Stimulation of chemotherapy-induced immunity by targeting IL-6 in the tumor microenvironment

by

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Submitted to the Department of Biology
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Abstract

Tumor formation and progression involves the growth and co-evolution of neoplastic cells in combination with microenvironmental stromal components. Transformed tumor cells initiate crucial changes in healthy tissues that convert their environment into one that supports and furthers cancer development. Consequently, heterogenous cell types within tumors and context dependent factors can influence and shape the therapeutic responses of these diseases. These interactions promote outgrowth of therapy-refractory malignancies, which are a recurrent and challenging problem when treating cancer patients in the clinic.

One of the most effective emerging approaches to safeguard patients from cancer recurrence is the stimulation and mobilization of the immune system against tumor cells. To this end, preclinical studies of a group of cytostatic and genotoxic agents has shown that these drugs exert their effects on cancer cells by, in part, boosting the functions of immune cells. However, the vast majority of these agents do not effectively engage the immune system when used as therapies for cancer patients. Activation of innate and adaptive immune responses against cancers rely on cell-to-cell communications regulated by cytokines. These soluble factors can also generate anti-inflammatory responses depending on their concentration and timing of exposure. Thus, detrimental immunosuppressive activity promoted by cytokines is one of the context-dependent factors that inhibit tumor-specific responses.

In my thesis, using an immune-competent mouse model of B-cell acute lymphoblastic leukemia, we describe how the microenvironmentally derived cytokine IL-6 inhibits anticancer immune responses generated by chemotherapy treatment. Specifically, we demonstrate that absence of IL-6 from tumor microenvironments leads to enhanced T-lymphocyte responses that culminate in the generation of long-term immunologic memory. These findings reveal one of the mechanisms by which microenvironmental changes brought upon by tumor cells result in therapy resistance and disease recurrence. Therefore, we present supporting evidence that unravelling the therapeutic potential of IL-6 pathway inhibition in combination with immune-stimulating therapies could improve care and treatment for various oncological indications.

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Title: Associate Professor of Biology

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Chapter 1

Introduction

Chapter 1, Part I: The mammalian immune system

1.1 An overview of the immune system

The immune system is a complex arrangement of soluble compounds, cells, and organs that protect against a vast array of intruders and foreign organisms. Entities that can cause disease and considered pathogenic are grouped into four different categories: viruses, fungi, parasites, and bacteria. Features shared within these pathogen groups are some of the exploitable characteristics that the immune system can recognize to attack and eliminate such pathogens. Whether these organisms can cause disease or not is largely defined by their pathogenic potential and by the integrity of the host immune system. Additionally, microenvironments in which immune responses occur can also influence their outcome. Therefore, initial responses by the immune system are dependent on pathogen types and the environments where they occur.

Physical barriers of the body are the first-line of defense against pathogens. Apart from the skin and mucous membranes, physical barriers include acidic and pH sensitive tissues like the stomach and sweat generated from perspiration. Additionally, soluble antimicrobial proteins secreted by epithelial cells can also act as physical barriers. It is only after breaching these physical barriers that immune pathways are triggered.

Distinct and specific types of immune responses are initiated after pathogen recognition. Host cells express soluble or membrane-bound receptors or recognition molecules that are engaged by recognizing specific chemical structures on or derived from pathogens. Additionally, microenvironmental cues and whether pathogens are inside or outside of host cells also helps

demarcate the most effective type of immune response. Integration of all of these signals results in an intracellular or extracellular cascade of events that lead to identification and destruction of pathogens. Engaging these immune pathways prompts the production of an assortment of soluble proteins that aide in identifying and destroying invaders (humoral immunity), and the creation and activation of a sophisticated arrangement of cells that can recognize and kill pathogens (cellular immunity).

Common foreign structures found on or expressed by pathogens are known as pathogen-associated molecular patterns (PAMPs). Vertebrates have evolved to express numerous cell surface and soluble pattern recognition receptors (PRRs) that can identify these PAMPs. PRRs are conserved proteins that are also constitutively expressed by several different immune cell types. These PRRs, together with immune pathways engaged by them, represent one of two arms of the immune response, known as innate immunity. However, if innate immune responses were our only defense against quickly evolving pathogens that avoid host detection, how could immune responses that recognize newly arising chemical structures or PAMPs be generated?

To counter this random production of new PAMPs, vertebrate immune systems have evolved to generate diversity when designing some of their recognition molecules. Generation of diversity is accompanied by expression of many identical copies of the same recognition molecule in a group of cells. Likewise, many different groups of cells with specifically diverse recognition molecules are generated at the same time. Theoretically, this approach has potential to generate a large cohort of cells capable of responding to any antigen or new PAMP that poses a threat.

