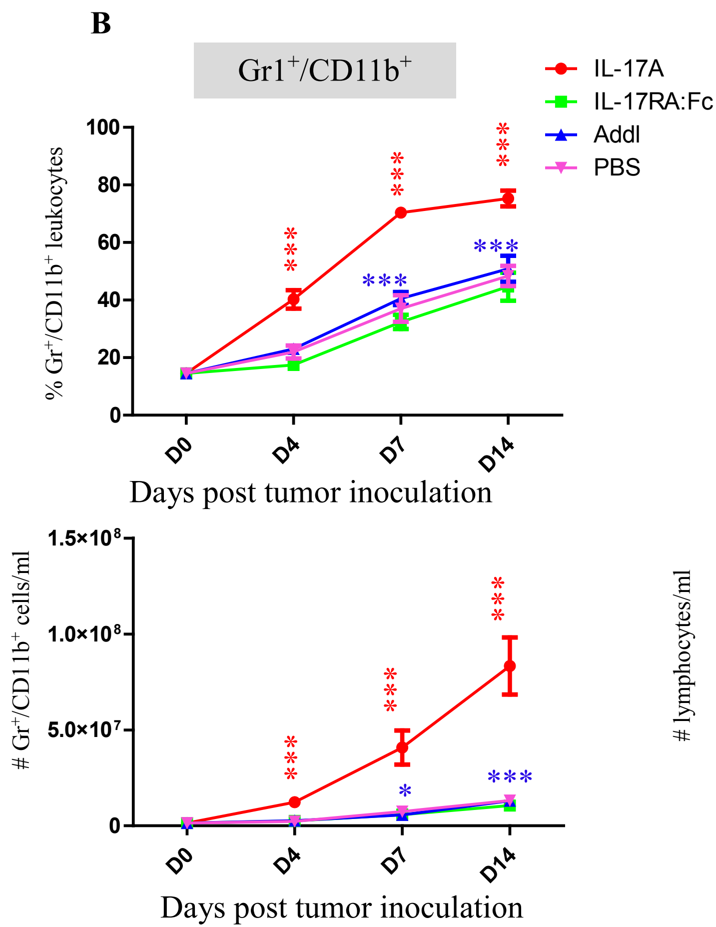
3.1.4. AdIL-17A transduction in 4T1 cells induces expansion of myeloid cells in PB

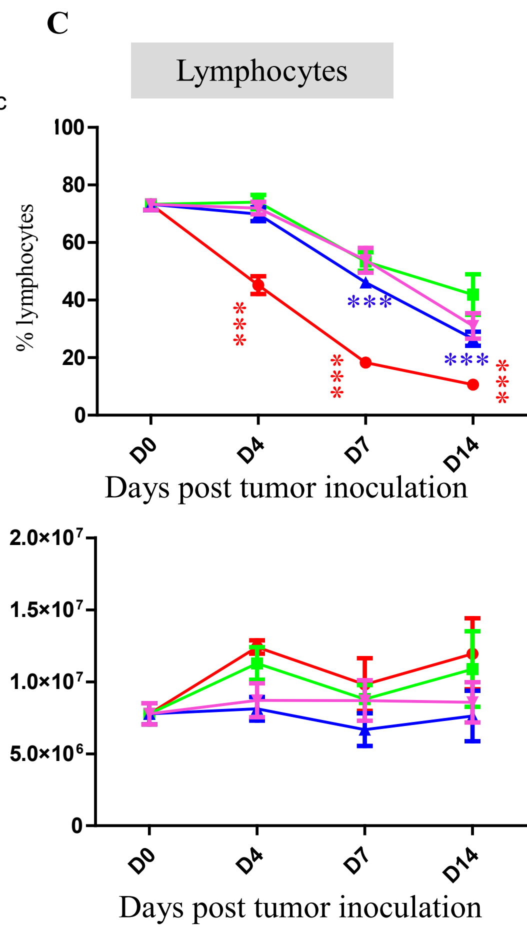
Having demonstrated an indirect role of AdIL-17A transduction in 4T1 cells in promoting tumor growth and lung metastasis, we conducted immune profiling in our model. Blood samples were collected from mice at different time points following tumor inoculation. Peripheral leukocytes were analyzed by flow cytometry. We found that $Gr1^+/CD11b^+$ myeloid cells accounted for approximately 16% of $CD45^+$ PB leukocytes in naïve mice. Upon 4T1 tumor inoculation as seen in Addl group (and PBS group), these cells progressively increased to 21%, 40% and 55% at days 4, 7 and 14, respectively (**Figure 7A, B**). Notably, AdIL-17A transduction in 4T1 cells significantly increased the frequency and the total number of $Gr1^+/CD11b^+$ cells in PB as early as four days following tumor injection ($P<0.001$; **Figure 7A, B**). Conversely, the frequency, but not the absolute number, of lymphocytes (T cells, B cells, and NK cells) was markedly reduced compared to the Addl control group ($P<0.001$; **Figure 7C**). Our results suggest that AdIL-17A transduction in 4T1 cells has a potent effect on the leukemoid reaction due to an increased production of myeloid, but not lymphoid, lineage cells.

Figure 7: AdIL-17A transduction in 4T1 cells induces expansion of myeloid cells in PB

Blood samples were collected from tail veins, at days 0, 4, 7, and 14, post tumor injection. The percentages of myeloid Gr1^{+}/CD11b^{+} cells and lymphocytes (includes CD19^{+} as marker for B cells, CD4^{+}, CD8^{+}, CD3^{+} and TCRβ as markers for T cells and NKp46 as marker for NK cells) out of CD45^{+} PB leukocytes were determined by flow cytometry; absolute cell numbers were determined by cell counting using hemacytometer. **(A)** Representative dot plots showing Gr1^{+}/CD11b^{+} cells in blood of naïve, AdIL-17A and Addl groups; gated on CD45^{+} cells. **(B)** Frequency and absolute number of myeloid Gr1^{+}/CD11b^{+} PB leukocytes in the CD45^{+} gate. **(C)** Frequency and absolute cell number of PB lymphocytes (B, T, and NK cells) in the CD45^{+} gate. Data are presented as the mean ± SEM of 10 mice per group pooled from 2 independent experiments. *$P<0.05$; ***$P<0.001$ versus Addl (vertical red stars), and versus Day 0 within Addl group (horizontal blue stars), as determined by two-way ANOVA with Bonferroni post-hoc analysis.


A
AdIL-17A
Naïve
Addl
D4
D7
D1
Grl
CD11b
2.71%
49.80%
47.29%
0.20%
2.77%
67.99%
28.91%
0.33%
1.28%
84.81%
11.19%
2.73%
5.02%
15.80%
78.07%
0.11%
4.99%
26.98%
67.89%
0.14%
4.90%
41.22%
53.71%
0.17%
5.49%
61.78%
32.48%
0.24%


B
Gr1+/CD11b+
IL-17A
IL-17RA:Fc
Addl
PBS
% Gr+/CD11b+ leukocytes
Days post tumor inoculation
Gr+/CD11b+ cells/ml
D0
D4
D7
D14

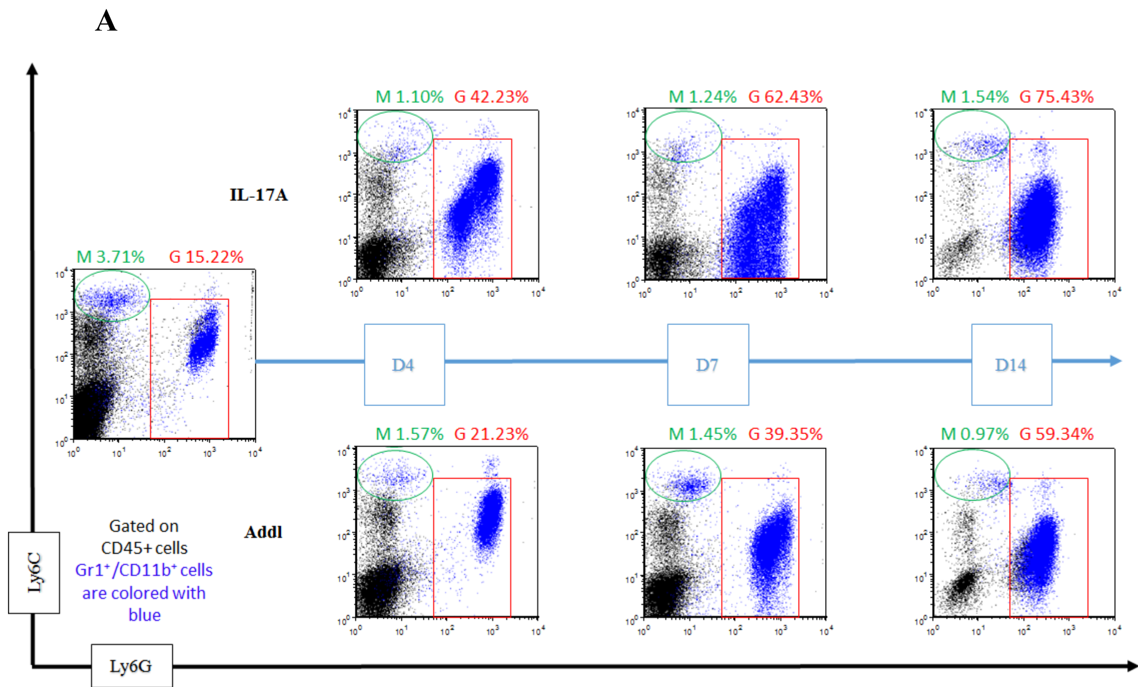

C
Lymphocytes
% lymphocytes
# lymphocytes/ml
Days post tumor inoculation
D0
D4
D7
D14

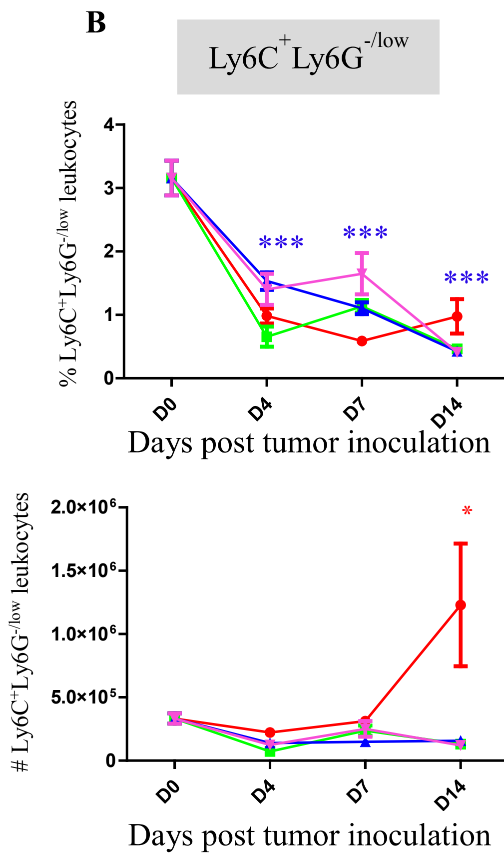
3.1.5. AdIL-17A transduction in 4T1 cells preferentially induces granulocytosis *in vivo*

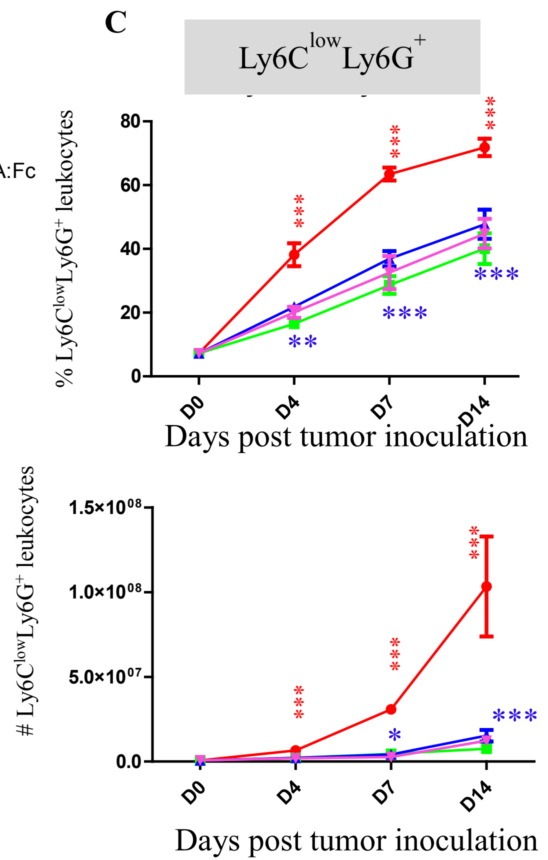
Arising from the fact that myeloid cells are a heterogeneous population of granulocytic and monocytic cells, we decided to analyze these two subpopulations in our model. A closer examination revealed that the main subpopulation induced in the AdIL-17A group were granulocytic cells ($CD11b^{+}Ly6C^{low}Ly6G^{+}$) that increased in frequency and absolute cell number (P<0.001; **Figure 8A, C**). In comparison, monocytic cells ($CD11b^{+}Ly6C^{+}Ly6G^{-/low}$) only increased in absolute cell number in the AdIL-17A group at a later time point (around two weeks) (P<0.05; **Figure 8B**). Overall, the AdIL-17A transduction in 4T1 cells preferentially induces expansion of granulocytic cells over monocytic populations.

Figure 8: AdIL-17 transduction in 4T1 cells preferentially induces expansion of granulocytic myeloid cells in PB *in vivo*

Frequency and absolute number of granulocytic and monocytic subpopulations in $CD45^+$ PB leukocytes. **(A)** Representative dot plots for two subpopulations in naïve, AdIL-17A and Addl groups; gated on $CD45^+$ cells, with $Gr1^+/CD11b^+$ cells coloured in blue; M=Monocytic myeloid cells (green circle) and G=Granulocytic myeloid cells (Red square). **(B)** Frequency and absolute number of monocytic cells ($CD11b^+Ly6C^+Ly6G^-$). **(C)** Frequency and absolute number of granulocytic population ($CD11b^+Ly6C^{low}Ly6G^+$). Data are presented as the mean ± SEM of 10 mice per group pooled from 2 independent experiments. *P<0.05; **P<0.01; ***P<0.001 versus Addl (vertical red stars), and versus Day 0 within Addl group (horizontal blue stars), as determined by two-way ANOVA with Bonferroni post-hoc analysis.


A
IL-17A
Addl
M 3.71% G 15.22%
M 1.10% G 42.23%
M 1.24% G 62.43%
M 1.54% G 75.43%
M 1.57% G 21.23%
M 1.45% G 39.35%
M 0.97% G 59.34%
D4
D7
D14
Gated on CD45+ cells
Gr1+/CD11b+ cells are colored with blue
Ly6C
Ly6G


B
Ly6C+Ly6G-/low
% Ly6C+Ly6G-/low leukocytes
# Ly6C+Ly6G-/low leukocytes
IL-17A
IL-17RA:Fc
Addl
PBS
Days post tumor inoculation

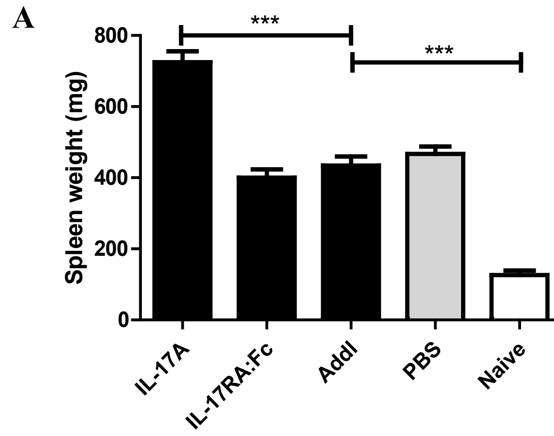

C
Ly6ClowLy6G+
% Ly6ClowLy6G+ leukocytes
# Ly6ClowLy6G+ leukocytes
Days post tumor inoculation

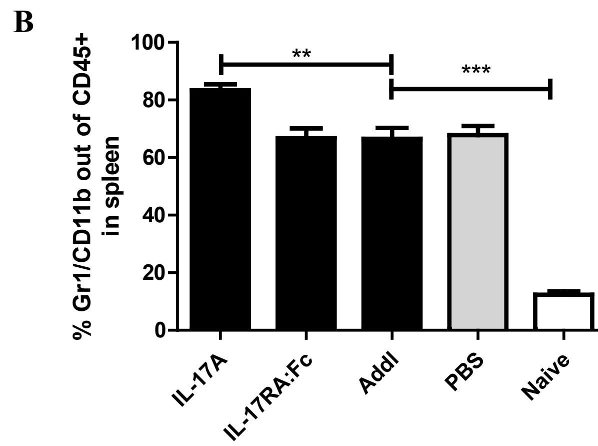
3.1.6. AdIL-17A transduction in 4T1 cells induces splenomegaly due to increased accumulation of Gr1$^+$/CD11b$^+$ cells

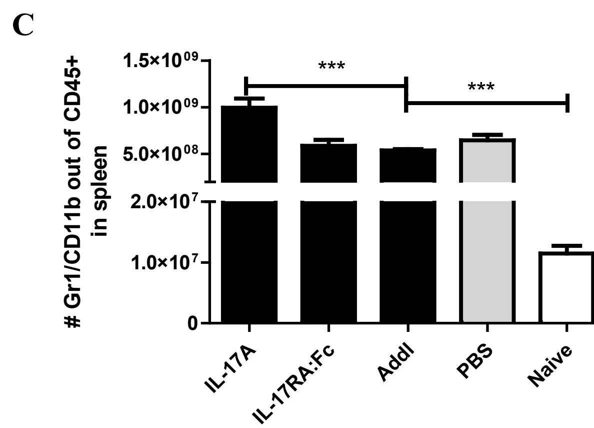
Having shown that AdIL-17A transduction in 4T1 cells induces myelopoiesis in PB, we further examined the immune cell profile in the spleen. Remarkably, the spleen size increased significantly in all of the groups (four fold in the control group compared to naïve spleen), but most significantly in the AdIL-17A transduction group, where they were 1.7 times bigger than the spleens in the Addl group at 17 days following tumor inoculation ($P<0.001$; **Figure 9A**). Analysis of the immune leukocyte profile revealed that, in all of the groups, the majority of leukocytes in tumor-bearing mice spleens were Gr1$^+$/CD11b$^+$ myeloid lineage cells, reaching over 60% of splenocytes compared to 10% in naïve mice. In agreement with the leukocyte profile in PB, the frequency and absolute number of these myeloid cells were significantly higher in the AdIL-17A group compared to the Addl group ($P<0.05$; **Figure 9B, C**). Collectively, results from our immune cell profiling in PB and spleen consistently demonstrate that 4T1 tumor inoculation stimulates myelopoiesis and this process is further enhanced by AdIL-17A transduction.

Figure 9: AdIL-17A transduction in 4T1 cells results in splenomegaly and accumulation of Gr1+/CD11b+ cells

Spleens were collected at the day of sacrifice (D17) and weighed. A single cell suspension was prepared and stained for flow cytometry. **(A)** Spleen weight in mg (n=10 per group pooled from 2 independent experiments; naïve group n=3). **(B)** Frequency of spleen myeloid cells after gating on CD45+ cells (n=10 per group pooled from 2 independent experiments; naïve group n=3). **(C)** Total number of spleen myeloid cells (n=10 per group pooled from 2 independent experiments; naïve group n=3). Data are presented as the mean ± SEM. *$P<0.05$; ***$P<0.001$ versus the Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.


A
***
***
800
600
400
200
0
Spleen weight (mg)
IL-17A
IL-17RA:Fc
Addl
PBS
Naive


B
**
***
100
80
60
40
20
0
% Gr1/CD11b out of CD45+ in spleen
IL-17A
IL-17RA:Fc
Addl
PBS
Naive


C
***
***
1.5×10⁰⁹
1.0×10⁰⁹
5.0×10⁰⁸
2.0×10⁷
1.0×10⁷
0
Gr1/CD11b out of CD45+ in spleen
IL-17A
IL-17RA:Fc
Addl
PBS
Naive

3.1.7. Characterization of serum cytokines

Having shown that AdIL-17A transduction in 4T1 cells has the ability to induce the production of other cytokines directly *in vitro* and enhances myelopoiesis *in vivo*, we collected serum samples from mice at different time points (naïve, and days 7 and 12 after tumor inoculation). We measured different cytokines and growth factors using a Luminex multiplex assay (see section 2.11). To our surprise, the serum level of IL-17A in AdIL-17A group at both day 7 and day 12 did not show any significant elevation compared to other groups (**Figure A 4A**). Nevertheless, AdIL-17A transduction in 4T1 cells significantly increased G-CSF production (approximately 3 fold) compared to the Addl group at day 7 post tumor inoculation ($P<0.05$; **Figure A 4B**). We did not see any impact of AdIL-17A transduction on serum levels of GM-CSF, IFN-γ, IL-13, IL-5, IL-6, TNF-α, IL-10, IL-22 and IL-1β compared to the Addl group (**Figure A 4C-K**). Levels of M-CSF, IL-12p70, IL-9, IL-2, IL-4, IL-23, and IL-27 were below the detection limit in all examined samples. Although AdIL-17R:Fc transduction in 4T1 cells showed no biological impacts on tumor growth and lung metastasis (**Figure 5A,B**), we observed a consistent trend for reduced serum levels of IL-17A, G-CSF, IL-13, IL-5, IL-6, and IL-10 at day 12 post tumor inoculation. Moreover, level of IFN-γ production at day 7 post tumor inoculation was significantly reduced compared to the Addl group ($P<0.05$; **Figure A 4D**). In addition, there was a significant increase of IL-5 level after two weeks of tumor injection in the Addl and PBS groups compared to naïve mice ($P<0.05$; **Figure A 4F**). These data suggest that, although AdIL-17A transduction in 4T1 cells did not result in over production of IL-17A in periphery *in vivo*, the IL-17A produced at the tumor microenvironment is clearly sufficient to enhance G-CSF production, which, in turn, promotes myelopoiesis *in vivo*.

3.1.8. AdIL-17A transduction in 4T1 cells stimulates potent immune suppression due to the changes in quantity but not quality of myeloid cell that become MDSCs

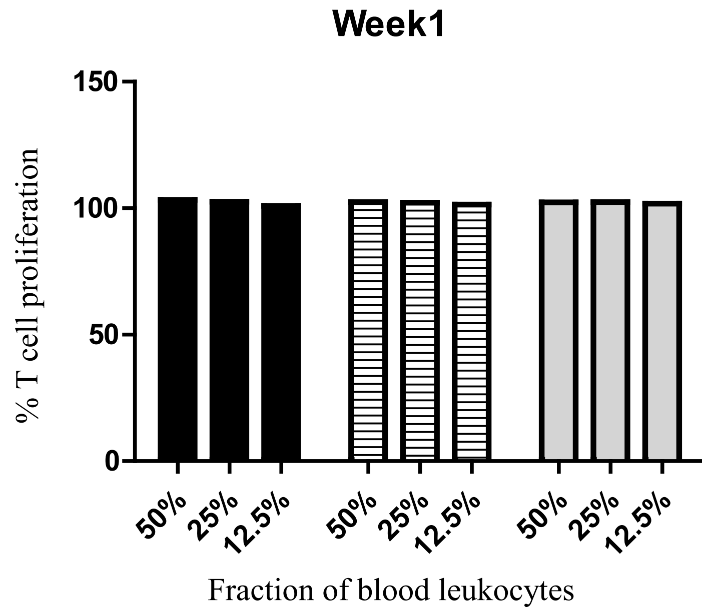
Prompted by the observation that increased tumor growth and lung metastasis were associated with an enhanced production of myeloid cells in the AdIL-17A group, we hypothesized that IL-17A-induced myeloid cells in 4T1 tumor-bearing mice were strong immune suppressors. To test the hypothesis, we first compared the overall suppressive activity of PB leukocytes from a fixed volume of blood collected from different adenovirus-transduced tumor-bearing mice. While the leukocytes circulating in PB became suppressive in the AdIL-17A group two weeks after tumor injection, no suppressive activity was observed this time point in the Addl and AdIL-17R:Fc groups (**Figure 10A)**. Subsequently, we examined the relative suppressive activity of sorted myeloid cells (granulocytic and monocytic populations) derived from the spleen of tumor-bearing mice in the adenovirus-transduced groups two weeks after tumor inoculation. Although both monocytic and granulocytic myeloid cells exhibited suppressive activities on T cell proliferation in a dose-dependent manner, the monocytic population displayed a higher potency compared to the granulocytic population (approximately 90% versus 50% suppression rate at 1:1 ratio, respectively) (**Figure 10B**). However, the potency of suppression by both populations was comparable among all adenovirus-transduced groups. Collectively, our results demonstrate that the IL-17A-induced $Gr1^{+}/CD11b^{+}$ myeloid cells display both phenotypical and functional characteristics of MDSC. However, the quality of both subsets of MDSCs is not altered by AdIL-17A transduction.

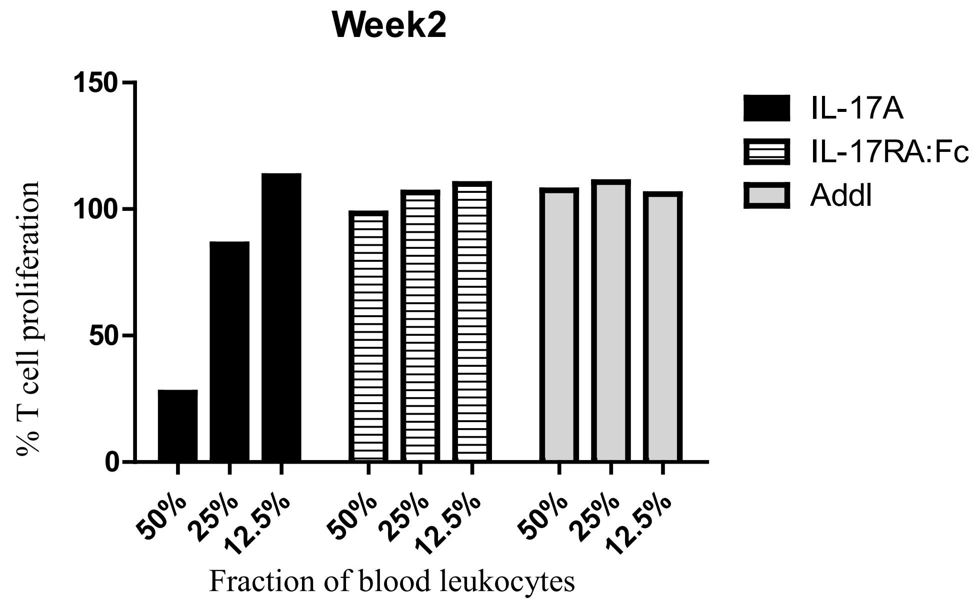
Figure 10: AdIL-17A transduction in 4T1 cells induces potent immune suppression due to the changes in quantity but not quality of myeloid cells that become MDSCs.

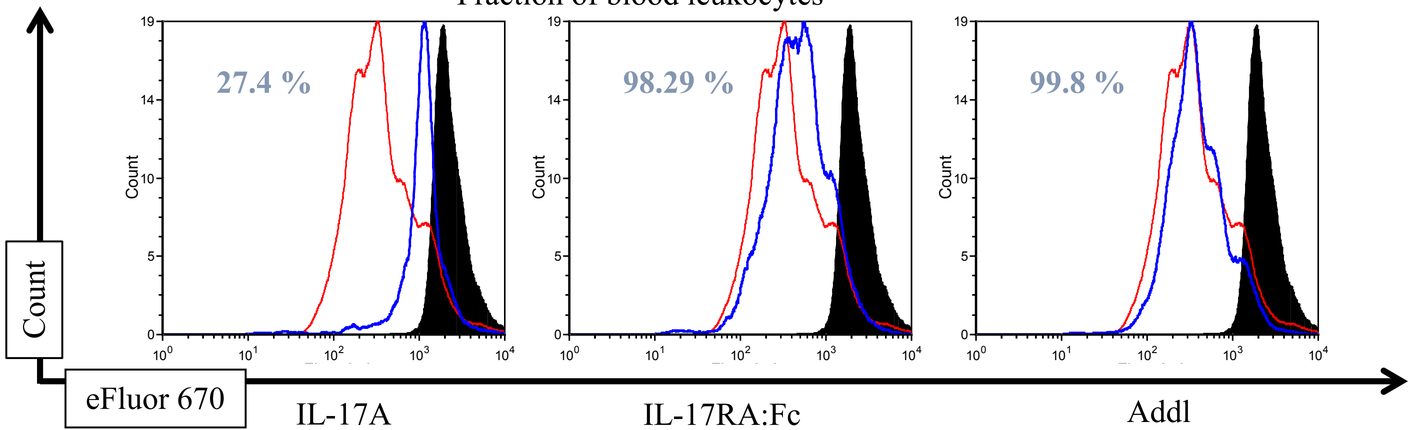
The effector function of $Gr1^{+}/CD11b^{+}$ cells and their subpopulations was tested by co-culturing leukocytes isolated from tumor-bearing mice (effector cells) with $2x10^{5}$ splenocytes isolated from DO11.10 mice (responder cells) in the presence of OVA-II peptide. **(A)** The suppression assay was performed 7 and 14 days after tumor injection; effector cells were tested at 50%, 25%, and 12.5%, of leukocytes recovered from 50 µL of PB from different adenovirus-transduced tumor-bearing mice. **(B)** The suppression assay was performed using sorted subpopulations of monocytic cells ($Ly6C^{+}Ly6G^{-}$) and granulocytic cells ($Ly6C^{low}Ly6G^{+}$), which were co-cultured with responder cells ($2x10^{5}$) at ratios of 1:1, 1:2 and 1:4. Representative histograms show T cell proliferation in the presence of myeloid effector cells (blue line) relative to the positive control (red line, no effector cells) and negative control (filled black, no OVAII peptide stimulation). Data shown here are from single experiment and represent a preliminary result. The number on each histogram represents the percentage of T cell proliferation that was calculated from the formula:

$$Proliferation\ (\%) = \frac{\text{Percentage of proliferated cells of sample X 100}}{\text{Percentage of proliferated cells in positive control}}$$