When antigens bind to these receptors, they prompt a clonal selection process that results in massive proliferation of antigen-selected cell clones. These newly selected and expanded clones are capable of destroying pathogens encountered by the initial clones. In contrast to genome encoded PRRs, these unique recognition receptors will vary from individual to individual and are not passed on to offspring.

The second arm of immune responses, also known as adaptive immunity, largely consists of these randomly generated but more antigen-specific responses. Both innate and adaptive immune systems cooperate to guard hosts from foreign invaders. Innate responses are rapid and less-specific, and their activation produces signals that are required to stimulate and engage adaptive immune responses. Adaptive responses can take days to develop but are highly specialized against antigens from pathogens. This second arm of the immune system mainly relies on B- and T-lymphocytes, cells with receptors that undergo diversity generation via DNA recombination. Therefore, adaptive immunity is slower because fewer cells express the specific receptors necessary for antigens of interest. Adaptive immunity is also slower because it relies on prior encounters to antigen, previous innate processes, and clonal selection.

Overall, adaptive immunity comprises of complex set of chemical signals and cells that generate specific and, ideally, long-lived responses that protect against future encounters with the same antigen. Indeed, the first exposure to antigen results in primary immune responses that conclude in the creation of memory cells. This immunologic memory provides the immune system with abilities to respond much more quickly to subsequent contact with the same pathogen. As such,

B- and T-lymphocyte memory cells are activated almost immediately with antigen re-encounter, and possess improved and specific eradication potential. Additionally, memory cells can remain in the host's body for decades after the first encounter with antigen. However, in some instances, one encounter with antigen is not enough to produce a sufficiently strong primary immune response that culminates with generation of memory cells. Sometimes two or more instances of antigen encounter are necessary to develop immunologic memory and guarantee a successful secondary immune response.

In the following sections of Part-I of this Chapter, I will expand the discussion on innate and adaptive immune responses by broadly reviewing how they function and are regulated. Additionally, I will present the main soluble factors and tissue components involved in complex immune responses. Finally, I will end the discussion with an introduction to a more insidious enemy of the immune system: transformed self-cells that acquire malignant properties.

1.2 Macro- and microenvironments of the immune system

Effective immune responses rely on co-localization of pathogens, cells of the immune system, and necessary signaling molecules. Therefore, for the immune system to be as effective as possible it has to be highly dispersed and mostly decentralized (Parkin & Cohen, 2001). To this end, specialized structures of immune activity are distributed throughout the body and positioned in key areas of significance to assist with sentinel activity. Although tissue-resident immune cells exist in most tissues, great numbers of white blood cells, particularly lymphocytes,

are in constant circulation throughout the body. These tissue-resident and circulating white blood cells mediate both innate and adaptive immune responses.

The most challenging problem for a disseminated system is coordinating and communicating what needs to occur for aggressive and properly directed immune responses. The communication needed for the cells involved in innate and adaptive responses is controlled by secreted messenger proteins known as cytokines (Dinarello, 2007). Whether membrane bound or soluble, these proteins bind to receptors on target cells and will typically stimulate cellular responses that range from proliferation, differentiation, and/or activation. Chemokines, a subset of cytokines, are particularly important since they relay the gradient of chemical stimulus necessary for recruiting target cells to a site of interest (Palomino & Marti, 2015). Often, more than one cytokine, chemokine, and soluble factor is involved in recruiting cells for pathogen elimination. These general communication and coordination events are illustrated in (**Figure 1.1**).