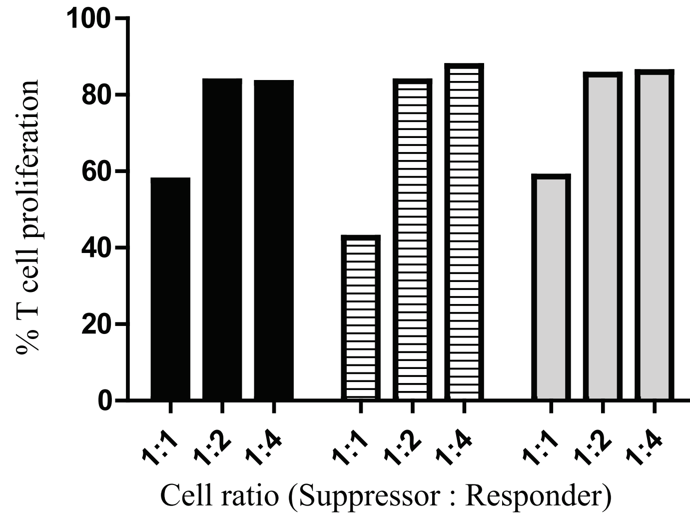
A

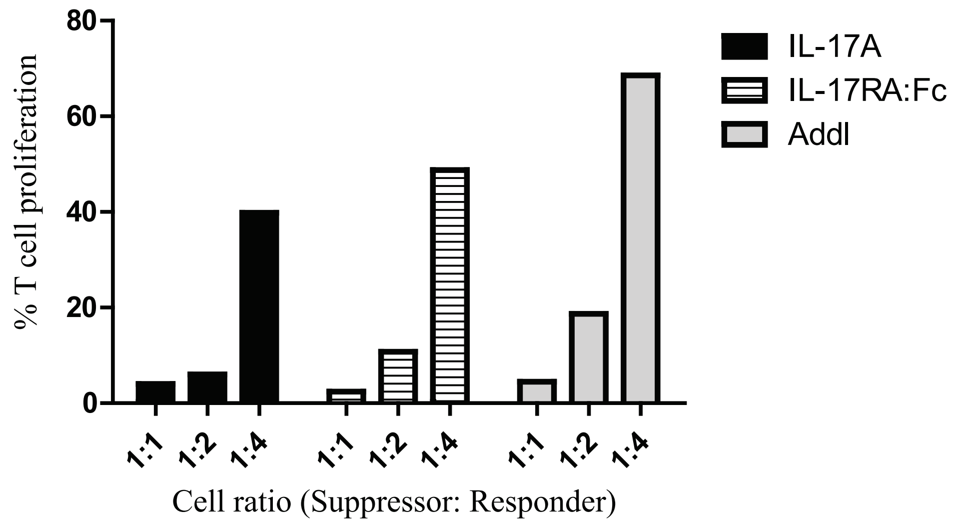


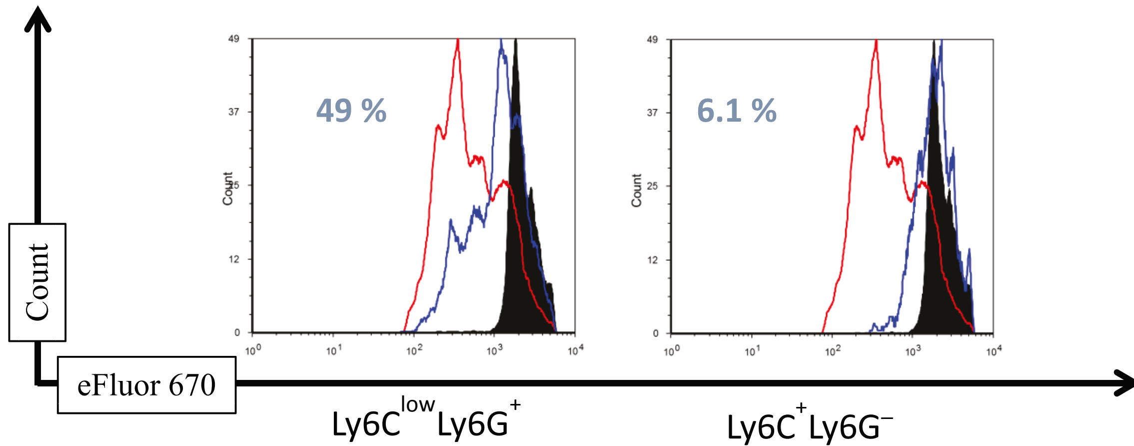




B





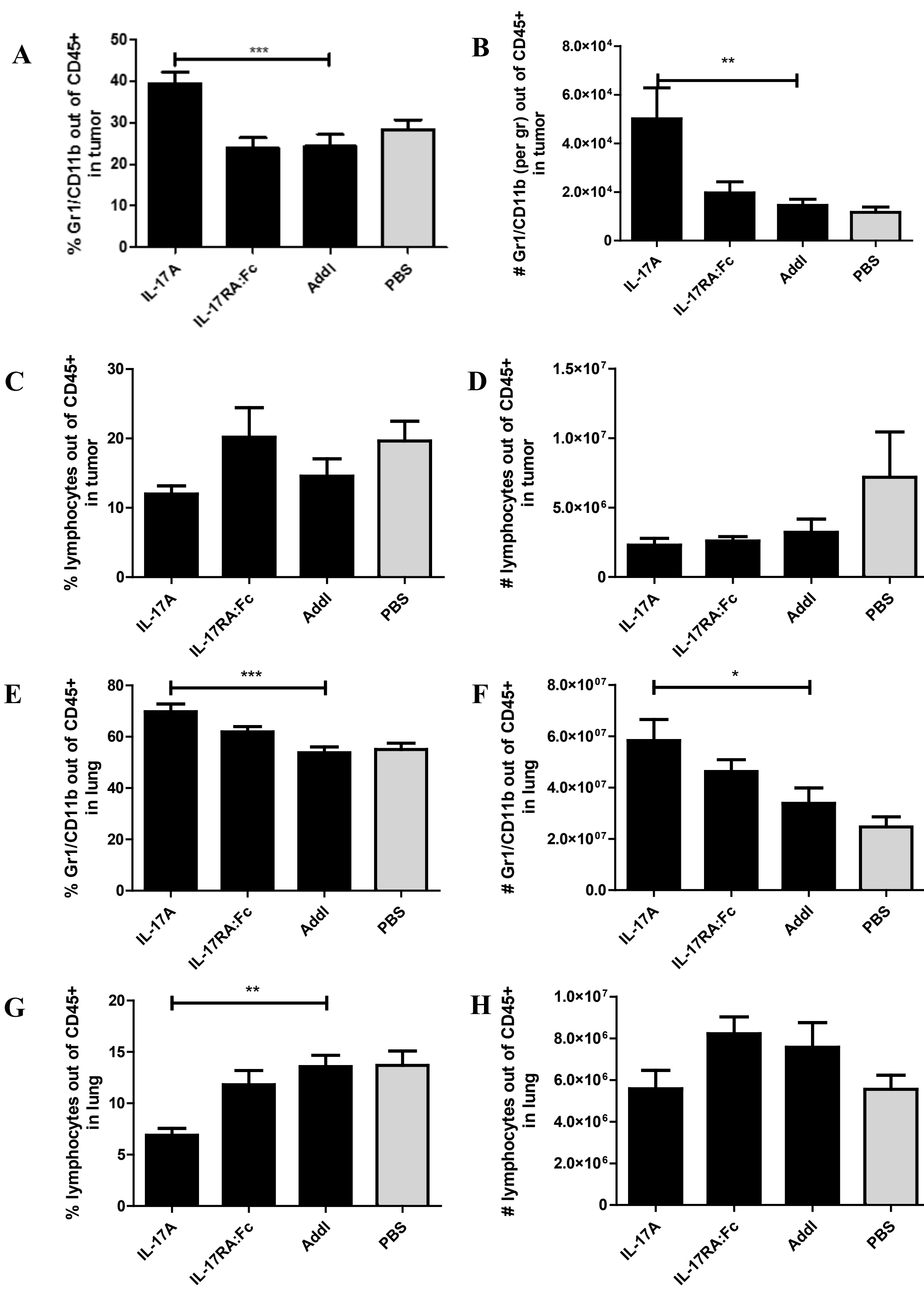


3.1.9. AdIL-17A transduction in 4T1 cells induces infiltration of $Gr1^{+}/CD11b^{+}$ cells within tumor and lung

The impact of AdIL-17A transduction in promoting expansion of MDSCs in PB and spleen prompted us to track these cells within primary and secondary tumor sites. Flow cytometric analysis revealed that the frequency and the absolute number of MDSCs were highly increased within the primary tumor site in the AdIL-17A group ($P<0.001$, $P<0.01$; **Figure 11A, B**). However, the total number of tumor-infiltrating lymphocytes was not different among groups, (**Figure 11C, D**). Similarly, MDSCs also significantly increased in frequency and total number within the secondary tumor site (the lungs) ($P<0.001$, $P<0.05$; **Figure 11E, F**). Conversely, a significantly reduced frequency and a trend for reduced total number of lymphocytes were found in the AdIL-17A group compared to the Addl group ($P<0.01$; **Figure 11G**). Collectively, it is conceivable that the infiltration of MDSCs in primary and secondary tumor sites contribute to enhanced tumor progression and lung metastasis in the AdIL-17A group.

Figure 11: AdIL-17A transduction in 4T1 cells induces enhanced infiltration of MDSCs within primary tumor and lung tissue site.

Tumors and lungs were collected at the day of sacrifice (D17), minced, digested, and single cell suspensions were stained for flow cytometry. **(A)** Frequency of tumor-infiltrating MDSCs after gating on $CD45^+$ cells (n=10 per group pooled from 2 independent experiments). **(B)** Total number of tumor-infiltrating MDSCs per gram of tumor (n=5 per group). **(C)** Frequency of tumor-infiltrating lymphocytes (T cells, B cells and NK cells) after gating on $CD45^+$ cells (n=5 per group pooled from 2 independent experiments). **(D)** Total number per gram of tumor-infiltrating lymphocytes (n=5 per group from single experiment). **(E)** Frequency of lung-infiltrating MDSCs after gating on CD45+ cells (n=5 per group pooled from 2 independent experiments). **(F)** Total number per gram of lung-infiltrating MDSCs (n=10 per group pooled from 2 independent experiments). **(G)** Frequency of lung-infiltrating lymphocytes (T cells, B cells and NK cells) after gating on CD45+ cells (n=9-10 per group pooled from 2 independent experiments). (H) Total number of lung-infiltrating lymphocytes (n=9-10 per group pooled from 2 independent experiments). Data are presented as the mean ± SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$ versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.

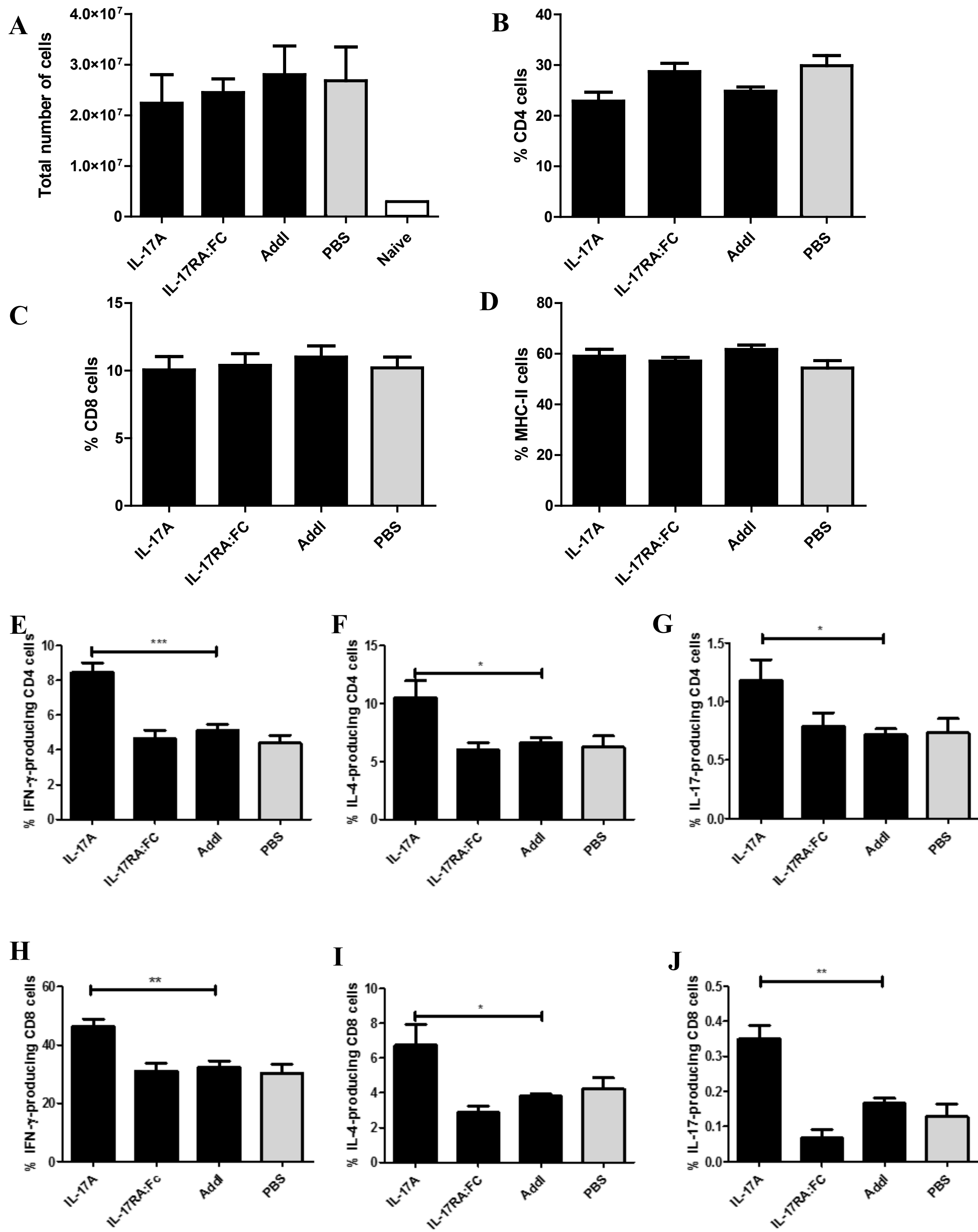

A
% Gr1/CD11b out of CD45+ in tumor
50
40
30
20
10
0
***
IL-17A
IL-17RA:Fc
Addl
PBS
B
Gr1/CD11b (per gr) out of CD45+ in tumor
8.0×10⁴
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
**
IL-17A
IL-17RA:Fc
Addl
PBS
C
% lymphocytes out of CD45+ in tumor
30
20
10
0
IL-17A
IL-17RA:Fc
Addl
PBS
D
lymphocytes out of CD45+ in tumor
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
IL-17A
IL-17RA:Fc
Addl
PBS
E
% Gr1/CD11b out of CD45+ in lung
80
60
40
20
0
***
IL-17A
IL-17RA:Fc
Addl
PBS
F
Gr1/CD11b out of CD45+ in lung
8.0×10⁰⁷
6.0×10⁰⁷
4.0×10⁰⁷
2.0×10⁰⁷
0.0
*
IL-17A
IL-17RA:Fc
Addl
PBS
G
% lymphocytes out of CD45+ in lung
20
15
10
5
0
**
IL-17A
IL-17RA:Fc
Addl
PBS
H
lymphocytes out of CD45+ in lung
1.0×10⁷
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
IL-17A
IL-17RA:Fc
Addl
PBS

3.1.10. AdIL-17A transduction in 4T1 cells induces enhanced-activation of T cell responses within TDLN

The observation that AdIL-17A transduction in 4T1 cells induced expansion/accumulation of immune suppressive MDSCs in multiple organ sites prompted us to examine the TDLN, the primary site for inducing anti-tumor immune responses. We found a remarkable swelling of the TDLN accompanied by increased cellularity in all of the groups (**Figure 12A**). Notably, the frequency of $CD4^+$ and $CD8^+$ T cells as well as $MHC\text{-}II^+$ APCs did not differ among the groups (**Figure 12B-D**). Moreover, there was no trace of $Gr1^+/CD11b^+$ myeloid cells in TDLN. However, intracellular cytokine staining assay revealed that the frequencies of IFN-γ, IL-4 and IL-17A-producing $CD4^+$ and $CD8^+$ cells were significantly greater in the AdIL-17A group compared to the Addl group ($P<0.001$, $P<0.01$, $P<0.05$; **Figure 12E-J**). Overall, our results demonstrate that AdIL-17A transduction in 4T1 cells induces enhanced-activation of CD4 and CD8 T cells in the TDLN without affecting tumor growth and lung metastasis.

Figure 12: AdIL-17A transduction in 4T1 cells induces enhanced activation of T helper responses within TDLN

TDLN were collected after 17 days post tumor injection. **(A)** Total TDLN cells (n=5 per group; naïve sample has n=1). **(B-D)** Frequency of CD4+, CD8+, and MHC-II+ APC cells within TDLN (n=5 per group pooled from 2 independent experiments). **(E-G)** Frequency of IFN-γ, IL-4 and IL-17A-producing CD4 cells within TDLN (n=5 per group pooled from 2 independent experiments). (H-J) Frequency of IFN-γ, IL-4 and IL-17A-producing CD8+ cells within TDLN (n=5 per group pooled from 2 independent experiments). Data are presented as the mean ± SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$ versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.


A
Total number of cells
4.0×10^7
3.0×10^7
2.0×10^7
1.0×10^7
0
IL-17A
IL-17RA:FC
Addl
PBS
Naive
B
% CD4 cells
C
% CD8 cells
D
% MHC-II cells
E
% IFN-γ-producing CD4 cells
***
F
% IL-4-producing CD4 cells
*
G
% IL-17-producing CD4 cells
*
H
% IFN-γ-producing CD8 cells
**
IL-17RA:Fc
I
% IL-4-producing CD8 cells
*
J
% IL-17-producing CD8 cells
**

3.1.11. AdIL-17A transduction induces CCR4 expression on 4T1 tumor cells and CCL17/CCL22 expression in the lung

The chemokine receptor CCR4 and its ligands CCL-17/CCL-22 have been shown to play an important role in 4T1 lung metastasis (155). We wondered whether this tissue-specific targeting mechanism was also induced in AdIL-17A-transduced 4T1 cells. Consistent with the hypothesis, 4T1 cells recovered from the lungs exhibited a significant increase in the frequency of $CCR4^+$ cells in the AdIL-17A group compared to the Addl control ($P<0.01$; **Figure 13A, B**). *In vitro*, 4T1 cells transduced with AdIL-17A had a significantly increased the frequency of $CCR4^+$ cells at 48 hrs post-transduction ($P<0.01$; **Figure 13C**), indicating a direct role of AdIL-17A transduction in promoting CCR4 expression in 4T1 cells. Next, we examined the expression of CCR4 ligands (CCL17 and CCL22) in the lungs of tumor-bearing mice 12 days after tumor inoculation. Both ligands were increased in the AdIL-17A group, although significance was only observed with CCL17 expression ($P<0.05$; **Figure A 5A, B**). Furthermore, stimulation of endothelial cell line bEnd.3 cells with recombinant IL-17A for 24 hrs significantly induced the levels of CCL17 and CCL22 mRNA compared to the PBS control ($P<0.0001$; **Figure A 5C, D**). These results suggest that AdIL-17A-transduction of 4T1 cells has the ability to induce CCR4 on 4T1 tumor cells directly and the corresponding ligands CCL17 and CCL22 at the lung tissue sites, thus, collectively promoting lung metastasis.

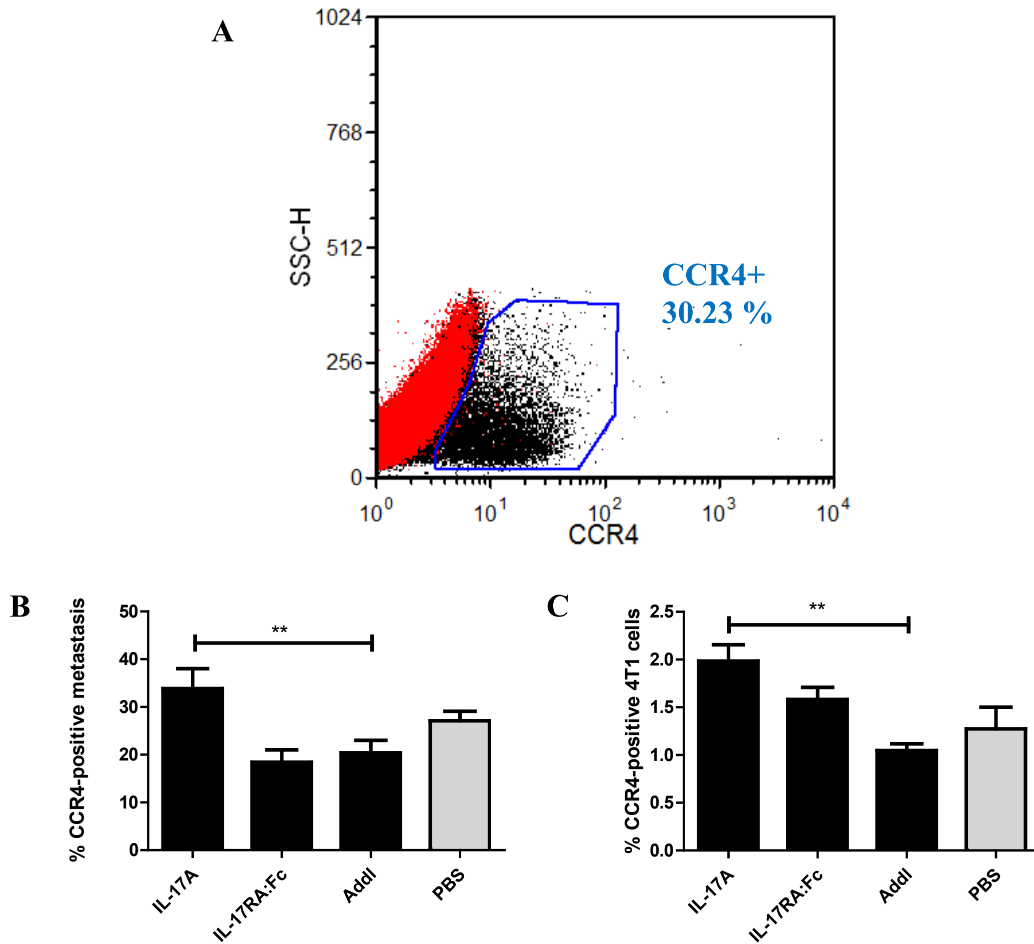


Figure 13: IL-17A induces lung metastasis via induction of CCR4 expression on 4T1 tumor cells.

(**A**) Representative dot plots showing $CCR4^+$ cells on lung metastasis; black dots (sample stained with PE-conjugated anti-CCR4 mAb), red dots (same sample stained with PE-conjugated isotype control) (**B**) CCR4 expression on metastatic colonies in the lungs was measured by flow cytometry (n=10 per group pooled from 2 independent experiments). (**C**) CCR4 expression on adenovirus-transduced and non-transduced 4T1 cells after 48 hrs of culture was measured by flow cytometry (n=5 per group from single experiment). Data are presented as the mean ± SEM. $^{**}P<0.01$; versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.

3.2. Primary Tumor with Ad-Injection Model

3.2.1. Intratumoral injection of AdIL-17A significantly induces tumor growth

An alternative model system to examine the role of the IL-17/IL-17R axis in breast cancer was developed as a part of my research. In this model, we sought to test the effects of AdIL-17A, or AdIL-17RA:Fc, on tumor growth and lung metastasis when injected locally prior to surgical resection of the tumor. Although the tumor was only exposed to the Ad vector treatment for 5 days, direct intratumoral injection of AdIL-17A significantly increased the size of tumor compared to the Addl control ($P<0.01$; **Figure 14**). This result, in conjunction with our previous model, collectively suggests a pro-tumor role of AdIL-17A, regardless of the route of induction.

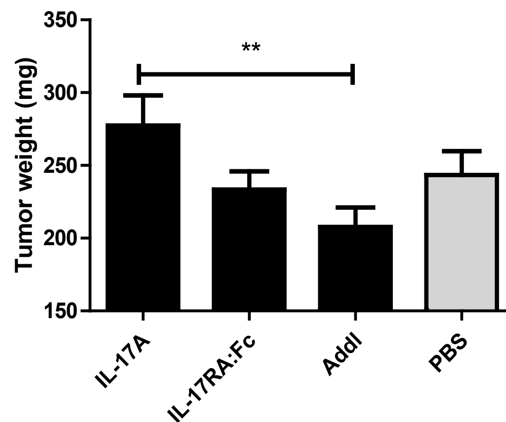


Figure 14: Intratumoral injection of AdIL-17A increases tumor growth

Mice were injected into the fourth mammary pad with $2x10^5$ cells of 4T1 cells. After 7 days tumors were injected with $5x10^8$ pfu of adenovirus vectors (AdIL-17A, Ad IL-17RA:Fc, Addl) or PBS^{++}. Tumors were resected surgically after 5 days of adenovirus treatment and weighed. (n=6 per experiment pooled from four independent experiments). Data are presented as the mean ± SEM. *$P<0.05$; **$P<0.01$ versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.

3.2.2. AdIL-17R:Fc treatment markedly increases lung metastasis after surgery

Treatment of metastatic breast cancer usually follows resection of primary tumor, which is the main source of metastasis. In our treatment model, established tumors were treated with adenovirus vectors or PBS prior to resection. To our surprise, treating mice with the IL-17A decoy receptor (AdIL-17RA:Fc) prior to resection significantly increased lung metastasis compared to the Addl group (**Figure 15**). In comparison, AdIL-17A injection in this model did not significantly affect lung metastasis. Collectively, these results demonstrate a paradoxical protective role of IL-17RA-mediated signal in controlling surgery-induced lung metastasis.

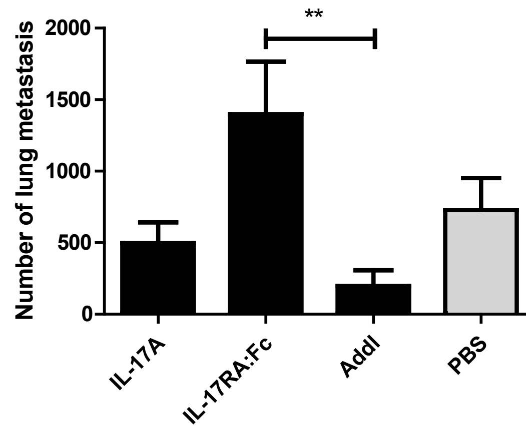


Figure 15: AdIL-17R:Fc treatment increases lung metastasis following surgery

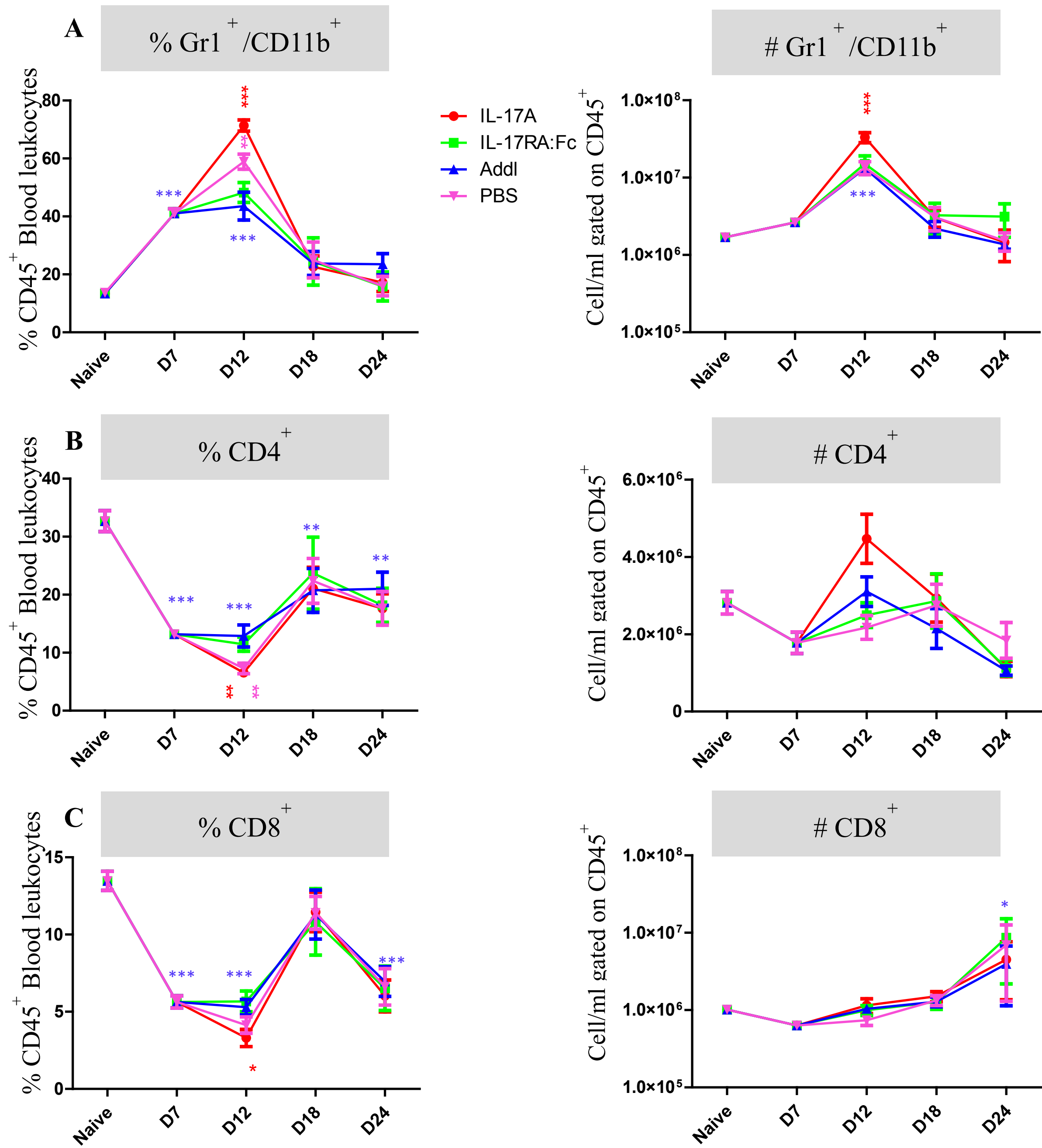
Tumors were injected with $5x10^8$ pfu of adenovirus vectors (AdIL-17A, Ad IL-17RA:Fc, Addl) or PBS^{++}. Tumors were surgically resected 5 days after Ad vector treatment. Number of lung metastasis was examined 26 days after tumor injection (16 days after resection surgery). Lungs were minced, digested and cultured in complete RPMI supplemented with 6-thioguanine, and metastasis colonies were counted 10-14 days later (n= 12-14/group from 3 independent experiments). Data are presented as the mean ± SEM. ***P*<0.01 versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis.

3.2.3. Intratumoral injection of AdIL-17A induces leukemoid reaction and resecting tumor reverses this reaction

Having studied the immune profile in the transduction model, we conducted a similar analysis in the tumor resection model. Our results showed that, $Gr1^+/CD11b^+$ cells significantly increased in frequency and number within 7 days post tumor injection ($P<0.001$; **Figure 16A**). Both the frequency and the total cell number increased significantly in the AdIL-17A group compared to the Addl group 5 days after treatment. Remarkably, this increase was quickly reversed to normal levels following tumor resection. In sharp contrast to the increased frequency of $Gr1^+/CD11b^+$ cells, the frequency of $CD4^+$ and $CD8^+$ T cells decreased significantly in the AdIL-17A group, but returned to normal levels after tumor resection. Throughout the course of experiment, we did not see a significant change in total number of $CD4^+$ and $CD8^+$ T cells (**Figure 16B, C**). Collectively, our results indicate that the IL-17/IL-17R axis has a very complex role in tumor progression. IL-17R signal is likely required during early stage of cancer, but become detrimental during late stage of cancer.

Figure 16: Intratumoral injection of AdIL-17A induces leukemoid reaction and resecting tumor reverses this reaction

(A) Frequency and absolute number of Gr1+/CD11b+ cells**.** **(B)** Frequency and absolute number of CD4+ cells. **(C)** Frequency and absolute number of CD8+ cells. Data are presented as the mean ± SEM (n=7-14 mice per group pooled from 5 independent experiments). **P*<0.05; ***P*<0.01; ****P*<0.001 versus Addl (vertical red stars), and versus Day 0 within Addl group (horizontal blue stars), as determined by two-way ANOVA with Bonferroni post-hoc analysis.