Figure 1.1

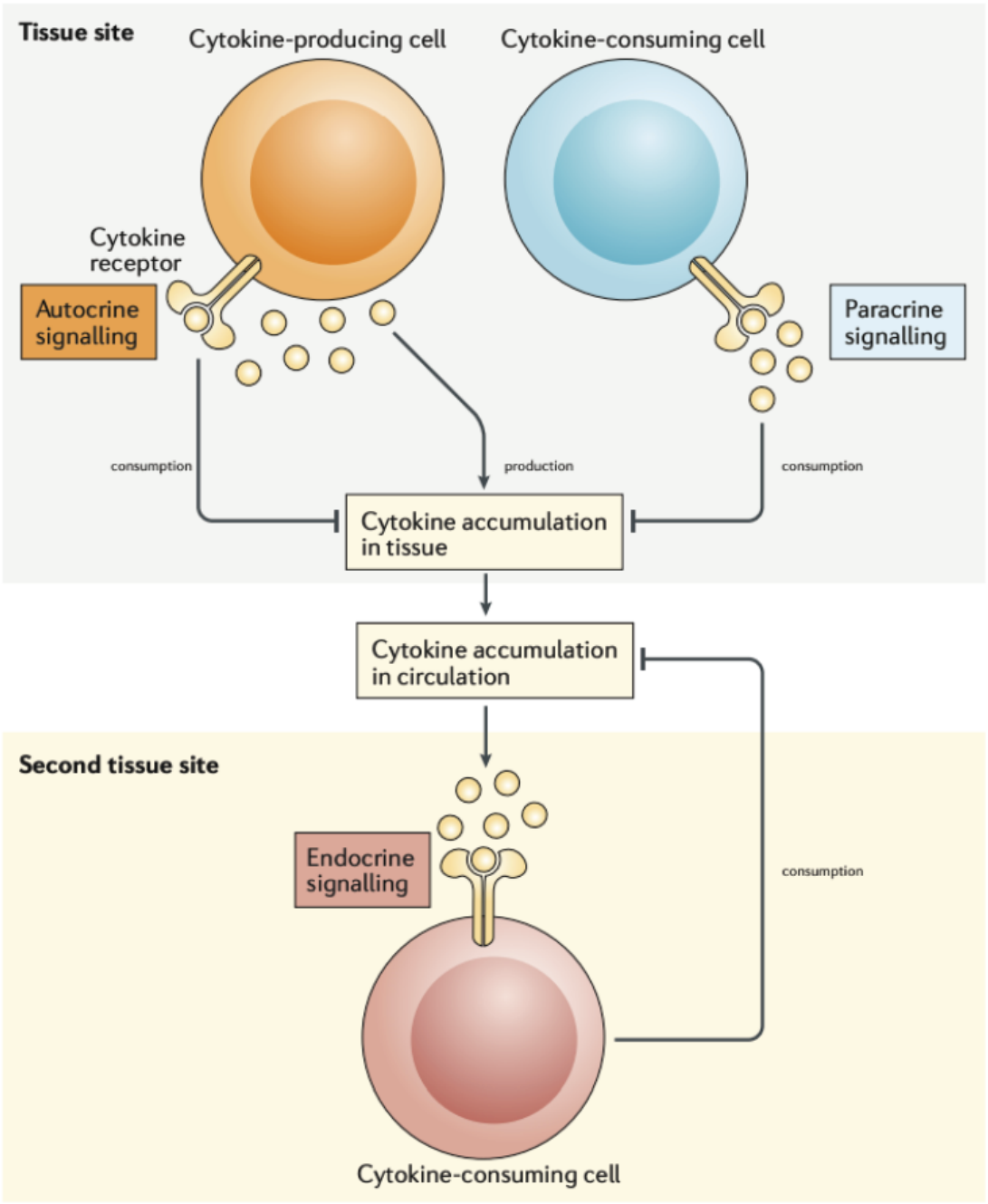
Tissue site
Cytokine-producing cell
Cytokine-consuming cell
Cytokine receptor
Autocrine signalling
Paracrine signalling
consumption
production
consumption
Cytokine accumulation in tissue
Cytokine accumulation in circulation
Second tissue site
Endocrine signalling
consumption
Cytokine-consuming cell

Figure 1.1 | Cytokine-mediated cell-to-cell communication. Cells from the immune system communicate by exchanging and perceiving secreted cytokines. Three modes of communication can occur: autocrine (cytokine signaling to self), paracrine (cytokine signaling to neighboring cells), and endocrine (cytokine signaling to distant tisseus through the circulation). Figure adapted from (Altan-Bonnet & Mukherjee, 2019).

Successful immune responses require precise and exact maneuvering of multiple cell types. Initial phases of these responses rely on innate immune components like neutrophils, monocytes, and macrophages to mount immediate lines of defenses against pathogens (Parkin & Cohen, 2001). Next, antigen presenting cells prime and activate lymphoid cells, which coordinate the ensuing antigen-specific adaptive immunity. Finally, memory from these adaptive responses is generated to respond to future exposures to the same antigen (Ratajczak et al., 2018). These highly coordinated and exact events are made possible by the specialized anatomy of the immune system and numerous white blood cell effectors. This anatomy includes primary and secondary lymphoid organs interconnected by two circulatory systems: the blood and lymphatic systems. All white blood cells necessary for effective immune responses are derived from a single cell type, the hematopoietic stem cell (HSC).

1.2.1 Hematopoiesis and cells of the immune system

HSCs are defined by the capacity to regenerate or "self-renew" and the ability to differentiate into diverse cell types, known as multipotency. However, there are limits to this multipotency, since with aging, the number and potential of self-renewing of HSCs is greatly reduced (Geiger et

al., 2014; Yang & de Haan; 2021). These cells originate in fetal tissues and reside primarily in the bone marrow of adult vertebrates, with smaller numbers found in adult liver and spleen. HSCs are an uncommon subset of tissue-specific stem cells, with tightly controlled proliferation and differentiation potential (Rieger & Schroeder, 2012).

Hematopoiesis is a process by which HSCs differentiate into mature blood cells, including both red blood cells (erythroid cells), and white blood cells (leukocytes). Under steady-state or homeostatic conditions – lack of pathogenic challenge – most HSCs are quiescent, and only a few of these cells are actively proliferating (Doulatov et al., 2012). Daughter cells can either retain the characteristics of their mother HSC and remain self-renewing, or they can further differentiate into other cell types. During infections or after radio- or chemo-therapy, there can be an increased demand for hematopoiesis and HSCs can proliferate in copious amounts.

There are several different types of HSCs. The various kinds of HSCs differ in their capacity to self-renew and their pluripotency ability to generate all blood cell types. Long-term HSCs (LT-HSCs) retain pluripotency throughout the lifetime of an organism and are the most quiescent stem cells. Cells derived from LT-HSCs that can generate all lineages with more frequency, but with reduced self-renewal capability, are known as short-term HSCs (ST-HSCs). Multipotent progenitors (MPPs) have a much more limited ability to self-renew but are still capable of generating all lineages (Doulatov et al., 2012).

As HSCs differentiate and progress into becoming MPPs they receive microenvironment cues for lineage commitment. These cells can either become myeloid or lymphoid progenitor cells, generally known as common myeloid progenitors (CMP) or common lymphoid progenitors (CLP), respectively. CMPs, as suggested by their names, give rise to myeloid cells, which are members of the innate immune system and are some of the first responders against pathogens or antigens. In one hand, cells including monocytes, macrophages, granulocytes, some subsets of dendritic cells, red blood cells and platelets, are generated from CMP cells. In the other hand, CLPs give rise to B- and T-lymphocytes, specific dendritic cell populations, and innate lymphoid cells. These latter populations of cells have roles in both innate and adaptive immunity, while B- and T-lymphocytes participate in antigen-specific adaptive immunity (Kondo et al., 1997; Akashi et al., 2000).

Seminal studies of stem cell biology using mouse models identified modifications in surface marker expression that were characteristic of the different stages HSCs go through while transitioning to specific cell lineages. Mouse HSCs were first isolated as a lineage-negative (Lin^-), c-Kit^+, $Sca1^+$ (LSK) population or LSK-HSCs (Spangrude et al., 1988; Ikuta and Weissman, 1992). Further classification identified that $CD34^-$ LSK cells retain attributes of long-term multilineage reconstitution and self-renewal (Osawa et al., 1996). Current models of lineage determination are illustrated in (**Figure 1.2**). The distinction of different environmental signals that stimulate HSC differentiation and the capacity to isolate purified HSCs has allowed for detailed analysis of transcriptional and epigenetic factors that drive HSCs in any of the possible developmental pathways available (Ivanova et al., 2002; Ramalho-Santos et al., 2002; Ji et al., 2010). Many

transcription factors identified with these approaches have also been shown to be involved in several developmental stages of hematopoiesis. However, the molecular and biochemical understanding of these transcription factors and pathways that underlie HSC function are continuing to grow.