A
% Gr1+/CD11b+
# Gr1+/CD11b+
IL-17A
IL-17RA:Fc
Addl
PBS
% CD45+ Blood leukocytes
Cell/ml gated on CD45+
Naive
D7
D12
D18
D24
B
% CD4+
# CD4+
C
% CD8+
# CD8+

Chapter 4. Discussion

Inflammation is an essential component of the immune response to infection and damage; however, chronic inflammation can be harmful and plays a role in the pathologies of many diseases, including cancer (152). Inflammation is capable of influencing all steps in tumor development, from initiation up until metastasis (152). In keeping with this role, some clinical trials have shown that the use of non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of breast cancer, indicating an important role of inflammation in tumor progression (278). IL-17A, which is a pro-inflammatory cytokine, has been linked to cancer based on its increased levels in the tumor microenvironment of different human and mouse cancers. IL-17A's function – and possible role in cancer – remains unclear. Although some reports suggest that IL-17A has a pro-tumor effect (279, 280), others have shown that it might support T cell-mediated tumor rejection (250, 281).

Studying the pro- and anti-tumor roles of IL-17A in animal models requires a reliable method for blocking its signaling pathway. Some studies have used IL-17A$^{-/-}$ mice to show the effect of endogenous IL-17A production (282), while others have used IL-17R$^{-/-}$ to eliminate the effects of IL-17 on cells (155, 170). In contrast, other studies have used different methods to neutralize IL-17A. Use of anti-IL-17A mAb or anti-IL-17R mAb can neutralize or block the effect of IL-17A cytokine, respectively (242). Furthermore, the extracellular region of IL-17R fused to IgG1 Fc can neutralize IL-17A cytokines (283). In mice, IL-17RA:Fc will block IL-17A, IL-17F, and IL17A/F, whereas IL-17RC:Fc will block only IL-17F and IL-17A/F (242). However, in humans, IL-17RA:Fc will block only IL-17A and, to a lesser degree, IL-17A/F, but not IL-17F, which can be blocked by IL-

17RC:Fc (242). Finally, another methodological approach is preventing the assembly of IL-17R is by using soluble peptides possessing the pre-ligand assembly domain (PLAD) of the targeted receptor (284).

Our laboratory focuses on studying the role of IL-17/IL-17RA axis in tumor development using B16 melanoma and 4T1 mammary carcinoma. To achieve our objective, we used adenovirus vectors as a gene delivery system to insert an IL-17A-encoding construct (to perform IL-17A-gain-of-function) or an IL-17RA:Fc-encoding genes (to perform IL-17A-loss-of-function) into cancer cells. The insertion of these genes within tumor cells will alter the levels of biologically active IL-17A within the tumor microenvironment. Using the same method, our laboratory has shown previously that over-expression of IL-17RA:Fc in B16 melanoma cells inhibited tumor growth by enhancing $CD4^+$ and $CD8^+$ T cell infiltration into the tumor site. This preliminary data have led us to hypothesise that the IL-17/IL-17R axis has a dominant role in mediating inflammation-induced tumor progression by subverting anti-tumor adaptive immunity.

In this study, we sought to confirm our findings in the 4T1 mouse mammary carcinoma model of breast cancer, and define the molecular and cellular mechanisms underlying the pro-tumor role of IL-17/IL-17R axis. The genes, responsible for IL-17A- or IL-17RA:Fc-production, were delivered into 4T1 cells by two routes: via adenovirus-transduction of 4T1 cells *in vitro* prior to injection, and via Ad-injection post 4T1 inoculation.

The 4T1 mammary carcinoma is a triple negative cell line (ER^-/PR^-/$HER2^-$) (285, 286), syngeneic in BALB/c mice. Orthotopic inoculation mimics human breast cancer with its highly metastatic properties involving the same target organs. While several

studies have shown that IL-17A has a pro-tumor role in the 4T1 mammary carcinoma model, with increasing levels of IL-17A during tumor progression, the mechanisms involved are not well-defined (245, 287, 288). Du *et al* (288) have shown that ectopic IL-17A has the ability to induce 4T1 tumor growth by promoting microvessel formation in tumor tissues, but the role of angiogenesis in tumor development itself is controversial because it opens the route for both anti- and pro-tumor immune cells to infiltrate within the tumor microenvironment. To date, our knowledge of the mechanisms by which the IL-17/IL-17R axis induces tumor progression remains basic. This is the first study to examine the involvement of IL-17A in innate and adaptive immune response in conjunction with tumor growth and metastasis in the 4T1 mammary carcinoma model.

4.1. Primary Tumor with Adenovirus-transduction Model

In our first model, the 4T1 cells were transduced prior to injection with AdIL-17A, AdIL17RA:Fc, or control virus Addl. As anticipated, AdIL-17A-transduced 4T1 cells produced high levels of IL-17A within tissue culture, compared to low – but detectable – levels of IL-17A in the control group. Although 4T1 cells are able to produce IL-17A in small amounts, Du *et al* (288) have shown that the main source of IL-17A within 4T1 tumors is TILs, with levels increasing during tumor progression. IL-17A has a strong effect on both tumor and stromal cells due to the ubiquitous expression of its heterodimeric receptor, IL-17R (85). The activation of NF-κB and MAPK as part of the IL-17R signaling pathway leads to the promotion of several genes responsible for the production of pro-inflammatory cytokines, chemokines and growth factors (242, 85). Consistent with these studies, we have shown that IL-17A produced from 4T1 cells positively stimulated these cells to produce several other cytokines including G-CSF, GM-CSF, M-CSF, IL-6, IL-10

and TNF in an autocrine manner (**Figure A 2**). These induced cytokines have several biological effects some of which are pro-tumor effect.

4.1.1. Tumor growth

4T1 cells transduced with different adenovirus vectors exhibited similar proliferation and viability *in vitro* compared to the non-transduced cells. Consistent with other studies (288), these data suggest that the adenovirus-vectors, IL-17A, and IL-17RA:Fc (as well as other stimulated cytokines) do not interfere with the cell-division or induce cell death through apoptosis or necrosis. In contrast to our finding, Nam *et al* (245) reported that IL-17A has a pro-survival effect on 4T1 cells by reducing apoptosis, an effect that synergizes with TGF-β. However, proliferation of adenovirus-transduced 4T1 cells did differ *in vivo*, suggesting that AdIL-17A-transduction within 4T1 tumors favors tumor growth via indirect mechanisms. As IL-17A-transduction induced expression of other cytokines, it is likely that these play a role in tumor development.

4.1.2. Lung metastasis

In addition to the ability of 4T1 cells to grow locally at the injection site, these cells are well known for their ability to metastasize to different organs including the lungs, liver, bone marrow, and brain within 1-2 weeks of implantation (289, 290). Broadly, several factors can affect the metastatic process at various points, including evading the immune system, invading the surrounding tissues, surviving in circulation, and colonizing targeted organs. With this in mind, the role of the IL-17A/IL-17R axis within the metastatic process was assessed and we observed a four-fold increase in lung metastasis in the IL-17A group as compared to the Addl group and the IL-17RA:Fc blocker group (**Figure 6B**).

One mechanism implicated in targeting metastasis to specific organs is homing via chemokine receptor/ligand interactions. Olkhanud *et al* (155) have shown that, only a proportion of the 4T1 cells that express CCR4 can metastasize to lungs, and that this depends on increased expression of the corresponding ligands, CCL17 and CCL22, in the lungs. These chemokines (CCL17 and CCL22) also attract CCR4$^+$ T_{regs}, which can kill NK cells directly using β-galactoside-binding protein, thus enhancing the 4T1 cells ability to survive the immunosurveillance response (155). To explore this relationship, we investigated the role of IL-17A on CCR4 and its ligands (CCL17 and CCL22) in our model. Only a proportion of metastases recovered from the lungs were CCR4$^+$ (20-40%), which indicate that they might have lost the expression of CCR4 within culture. However, AdIL-17A group exhibited a higher proportion of CCR4$^+$-4T1 cells *ex vivo* (**Figure 13A**). Moreover, we found that IL-17A and other stimulated cytokines *in vitro* directly increase the proportion of 4T1 cells that are CCR4$^+$ (**Figure 13B**). Correspondingly, we found that lungs of the IL-17A group have significantly higher levels of the CCR4 ligands CCL17 and CCL22 (**Figure A 5A, B**). Our *in vitro* results show that IL-17A has the ability to stimulate endothelial cells directly to express CCL17 and CCL22 (**Figure A 5C, D**). Beside the positive correlation between IL-17A and the CCR4/ CCL17, CCL22 axis, other studies have shown that IL-17A is also essential to induce different chemokines, such as CXCL1, CXCL2 and CXCL5 within the inflammatory sites (291). This in turn mediates the recruitment of neutrophils, which are associated with a poor prognosis in several types of cancer (292). Collectively, our data suggest that IL-17A is one of the essential components required for the metastatic process, via its direct role in stimulating the expression of the

chemokine receptor CCR4, its requirement for 4T1 metastasis and T_{regs} chemo-attractant to lungs, as well as its ligands (CCL17 and CCL22) in targeted organs.

4.1.3. Immune response

One of the remarkable properties of the 4T1 mammary tumors in BALB/c mice is the induction of a leukemoid reaction, wherein myeloid cells outside the bone marrow (268). This reaction has been reported not only in animal models but also in human cancers (293 - 296). Although the leukemoid reaction happens rarely in breast carcinomas (297), it has nonetheless been associated with a bad prognosis (298). $Gr1^+/CD11b^+$ cells are a heterogeneous population composed of granulocytic ($CD11b^+Ly6C^{low}Ly6G^+$) and monocytic ($CD11b^+Ly6C^{high}Ly6G^-$) populations. In animal tumor models, an excess of granulocytes in the host is thought to induce metastasis (299). Wu *et al* (300) have shown that human neutrophils assist the human breast tumor cell line MDA-MB-231 in trans-endothelial migration *in vitro*, thus enhancing metastasis. In the literature, IL-17A is well known to have a potent effect on granulopoiesis and neutrophil recruitment through induction of G-CSF, GM-CSF, M-CSF, and IL-6-dependent and independent mechanisms (170, 301, 302, 242, 303). Moreover, Marigo *et al* have shown that GM-CSF, G-CSF, and IL-6 allow the generation of MDSCs *in vitro* from mice and human bone marrow precursors (304).

Consistent with these studies, we have shown that IL-17A induces 4T1 cells to produce G-CSF, GM-CSF, M-CSF, IL-6, and IL-10 in an autocrine manner (**Figure A 2**). Although these cytokines are secreted to high levels in tissue culture, only G-CSF was found to be significantly higher in serum samples in the AdIL-17A-transduced group (**Figure A 4**). G-CSF is a potent stimulator of granulopoietic progenitors in bone marrow

and extra-medullary organs to increase production of granulocytes (303, 305, 306). As expected, IL-17A has been shown to induce an early and rapid expansion of myeloid cells ($Gr1^{+}/CD11b^{+}$) in PB and the spleen, most of which were granulocytes that have possibly expanded under the influence of G-CSF.

It has been reported that $Gr1^{+}/CD11b^{+}$ cells exhibit suppressive activity against T cells in a number of diseases, including cancer, inflammation and trauma, such that they have been termed myeloid-derived suppressor cells (MDSCs) (306). In our study, these cells increased excessively in PB – especially in the IL-17A group – where they exhibited an increased suppressive activity after two weeks of tumor inoculation compared to the Addl group. However, both granulocytic and monocytic populations in the spleen were able to suppress T cell proliferation (monocytes were more potent), and this effect was observed across all transduction groups. These data suggest that the increased suppressive activity in the AdIL-17A group was due to an increase in the quantity of MDSCs, and not a result of alterations in their quality among the groups.

The increased frequency and absolute numbers of MDSCs in PB and spleen in the IL-17A group were accompanied by increased infiltration of these cells within the tumor microenvironment and in the lungs compared to the Addl group. As discussed earlier, the infiltration of these MDSCs in primary and secondary tumor sites could be due to IL-17A-dependent-induction of chemokines that can attract myeloid cells to inflammatory sites, such as CXCL1, CXCL2 and CXCL5 (291, 292). The increased infiltration of MDSCs within primary and secondary tumors could be one of the mechanisms by which IL-17A promotes tumor growth and metastasis. Furthermore, there have been reports that MDSCs can cross talk with T_{regs}, which are also immunosuppressor cells that favor pro-tumor

effects (307). In addition, naïve T cells differentiate into T_{regs} in the presence of TGF-β, which can be produced by MDSCs (308); however, with the presence of IL-6 and IL-1β, both naïve T cells and T_{regs} can differentiate and polarize to become Th17 (309). We have also shown that IL-17A is able to stimulate 4T1 cells to produce TNF *in vitro*. It has been reported that TNF-α can support T_{reg} proliferation through TNFR2, which is highly expressed on these cells (310, 311). Together with MDSCs, T_{regs} are hypothesised to contribute to the establishment of an immunosuppressive tumor microenvironment in our model. However, the effect of AdIL-17A-transduction on T_{regs} needs to be clarified in the future.

Arising from the importance of TDLN in the anti-tumor response, we investigated the effects of AdIL-17A on the immune profile within TDLN. Although blood-borne neutrophils are excluded from tracking within lymphatic circulation, there have been some studies indicating that neutrophils can accumulate in lymph nodes following pathogen-mediated (312), and tumor-induced inflammation (313). However, our data showed contradictory results as there were no trace of myeloid cells ($Gr1^+/CD11b^+$) within TDLN in all groups. Moreover, there was no difference among the groups in the frequency and absolute number of lymphocytes as well as $MHC\text{-}II^+$ APCs. Although the frequency and absolute number of $CD4^+$ and $CD8^+$ T cells were comparable among groups, the frequencies of $CD4^+$ effector cells (Th1, Th2, and Th17) and $CD8^+$ effector cells (Tc1, Tc2, and Tc17) were significantly higher in the AdIL-17A-transduced group.

Some of these activated cells, like Th1 and CTLs (Tc1), are well characterized to have an anti-tumor response, which was consistent with studies that have shown an anti-tumor role of IL-17A in some types of cancer through the induction of CTLs (250). IL-

17A is able to increase IL-6 production in the tumor microenvironment, and IL-12 secretion from macrophages; both IL-6 and IL-12 activate specific tumor-induced CTL (314). However, although IL-17A induced the frequency of IFN-γ-producing cells (Th1 and Tc1) within TDLN, the levels of IFN-γ in periphery were low in the AdIL-17A and AdIL-17RA:Fc groups. The decrease in IFN-γ levels in the IL-17RA:Fc group could be due to blocking of the IL-17A signalling pathway. This is consistent with a previous study by Kryczek *et al* (282), which has shown that endogenous IL-17A is essential for tumor specific T-cell immunity using an MC38 colon cancer cell line in C57BL/6 mice.

4.1.4. Conclusion

The summary of the primary tumor with adenovirus-transduction model is outlined in **Figure 17**. IL-17A is able to induce synthesis of several hematopoietic cytokines, including G-CSF, GM-CSF, and M-CSF directly from tumor cells. Together, these factors can stimulate bone marrow to increase production of myeloid cells, which can proliferate in other organs like the spleen. Along with other cytokines like IL-6, IL-10, and TNF that are also induced directly by IL-17A, these factors can promote tumor proliferation (315 - 317), and increase MDSC accumulation and function (318). The infiltration of these MDSCs within primary tumors may enhance tumor growth through inhibition of the anti-tumor responses. Moreover, the infiltration of MDSCs within several other organs, such the lungs, could make the microenvironment ideal for metastasis and evasion of the immune response, leading to formation of secondary tumors. Furthermore, the induction of CCR4 on tumor cells and its ligands (CCL17 and CCL22) in the lung, could be one of the mechanisms by which IL-17A facilitates the homing of 4T1 cells into lungs (targeted organs), and thus enhanced formation of secondary tumors.