Figure 1.2

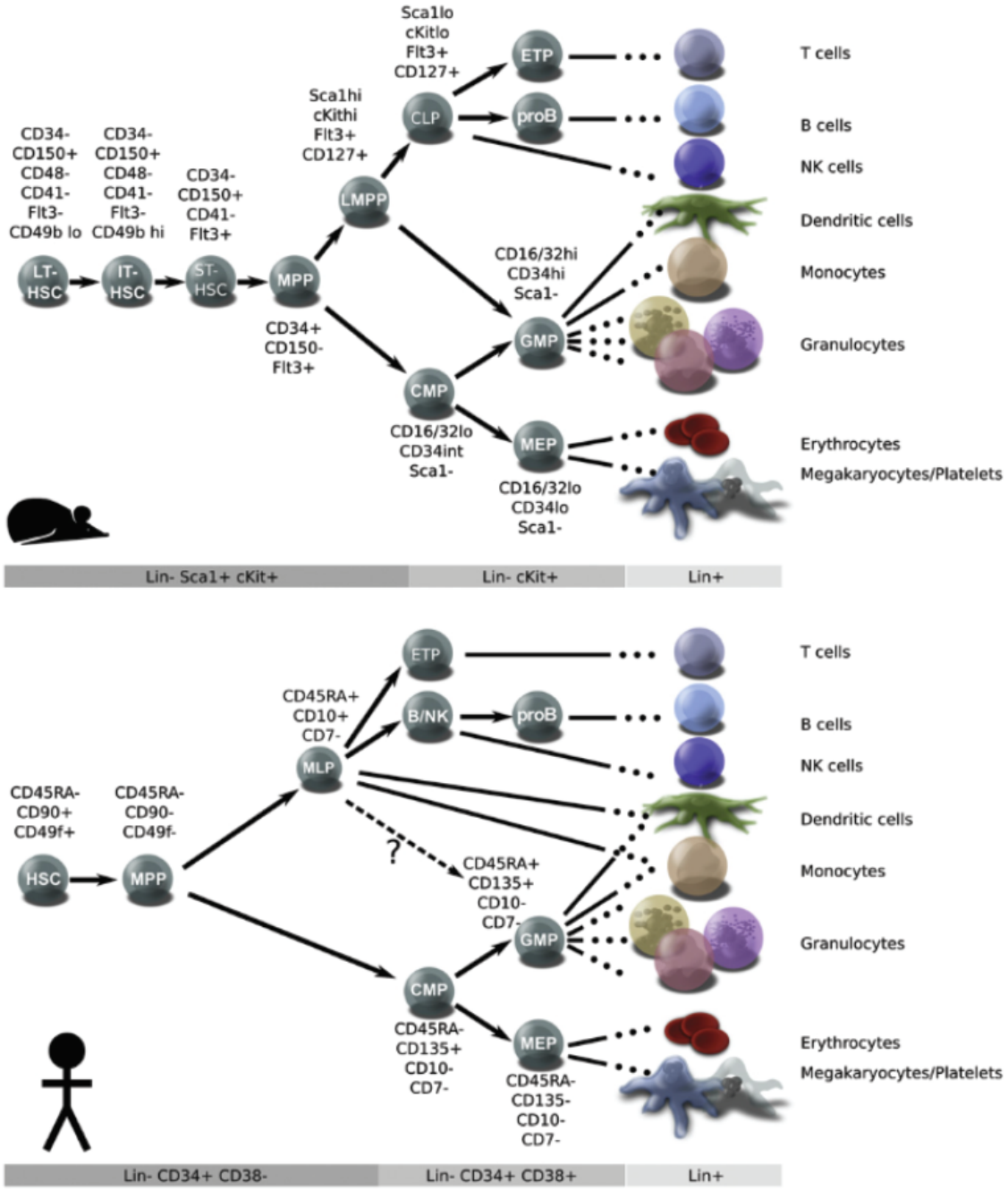
Sca1lo
cKitlo
Flt3+
CD127+
ETP
T cells
Sca1hi
cKithi
Flt3+
CD127+
CLP
proB
B cells
NK cells
CD34-
CD150+
CD48-
CD41-
Flt3-
CD49b lo
CD34-
CD150+
CD48-
CD41-
Flt3-
CD49b hi
CD34-
CD150+
CD41-
Flt3+
LMPP
Dendritic cells
CD16/32hi
CD34hi
Sca1-
Monocytes
LT-HSC
IT-HSC
ST-HSC
MPP
GMP
Granulocytes
CD34+
CD150-
Flt3+
CMP
CD16/32lo
CD34int
Sca1-
MEP
Erythrocytes
Megakaryocytes/Platelets
CD16/32lo
CD34lo
Sca1-
Lin- Sca1+ cKit+
Lin- cKit+
Lin+
CD45RA+
CD10+
CD7-
ETP
T cells
B/NK
proB
B cells
NK cells
MLP
CD45RA-
CD90+
CD49f+
CD45RA-
CD90-
CD49f-
Dendritic cells
?
CD45RA+
CD135+
CD10-
CD7-
Monocytes
HSC
MPP
GMP
Granulocytes
CMP
CD45RA-
CD135+
CD10-
CD7-
MEP
Erythrocytes
Megakaryocytes/Platelets
CD45RA-
CD135-
CD10-
CD7-
Lin- CD34+ CD38-
Lin- CD34+ CD38+
Lin+

Figure 1.2 | Hematopoietic lineage hierarchies in adult mouse and human. Major classes of stem and progenitor cells describe in the text are shown, and defined by cell surface markers. Long-term (LT), intermediate-term (IT), short-term (ST), lymphoid-primed multipotent progenitors (LMPPs), multilymphoid progenitors (MLPs), earliest thymic progenitors (ETPs), granulocyte-monocyte progenitors (GMPs), and megakaryocyte-erythrocyte progenitors (MEPs). Figure adapted as is from (Doulatov et al., 2012).