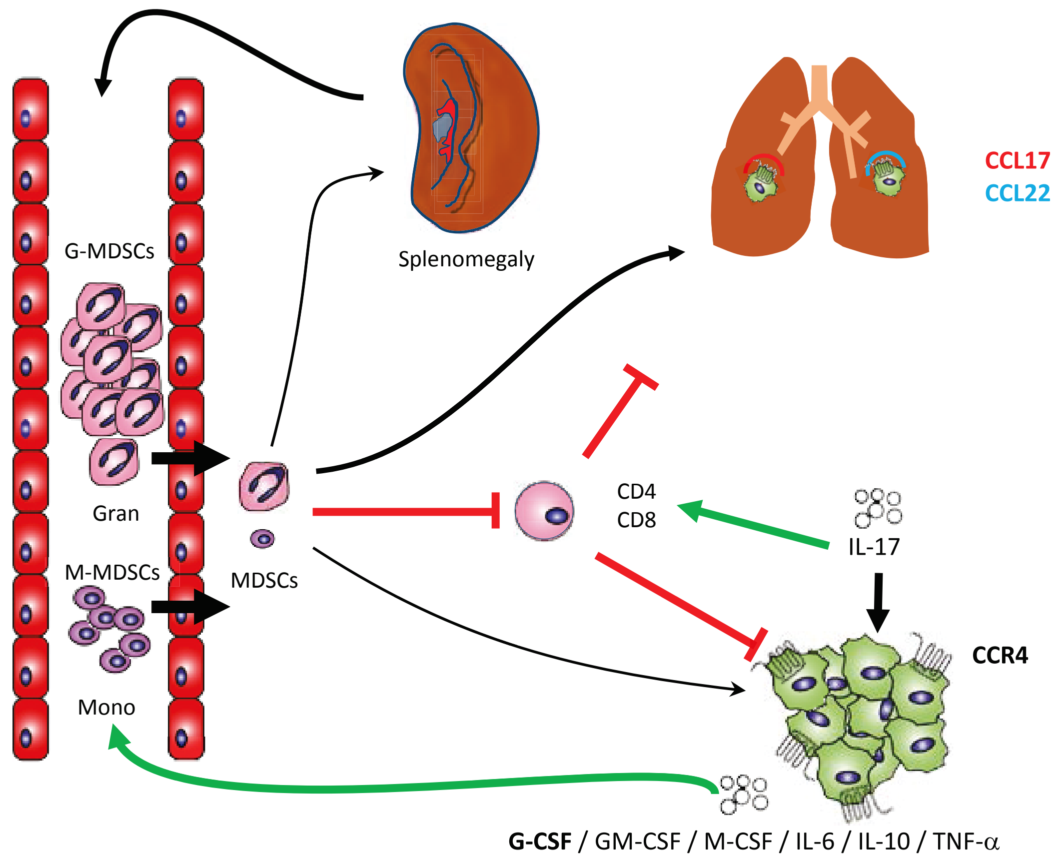


Figure 17: Schematic summary of biological effects of AdIL-17A-transduction in 4T1 tumor microenvironment

IL-17A overproduction within 4T1 tumor microenvironment induces tumor growth through different mechanisms. IL-17A induces tumor cells to produce several cytokines, like G-CSF, GM-CSF, M-CSF, IL-6, IL-10 and TNF, as well as chemokine receptor CCR4. These factors favor the induction of tumor growth. Remotely it increases the expression of CCL17 and CCL22 (CCR4 ligands), which chemoattract CCR4^{+} 4T1 cells into the lung. IL-17A and the stimulated cytokines induce the expansion of MDSCs in peripheral blood with increased infiltration of these cells within the spleen, the tumor, and the lung. Although, IL-17A is able to increase the frequency of activated effector CD4^{+} and CD8^{+} T cells, some of which have anti-tumor effects, in the TDLN, the increase of suppressor cells like MDSCs can subvert their effects within primary and secondary tumor sites.

4.2. Primary Tumor with Adenovirus-injection Model

Several recent reports based on clinical trials and animal models have suggested that the combination of immunotherapy with other treatments, like surgery, could have a beneficial role in eliminating tumors (319 - 321). It should be no surprise that combining surgery with an anti-tumor immunotherapy can have a better outcome than surgery alone (322). Similar to the controversy about the role of IL-17A in tumor progression (sometimes in the same animal model) (228, 323), there has been also contradictory results regarding the effectiveness of primary tumor resection in eliminating the metastatic disease (324 - 326). Fisher *et al* (324) have shown that the stress caused by surgical resection of a primary tumor could induce metastasis proliferation. Moreover, Folkman *et al* (325) have suggested that some primary tumors are able to reduce the growth of their metastasis by inhibiting angiogenesis; thus, the resection of these tumors can cause rapid tumor recurrence or explosive growth of metastases. In contrast to these studies, Rashid *et al* (326) have shown that resection of the primary tumor improves survival by reducing the overall tumor burden, preventing the primary tumor from shedding more metastatic cells.

Recent clinical trials have combined immunotherapy with conventional tumor therapies, such as using IL-2 and IFN-α in the reducing tumor burden of renal cell carcinoma prior to surgery (322). Arising from the controversy in both the role of primary tumor resection and IL-17A in tumor progression, we used a resection model to test the role of AdIL-17A or its IL-17RA:Fc blocker in the context of combined immunotherapy and surgery.

4.2.1. Tumor growth, metastasis, and immune profile

We found that intratumoral injection of AdIL-17 induced primary tumor progression compared to Addl group, but did not increase metastasis. Surprisingly, the highest metastatic rate was observed in the IL-17RA antagonist group. These results differed from those seen in the absence of tumor resection, suggesting that the mechanisms underlying the metastatic process may differ between models.

Having observed an excess IL-17A-mediated expansion of myeloid cells, which function as MDSCs in the transduction model, we investigated the kinetics of myeloid and lymphoid cells in the resection model. As anticipated, intratumoral injection of AdIL-17A significantly increased the frequency and numbers of myeloid cells in the PB. However, after surgery, the level of these cells returned to normal values. In contrast, the frequency of $CD4^+$ and $CD8^+$ cells significantly declined after one week of tumor inoculation then declined further after injection of AdIL-17A and PBS compared to the control group and IL-17RA:Fc group, and then returned to normal levels after surgery. Rashid *et al* (326) reported similar results in a 4T1 tumor model, showing that surgery to remove primary tumor was able to decrease MDSCs, increase $CD4^+$ and $CD8^+$ T cells, and improve survival.

We know that 4T1 tumors can start to metastasize within one week following tumor transplantation, so by the time of surgery (day 12) all groups are expected to have metastasis to distant organs.

Treatment with AdIL-17A boosted the production of $Gr1^+$/$CD11b^+$ cells, but these cells might not have been fully transformed into MDSCs. The likely reason, as supported by several reports regarding the plasticity of MDSCs (60), is that tumor resection removed

source of IL-17A as well as other cytokines and growth factors required for development of MDSCs. These reports suggest that the immunostimulatory or immunosuppressive functions of MDSCs depend largely on tumor-derived cytokines (60). It may also be that chronic vs acute inflammations produce opposing functions by MDSCs (60). In fact, chronic inflammation is more accepted to have a pro-tumor effect, and favors the induction of MDSCs (61). In contrast, although acute inflammation might show increased levels of MDSCs like in sepsis (327), and viral infections (328), there have been reports that acute inflammation could favor the induction of antigen presentation, thus making myeloid cells less likely to become MDSCs (60). For instance, several reports have shown that IL-12, IFN-γ, and TNF-α can transform MDSCs into APCs (329, 330).

4.2.2. Conclusion

The summary of the primary tumor with adenovirus-injection model is outlined in **Figure 18**. The injection of AdIL-17A with consecutive surgery might have induced a transient acute inflammation that converted myeloid cells into APCs but not into MDSCs before their level went back to normal, thus enhancing the immune system and ultimately reducing lung metastasis. Moreover, as seen previously, IL-17A significantly increased the frequency of effector T cells; some of these cells have anti-tumor effects (e.g., Th1 cells and CTLs), which were suppressed due to the existence of MDSCs. However, primary tumor removal removed the source of several immune suppressive and tumor-inducing factors. Thus, the anti-tumor immune cells, which have encountered tumor antigens and been activated within the TDLN, are no longer suppressed and are able to eliminate the circulating or infiltrating tumor cells in distant organs. On the other hand, blocking IL-17A in the Ad-IL17RA:Fc group leads to low numbers of myeloid cells that can become APCs

after surgery, as well as low levels of IFN-γ-producing cells, such that after tumor removal the immune response might not be sufficient to eliminate the metastatic cells, which resulted in high metastasis.

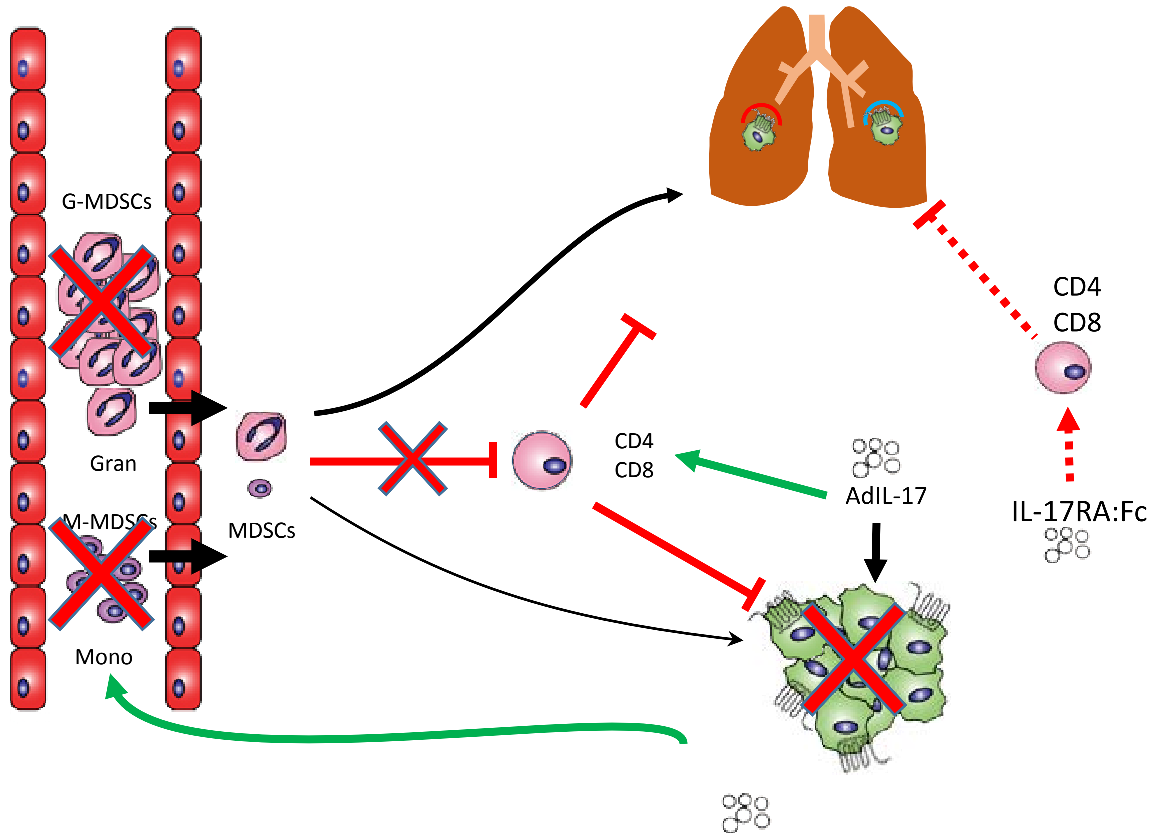


Figure 18: Proposed model of AdIL-17A/AdIL-17RA:Fc immunotherapy in combination with surgery

AdIL-17A injection within a 4T1 tumor microenvironment plays a pro-tumor role that induces tumor growth and expansion of MDSCs in peripheral blood. However, surgical resection of the tumor and the TDLN reduces the frequency and absolute number of MDSCs to their normal levels, leading to the elimination of suppression on activated effector $CD4^+$ and $CD8^+$ T cells, which may lead to reduced lung metastasis. In contrast, blocking IL-17A exhibits higher rates of lung metastasis, which is likely the result of reduced activation of effector $CD4^+$ and $CD8^+$ T cells.

4.3. Study limitations

4.3.1. Mouse model

Mouse models have offered a tremendous insight into the mechanisms underlying breast cancer progression and metastasis. However, despite the convenience of mouse models in the basic cancer research and in challenging several treatments, there are several limitations of these models. Obviously, the mechanisms underlying tumorigenesis are different between human and mouse, such as the differences in the cell-intrinsic and cell-extrinsic factors that control cancer development. An example of the cell-intrinsic factors is the increased activity of the telomerase enzyme in mice (331). Thus, cells transform more easily and require fewer mutations for malignant transformations compared to humans (331). Regarding the cell-extrinsic factors, there are substantial differences between mice and humans in the immune system development, composition, activation, and response to stimulus in both innate and adaptive responses. The distribution of lymphocytes and neutrophils between mice and humans is quite different; human is neutrophil rich (50–70% neutrophils, 30–50% lymphocytes), whereas mice are rich in lymphocytes (75–90% lymphocytes, 10–25% neutrophils) (55, 332). Besides that, BALB/c mice, which have been used in this study, generally have polarization of immune system toward Th2 compared to other strains like C57BL/6 mice (333); this might allow tumor cells to grow more progressively than in other strains.

4.3.2. *In vitro* vs *in vivo* characteristics

One of the major limitations in this study was the use of a mammary carcinoma cell line instead of primary cells. Cell lines are more adapted to culture environment, thus, they might lose tissue-specific functions and acquire a phenotype different from cells *in vivo,*

whereas primary cells (isolated from tumors) might still have an *in vivo*-suitable phenotype (334). In our model, although the use of 4T1 mammary carcinoma has some advantages in studying the late stage of breast cancer, it has several limitations. 4T1 mammary carcinoma is a triple negative cell line ($ER^-/PR^-/HER2^-$), which can mimic only triple-negative breast cancers, such as basal-like breast carcinoma, but they cannot be used to model other types of breast cancer that have variable levels of these receptors. Furthermore, 4T1 cells tend to be aggressive and grow rapidly over a short time frame, so their proliferation does not resemble most breast cancers. Moreover, 4T1 cells are passaged more than once in culture, so *in vitro* characteristics, such as proliferation, heterogeneity, and secretion of several factors, might not be similar to *in vivo* due to the lack of extracellular matrix that shapes the tumor cells. The same limitation applies to the study of MDSCs suppressive activity on TCR-transgenic T cells that are stimulated by OVA-II peptide *in vitro*. This stimulation is commonly accepted for *in vitro* proliferation and suppression assays; however, it represents a single method out of the complex interactions of T cells with MDSCs and other suppressor cells *in vivo*. Moreover, OVA-II peptide stimulates T cells in an antigen-specific manner, but we have not tested the effect of MDSCs suppressive activity on the proliferation of non-specifically activated T cells, like using αCD3 and αCD28 to stimulate T cells. Other cells that might be responsible of T cell suppression in tumor-bearing mice could be Tregs and TAMs; these cells can reciprocally cross talk with MDSCs to create a suppressive microenvironment. In our study, we did not focus on studying other immunosuppressor cells rather than MDSCs. However, we have shown that IL-17A has the ability to induce CCL17 and CCL22 in lung, which might attract $CCR4^+$-T_{regs} as well as $CCR4^+$-4T1 cells, but there might be other chemokine receptors worth to be studied as

well, such as CCR2, CCR7, and CXCR2, which are intermediate to attract MDSCs, Tregs, and TAMs, respectively (152 - 155).

4.3.3. Monocytes expansion after two weeks of tumor inoculation

The late expansion of monocytes in peripheral blood was not accompanied by an increase in the level of IL-17, GM-CSF, and M-CSF. As discussed above (see section 4.3.2), this could be to the short half-life of these cytokines in peripheral blood. This should be addressed in the future by investigating the effect of IL-17A on differentiation of bone marrow precursor cells collected from BALB/c mice.

4.3.4. CCR4 level on lung metastasis

Olkhanud *et al* (155) have shown that only $CCR4^+$ 4T1 cells can metastasize to lungs. However, in our transduction model, only a proportion of metastatic cells were $CCR4^+$ (20-40% of 4T1 cells), indicating that our cells might have lost the expression of CCR4 during the long time of incubation (10-14 days). The low number of tumor cells collected from lungs at the day of sacrifice, the lack of specific marker of tumor cells, and the expression of CCR4 on other cells (like T_{regs}) have prevented us from investigating the level of CCR4 on tumor cells directly after sacrifice.

4.3.5. Suppression assay

In this study, the suppression assay results were obtained from a single experiment representing a preliminary data. However, the use of OVA-II peptide to stimulate T cells can investigate the ability of MDSCs to suppress antigen-specific activated T cells, but does not investigate the suppression of MDSCs of non-specifically activated T. Moreover, we have not investigate the difference among groups in the level of APCs in the blood

samples; APCs, such as DCs, can also activate T cells that might interfere with the OVA-II peptide and the suppressive activity of MDSCs.

4.3.6. Activation of T cells in TDLN

IL-17A has shown to increase the frequency of activated effector T cells within the TDLN. Even though we did not find any increase in APCs within TDLN, but the possible mechanism for this increase still not investigated well. Increased frequency of activated T cells could be either due to local expansion of activated T cells or due to increased infiltration of previously activated T cells elsewhere. This might require further investigations to address the role of IL-17A on activation and proliferation of T cells.

4.3.7. Adenovirus vector

In this study, the adenovirus-vector that has been used is from a first generation vector that has some undeleted viral genes left. Although the vectors used in our models have no direct effect on 4T1 cell proliferation and viability *in vitro*, the empty vector reduced tumor burden significantly *in vivo*, which could be due to the induction of immunity against *de novo* synthesis of viral proteins (335). Another limitation of the adenovirus first generation is that it rarely integrates into the host genome, leading to a transient gene expression of the transferred construct. The transient-expression could be one of the explanations for not detecting high levels of IL-17A within the host after one week of inoculation with AdIL-17A-transduced 4T1 cells. Moreover, the expression might be inhibited by immune response, thus, in future work, we wanted to isolate tumor cells from *in vivo* and culture it *in vitro* then check IL-17A production. In addition, the existence of many cells that express IL-17R, might leads to an increase absorption of IL-17A locally.

Another suggestion could be due to a short half-life of the IL-17A *in vivo* compared to *in vitro* (336).

4.3.8. Blocking IL-17A

As discussed previously, there are several methods to block IL-17A, such as using neutralizing antibodies or using soluble receptor in order to block IL-17A/IL-17R interaction (see section 1.2.3.2). In our study, the use of IL-17RA:Fc to block IL-17A signalling pathway did not show significant biological effects compared to the control virus except for increasing metastasis in the surgery model. This raises questions about the efficacy of our method to block IL-17A.

4.3.9. Surgery

The surgical resection of tumors revealed contradictory roles for the IL-17A/IL-17R axis in lung metastasis as compared to the non-surgical model. However, surgery was not the only variable between the two models, limiting the ability to claim that the surgery alone was responsible for this observation. Although, both adenovirus transduction and injection in 4T1 cells and primary tumors, respectively, have shown a pro-tumor results regarding tumor growth, thus it could be the surgery or the adenovirus delivery method the variables that might have affected the metastatic results.

4.4. Future research

4.4.1. Mouse strains and mammary carcinoma

We have studied the effect of IL-17A using the 4T1 cells with BALB/c mice, thus, it is important to compare the effect of IL-17A on tumor progression using other strains, with syngeneic mammary carcinoma cells. For example, the E0771 cell line, syngeneic in C57BL/6 mice, could be used to test our methodology using a cell line that is ER-positive,

which might resemble several types of breast cancer that are affected by estrogen levels (337). Comparing 4T1 and E0771 not only investigate the difference between ER- and ER+ cell line, rather it will also help to identify the role of IL-17A in tumor progression between two strains that have different characteristics in the cell line used and the immune response.

4.4.2. Investigating other immune cells

In the current study, we have focused on the role of IL-17A on one population of suppressive cells, MDSCs. However, it might be important to address the role of IL-17A on other suppressor cells, such as Tregs and TAMs, and differentiate how they infiltrate within primary and secondary tumors as well as in lymphoid organs.

4.4.3. *In vitro* and *in vivo* suppression assays

In vitro evaluation of MDSCs has shown the ability to suppress antigen-specific activated T cells *in vitro*; this experiment has to be paralleled with the study of MDSCs suppressive activity on non-specifically activated T cells, such as activation with αCD3 and αCD28. Moreover, the suppressive activity of organ-infiltrated MDSCs and other suppressor cells must be compared, such as MDSCs infiltrated within tumor, lung, and spleen, to investigate the difference between them in the context of T cell suppression activity.

The suppressive activity can also be assessed *in vivo* by labelling T cells with an intracellular dye then adoptively transfer them to the tumor-bearing mice. The proliferation of labelled-T cells can be assessed by collecting blood at different time points and evaluating their proliferation depending on the reduction of the internal dye using flow cytometry. This will be more accurate to estimate the suppressive activities that are more complex *in vivo* rather than *in vitro*.

4.4.4. Design new vectors

The adenovirus that has been used in this model cannot integrate within the genome of host cells, leading to a transient gene expression of the transferred construct. This suggests that looking for other vectors that can favor longer-term of gene expression and induce less immune response, such as helper-dependent adenovirus or lentivirus (338, 339). Beside the long-term advantages of the third generation of the adenovirus, it is also less immunogenic due to the lack of all viral genome, thus, leading to less immune response toward the adenovirus-infected cells. In addition, it could be useful to use adenovirus encoding green fluorescent protein (GFP) to enable the tracking of viral infection within primary and secondary tumors, as well as in culture.

4.4.5. Perform sham surgery

Cancer surgery is still the preferential method in most solid tumors. However, the controversial outcome of surgery has been reported in several types of cancer (340, 341). In our model, surgery has changed the outcome of IL-17A vs IL-17A:Fc treatment from pro-tumor to anti-tumor and vice versa, respectively. In the future work, we should address the role of surgery itself using both models (Ad-transduction and Ad-injection). Surgery must be addressed within the transduction model; mice must undergo surgery at different time points and the metastatic numbers must be compared to mice that have not encounter surgery and others that have only sham surgery. In the treatment model, the experiment must be repeated also and mice have to be compared with and without surgery as well as doing sham surgery for some mice. This will enable us to evaluate the effect of surgical resection of IL-17A-enriched and IL-17A-low tumors on the metastatic process.

APPENDIX: SUPPLEMENTARY DATA

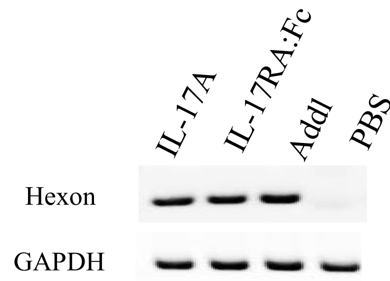


Figure A 1: *In vitro* characterization of 4T1 cells upon transduction with different adenovirus constructs

4T1 cells were divided into four groups, each group was transduced with a different adenovirus vector (AdIL-17A, Ad-IL17RA:Fc, or Ad170) with MOI=200 and one group only was treated with PBS^{++} as a control. Hexon and GAPDH DNA in adenovirus-transduced and non-transduced 4T1 cells. This experiment was conducted by Mr. Chi Yan.

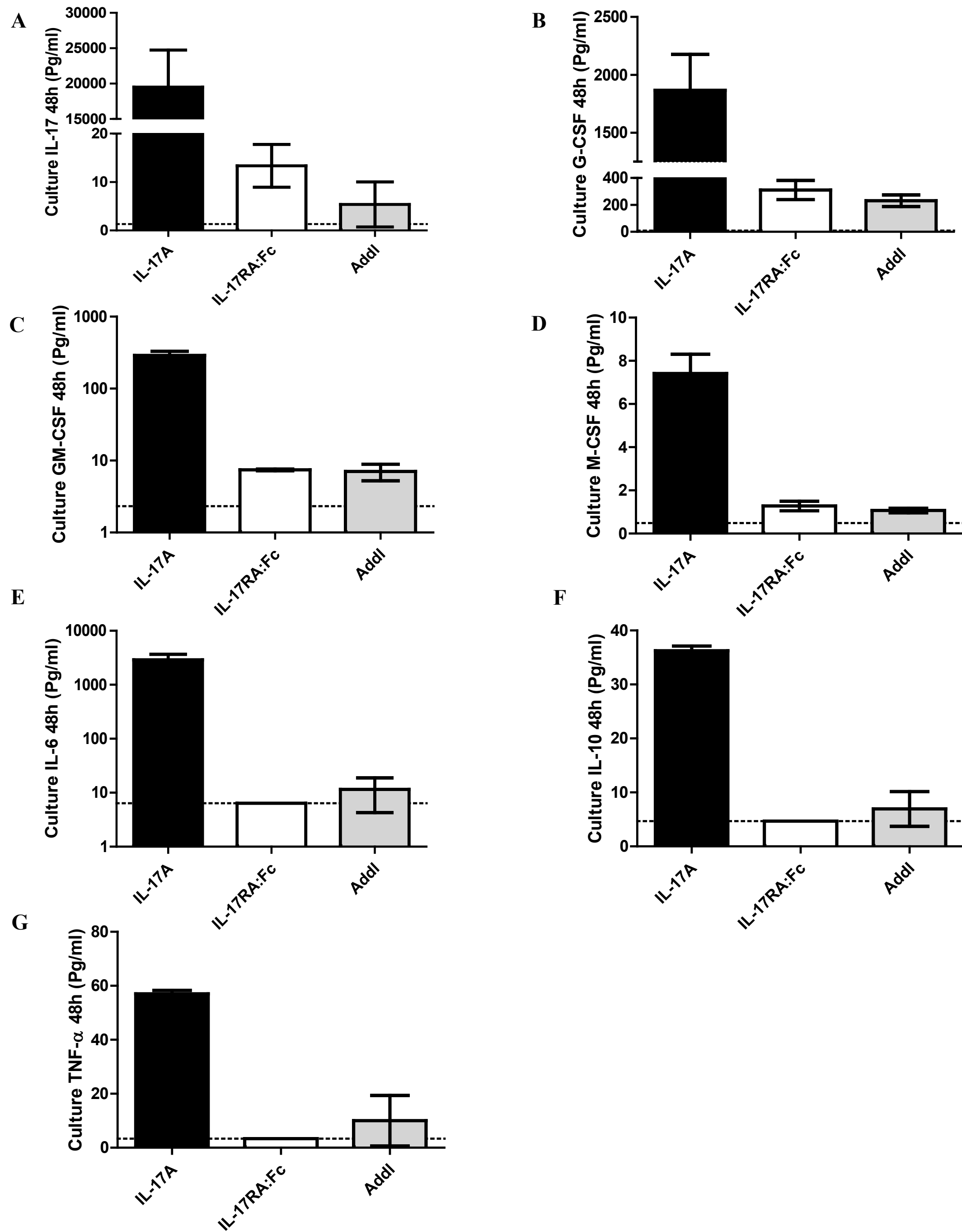


Figure A 2: AdIL-17A transduction in 4T1 cells induces cytokine secretion from 4T1 cells

(A-G) Levels of IL-17A, G-CSF, GM-CSF, M-CSF, IL-6, IL-10, and TNF-α in the supernatant of 4T1 cultures, by Bioplex assay, 48 hrs after adenovirus-transduction. Data are presented as the mean ± SD from duplicate samples in a single experiment. This piece of data was generated by Mr. Chi Yan.

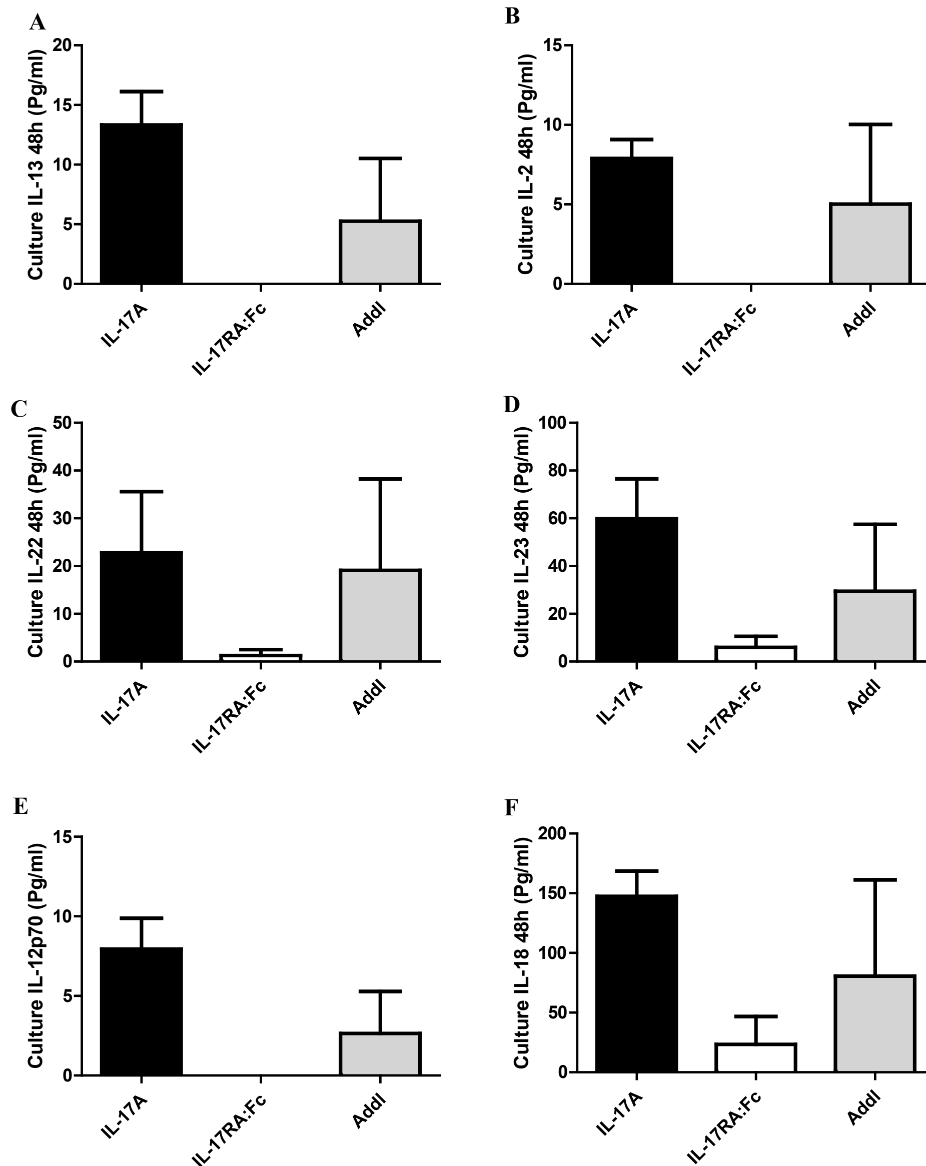
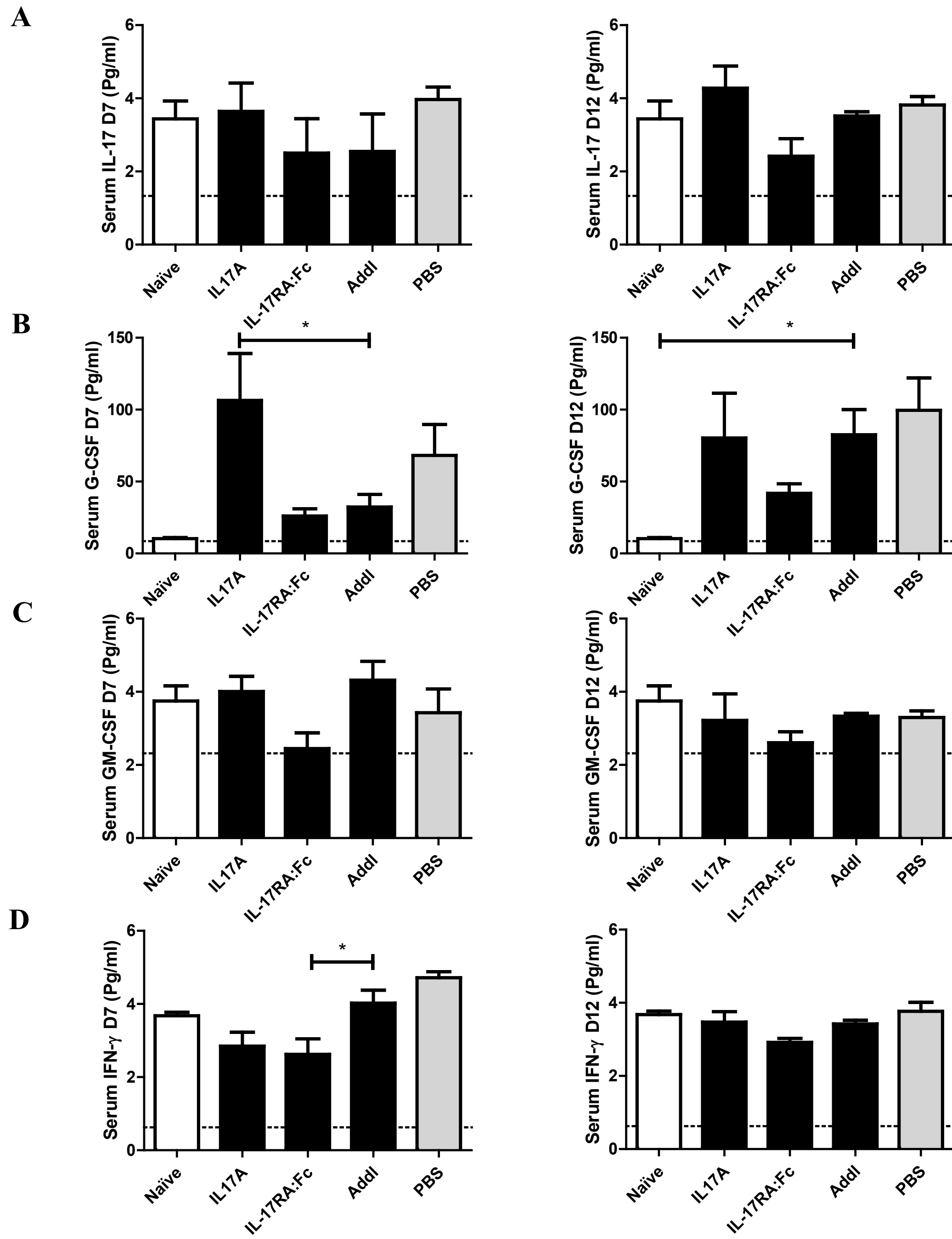