1.2.1a Myeloid lineage cells

Erythroid cells and megakaryocytes

Erythrocytes or red blood cells have high concentrations of hemoglobin and deliver oxygen to all cells and tissues while circulating through blood vessels. Although erythrocytes primarily labor in oxygen exchange, they also play a role in innate immunity by expressing surface receptors that bind antibodies which are later cleared by scavenging macrophages. Additionally, these cells can produce compounds like nitric oxide which can directly damage pathogens. In turn, damaged red blood cells may also release damage signals to prime other innate immune responses. Finally, megakaryocytes are large bone marrow-residing myeloid cells that produce many platelets – small cells or cell fragments that participate in the process of blood clotting. Clots form a physical barrier that may also help in preventing further dissemination of pathogens (Frame et al., 2013).

Granulocytes

Often first responders during an immune response, granulocytes encompass neutrophils, basophils, eosinophils, and mast cells (Friedman, 2002). All granulocytes have multilobed nuclei

and granulocyte subtypes differ by the staining patterns of their cytoplasmic granules. When these cells are activated in response to pathogenic insults, their cytoplasmic granules, which are membrane-bound vesicles containing proteins with distinct functions, are released and indirectly and directly help in inhibiting pathogenic activities (Borregaard, 2010). Additionally, these cells help shape and influence necessary adaptive immune responses.

Myeloid antigen-presenting cells

Phagocytic cells by excellence, monocytes, macrophages, and dendritic cells are professional antigen-presenting cells (pAPCs) indispensable for connecting the innate and adaptive immune systems (Geissmann et al., 2010). Activated at first contact with antigen at sites of infection, these cells travel to lymph nodes and display peptides from pathogens to T-lymphocytes. Although all cells are capable of presenting peptides on MHC class-I molecules, pAPCs are also capable of presenting peptides on MHC class-II molecules. Therefore, after coming into contact with antigens, essential roles of pAPCs include production and secretion of proteins that attract and activate other immune cells. pAPCs phagocytose pathogens or antigens and digest their foreign proteins into peptides. These peptides are then presented on the surface membrane of pAPCs via either MHC class molecules – through both conventional and unconventional endogenous and exogenous antigen presentation (Lee et al., 2020).

Dendritic cells (DCs), are the most potent pAPCs for naïve T-lymphocytes. DCs receive their name due to long membranous extensions that resemble the dendrites of nerve cells. These membranous processes are capable of extending and retracting, which increases the surface area

DCs have available for sampling both for antigens and T-lymphocytes. Particularly characteristic of DCs is how they sample for antigens in tissues but present them more efficiently in lymph nodes, initiating adaptive immune responses (Bajénoff et al., 2007). Immature DCs take up antigen in peripheral tissues by three different mechanisms: receptor-mediated endocytosis, by pinocytosis – interstitial space liquid filtering performed by cells – or by engulfing antigens through phagocytosis.

Antigen-stimulated DCs mature and upregulate expression of costimulatory surface molecules required for proper and optimal activation of T-lymphocytes (Banchereau et al., 2000). Maturation also prepares DCs for entry into lymphatic or blood vessels and migration into the closest lymph node, where they present antigen to naïve T-cells. Additionally, DCs are capable of performing a process termed cross-presentation, which combines actions from endogenous and exogenous pathways of antigen presentation (Jung et al., 2002; Joffre et al., 2012; Alloatti et al., 2016). In this process, exogenous antigens internalized by DCs gain access to endogenous presentation pathways and become associated with MHC class-I molecules, leading to activation of antigen-specific lymphocytes.

1.2.1b Lymphoid lineage cells

Lymphocytes are the main components of adaptive immune responses and sources of immunologic memory. Lymphocytes are made up of three major populations: innate lymphoid cells (ILCs) – which include natural killer (NK) cells, – B-lymphocytes (B-cells), and T-lymphocytes (T-cells) (Artis & Spits, 2015). These cells circulate blood and lymphatic vessels and constantly

migrate to and surveil tissues like intestine linings, airways, and reproductive tracts, among others.

Lymphocyte subsets possess similar physical characteristics. Thus, researchers rely on profiling of their surface proteins to differentiate lymphocyte subpopulations. Using the cluster of differentiation (CD) nomenclature, which labels the surface proteins expressed on cells, different subtypes of lymphocytes can be identified. Depending on the developmental and activation state of B- and T-lymphocytes, many different CD proteins are expressed on their surface. Additionally, each B- and T-cell expresses an antigen-specific receptor on their surface, the B- or T-cell receptor, respectively (Germain, 2002; Eibel et al., 2014). From lymphocyte-to-lymphocyte, there is remarkable diversity within their surface expressed antigen-specific receptors. However, all copies of antigen-specific receptors on the surface of one single B cell or T cell are identical in structure and specificity (Roth, 2014). When prompted to proliferate and clonally expand, all progeny from one specific lymphocyte shares the same antigen-specific receptor as their mother cell. At any given moment, there are tens to hundreds of thousand different antigen-specific B- and T-cell clones circulating around the body.

Innate lymphoid cells

Although derived from common lymphoid progenitors, innate lymphoid cells do not express antigen-specific receptors (Varadé et al., 2021). Many ILCs are first responders against insults in epithelial and mucosal tissues, and are distinguished by their cytokine secretion profile. ILCs are divided into three groups (ILC1-3), but much of this field is still under active investigation

(Gasteiger & Rudensky, 2014; Artis & Spits, 2015). Cytotoxic natural killer cells are the most studied member of ILCs. NK cells are very efficient at killing target cells via release of their cytotoxic granules. These cells are capable of recognizing and attacking target cells that lack expression of MHC class-I molecules on their surface. Also, NK cells express receptors against the Fc portions of antibodies – Fc receptors or FcRs – that allow them to come into close contact with target cells and induce cell death by a process known as antibody-dependent cell cytotoxicity (ADCC) (Vivier et al., 2018).

B lymphocytes

B cells were found to mature in the bursa of Fabricius in birds, which explains their one letter designation. In mice, humans, and other mammals, the major site of maturation for B cells is the bone marrow. Mature B cells express membrane-bound antibodies, also known as B-cell receptors (BCRs), and each B cell expresses a surface immunoglobulin with unique antigen specificity that can recognize soluble or particulate antigen. The ability of B cells to bind antigen can also be greatly improved by a process called somatic hypermutation. These cells are also capable of undergoing antibody class switching, a process that generates antibodies of several distinct functional classes that can interact with different effector molecules (Wang et al., 2020).

Activated B lymphocytes may additionally act as pAPCs by internalizing antigens with their antigen-specific BCRs. By presenting antigens, activated B cells help in activating T lymphocytes with the expression of costimulatory molecules on their surface. Likewise, B cells receive help from these T cells in the form of cytokine stimulations. Such stimulus induces B cell differentiation

into memory and plasma cells, the latter which are responsible for massive secretion of antigen-specific immunoglobulins (Wang et al., 2020).

T lymphocytes

Like B cells, T cells derive their one letter designation from their site of maturation, the thymus. As well, T cells express unique antigen-specific receptors, known as T-cell receptors (TCRs). Unlike BCRs, TCRs can only recognize processed pieces of antigen – peptides – that are bound to and presented by MHC molecules. MHC-peptide complexes allow cells to showcase on their surface both self- and non-self-antigens for T cells to browse from. MHC class-I molecules are expressed by virtually all nucleated cells and class-II molecules are principally expressed by pAPCs (Kumar et al., 2018).

T cells are divided into two major types, T helper (T_H) cells and T cytotoxic (T_C) cells. These cell types are distinguished from each other by surface expression of the membrane glycoproteins CD4 and CD8, respectively. T cells displaying CD4 will recognize antigen-MHC class-II complexes and T lymphocytes displaying CD8 will recognize antigen peptides loaded on MHC class-I molecules. When binding to peptide-MHC complexes occurs, naïve $CD8^+$ T cells become activated, proliferate, and differentiate into cytotoxic T lymphocytes (CTLs). CTLs monitor cells of the body in search for non-self-antigen displaying targets such as virus infected cells, and tumor cells.

Naïve $CD8^+$ T cells rely on help from mature $CD4^+$ T cells for proper proliferation and differentiation. Activated $CD4^+$ T cells differentiate into a variety of T_H subtypes like T helper type 1 (T_H1), and T helper type 2 (T_H2) cells, among others. T_H1 cells help to regulate responses against intracellular pathogens, while T_H2 cells help fight extracellular pathogens like bacteria. By producing different sets of cytokines, each $CD4^+$ T_H cell subtype aides in activation of B cells, T_C cells, and some myeloid cells. Ultimately, activated helper subtypes are defined by the types of threats or pathogens that initiated the immune responses (Luckheeram et al., 2012).

Finally, a specific subset of $CD4^+$ T cells, that can either arise during maturation in the thymus or is induced at sites of immune responses, adds to the diversity of T cell subpopulations. Regulatory T cells (T_{REGS}), recognized by presence of CD4 and CD25 molecules on their surface, along with expression of the transcription factor FoxP3, have the unique ability of inhibiting immune responses (Togashi et al., 2019). By binding to self-proteins with high affinity, T_{REG} cells defend against autoreactive immune responses and help in limiting overaction and hyperactivation of other T cell subtype responses.