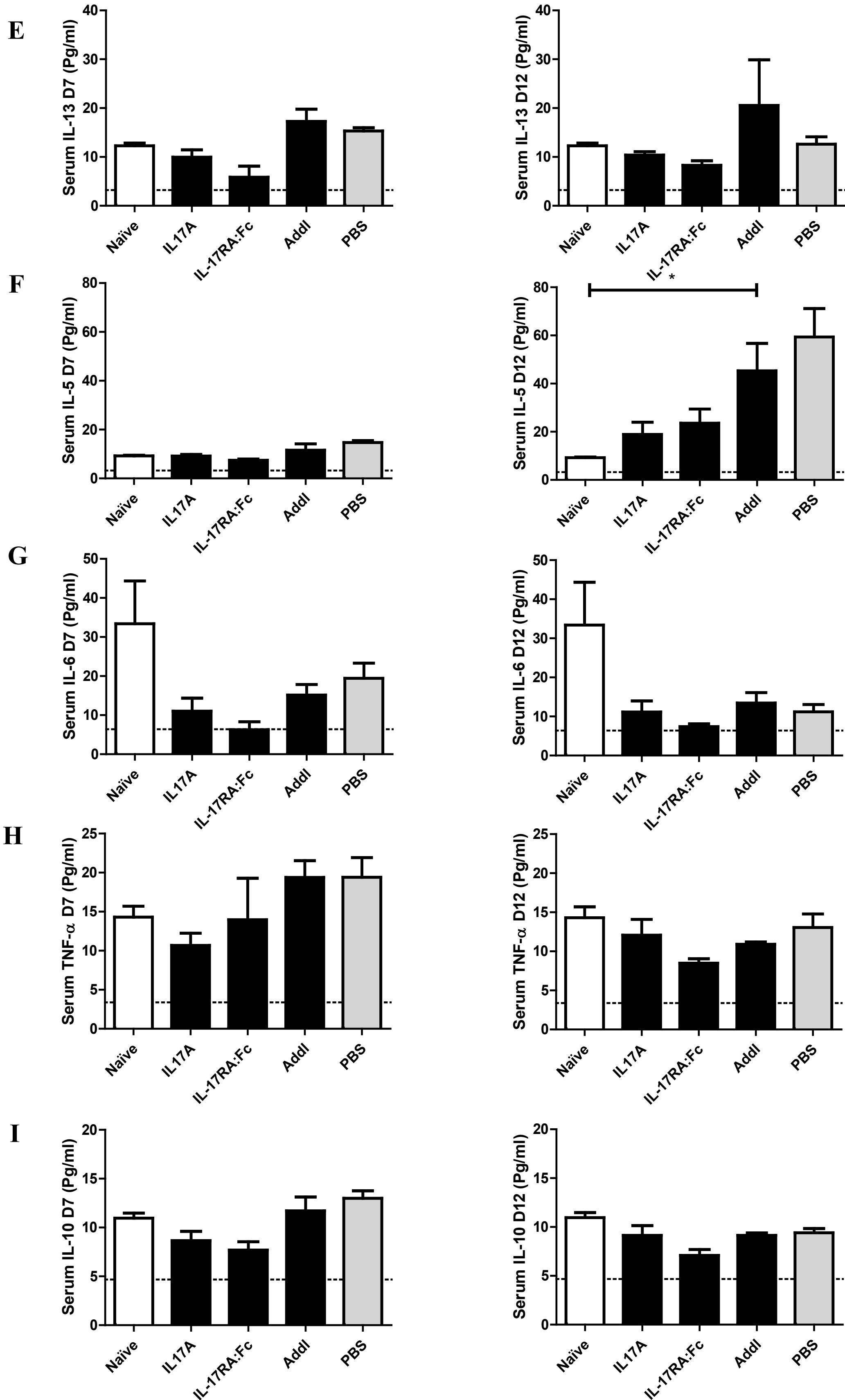
Figure A 3: AdIL-17A transduction in 4T1 cells induces cytokine secretion from 4T1 cells

(A-G) Levels of IL-13, IL-2, IL-22, IL23, IL-12p70, and IL18 in the supernatant of 4T1 cultures, by Bioplex assay, 48 hrs after adenovirus-transduction. Data are presented as the mean ± SD from duplicate samples in a single experiment. This piece of data was generated by Mr. Chi Yan

Figure A 4: AdIL-17A transduction in 4T1 cells significantly stimulates serum G-CSF level.

(A-K) Concentration (pg/mL) of IL-17A, G-CSF, GM-CSF, IFN-γ, IL-13, IL-5, IL-6, TNF-α, IL-10, IL-22 and IL-1β in the sera of naïve and tumor-bearing mice after 7 and 12 days of tumor inoculation Data are presented as the mean ± SEM of 5 mice from a single experiment. *$P<0.05$ versus the Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis. This piece of data was generated by Mr. Chi Yan.


A
Serum IL-17 D7 (Pg/ml)
Serum IL-17 D12 (Pg/ml)
B
*
Serum G-CSF D7 (Pg/ml)
Serum G-CSF D12 (Pg/ml)
C
Serum GM-CSF D7 (Pg/ml)
Serum GM-CSF D12 (Pg/ml)
D
Serum IFN-γ D7 (Pg/ml)
Serum IFN-γ D12 (Pg/ml)
Naïve
IL17A
IL-17RA:Fc
Addl
PBS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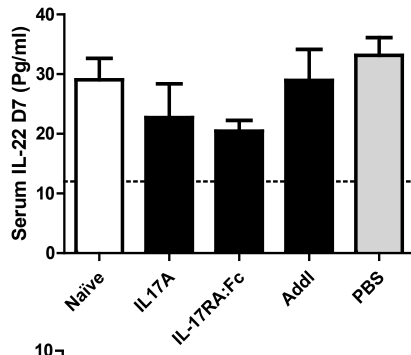

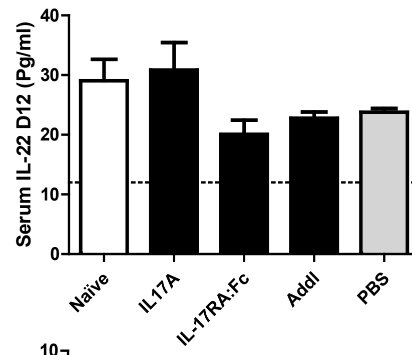

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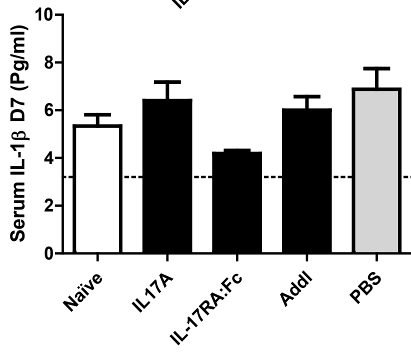

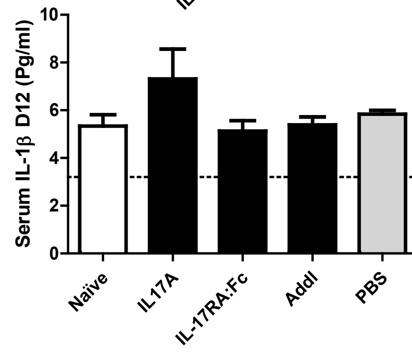

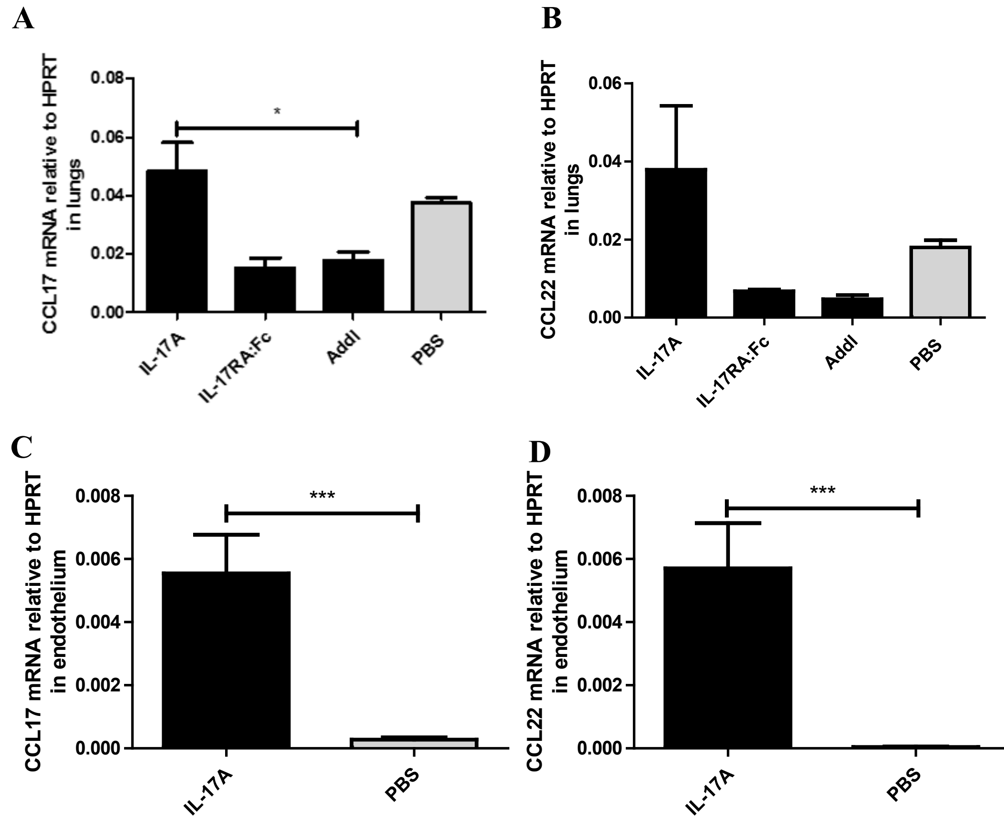

Figure A 5: IL-17A induces lung metastasis via induction of CCR4 expression on 4T1 tumor cells and CCL17 and CCL22 in the lungs.

(A/B) The levels of CCL17-mRNA and CCL22-mRNA in the lungs were measured by RT-PCR and expressed relative to HRPT-mRNA. (n=3-5 per group pooled from 2 independent experiments) Data are presented as the mean ± SEM. *P<0.05; **P<0.01; ***P<0.001 versus Addl as determined by one-way ANOVA with Dunnett's post-hoc analysis. **(C/D)** The levels of CCL17-mRNA and CCL22-mRNA in bEnd.3 primary endothelial cell line were measured by RT-PCR (n=6 per group from single experiment) and expressed relative to HRPT-mRNA after treatment with recombinant IL-17A (100 ng/mL). Data are presented as the mean ± SEM. *P<0.05; ***P<0.001 versus PBS as determined by t-test. The data presented here were obtained by Mr. Simon Gebremeskel.

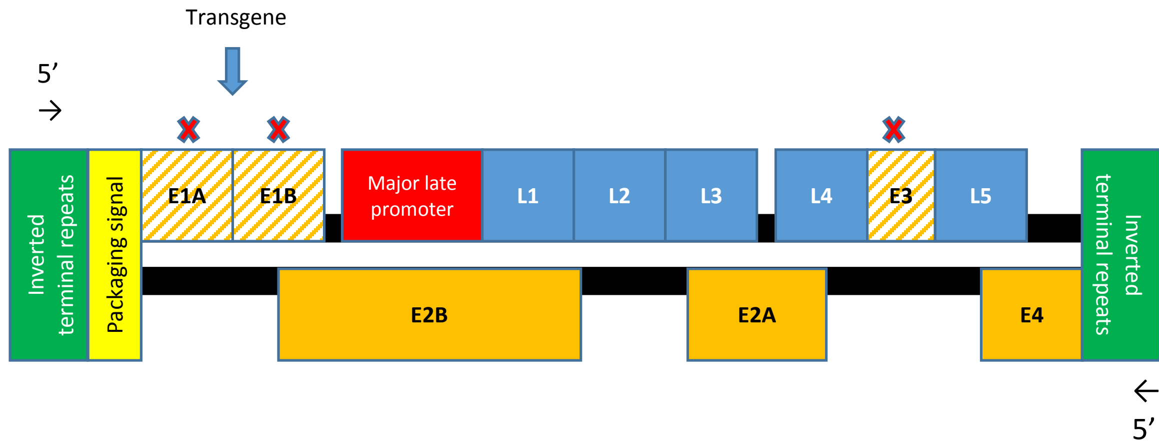

Figure A 6: Transcription map of human adenovirus serotype 5

The genome of human adenovirus serotype 5 is divided into early genes (E1-E4), five late genes (L1-L5), inverted terminal repeats (ITRs) that are involved in viral DNA replication, and the packaging signal that is involved in packaging of the genome into virion capsids. This figure is adapted from *Journal of Genetic Syndromes & Gene therapy,* (2011); S5-001.

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