NKT cells

NKT cells possess features of both innate and adaptive immune cells. Some NKT cells express CD4 on their surface while all have TCRs. Unlike most T cells, NKT versions of TCRs are less diverse and do not recognize protein peptides (Benlagha et al., 2002), instead TCRs on NKT cells recognize lipids and glycolipids. These cells also contain receptors associated with NK cells, like the ones discussed above. Although the exact roles of these cells remain to be fully understood, we know

that activated NKT cells are involved in direct cytotoxic activity and in secretion of cytokines to both enhance or suppress immune responses. Their involvement in autoimmunity and cancer has also been documented (Bae et al., 2019).

1.2.2 Primary lymphoid organs

HSCs reside in specialized microenvironments with supportive cells. These microenvironments nurture stem cells throughout all their stages like proliferation, differentiation, survival, and trafficking. While HSCs can be found circulating in blood vessels, the main and primary site of hematopoiesis through adult life is the bone marrow. Many differentiated cells derived from HSCs also reside in these specialized microenvironments. For example, in both mice and humans, bone marrow microenvironments support maturation of B lymphocytes. However, T lymphocyte precursors leave the bone marrow and complete their maturation in the thymus (Thapa & Farber, 2019).

1.2.2a Hematopoiesis in the bone marrow

The site of hematopoiesis changes during embryonic development, shifting several times before transitioning into the bone marrow, the adult stem cell niche. Hematopoiesis begins with precursor cells in the yolk sac of an embryo. Fetal HSCs capable of generating all blood cell types are found close to the kidneys, in the aorta-gonad-mesonephros (AGM). Mature HSCs are also found in the fetal placenta and fetal liver, with placental pools of HSCs eventually diminishing as the numbers of HSCs in the liver expand. HSCs first traffic to the bone marrow at later stages of fetal development and most bones in the body are haematopoietically active until around the

age of 18. At this stage, HSCs mostly reside in the vertebrae, ribs and sternum, skull, pelvis, and bones in the arms and legs like the humerus and femur, respectively (Rieger & Schroeder, 2012).

The marrow, or medullary cavity, is a non-hard sponge-like matrix filled with stromal cells that provides structure and support for hematopoiesis. Stromal cells in the marrow include: endothelial cells lining blood vessels, perivascular cells with varied functions, sympathetic nerves for signal transmission and communication, macrophages that regulate activities of other niche cells, and osteoblasts which generate bone tissue (Al-Drees et al., 2015). Generally, most HSCs remain quiescent while some divide and differentiate into progenitor cells that replenish both myeloid and lymphoid lineages. Progenitor B lymphocytes are commonly found in association with osteoblasts and complete most of their development within the bone marrow. More mature B cells eventually exit the bone marrow to complete their maturation in the spleen. Meanwhile, progenitors of T lymphocytes exit the bone marrow at very early stages and complete their development in the thymus (Al-Drees et al., 2015).

1.2.2b The thymus and T-cell maturation

T cell development is not complete until T cells undergo selection in the primary lymphoid organ of T cell maturation (Geenen, 2021). After T cell progenitors, known as thymocytes, arrive via circulation to the thymus, they progress through multiple microenvironments and developmental stages to become fully functional T cells. Here, with support from thymic epithelial cells, thymocytes generate unique TCRs that are selected to maturity based on their reactivity with self-peptide-MHC complexes (Miller, 2011). These developing thymocytes are

screened for their capacity to recognize self-MHC molecules (positive selection), and against autoreactivity (negative selection), which eliminates thymocytes with TCRs that bind self-MHC complexes with high affinity. This selection process is very stringent and most thymocytes do not survive all these stages of development, with a majority of cells dying because they have low affinity for self-MHC molecules (Thapa & Farber, 2019).

Expression of CD4 and CD8 molecules is used to distinguish thymocytes at different stages of development. Double negative (DN) thymocytes lack expression of both surface molecules and are at the most immature stage of development. After entering one of the distinct microenvironments of the thymus, DN thymocytes upregulate expression of both CD4 and CD8 molecules, becoming double positive (DP) cells. As T cells mature, they become single positive (SP) by losing expression of one molecule or the other (Thapa & Farber, 2019). $CD8^+$ T cells become cytotoxic killer cells and $CD4^+$ T cells develop into helper cells. The final steps of maturation occur in the periphery or circulation, once these SP T-cells exit the thymus, they surveil the body for antigen presentation in secondary lymphoid organs like the spleen and lymph nodes (Klein et al., 2014).

1.2.3 Secondary lymphoid organs

Many cells of the immune system encounter antigen and formally initiate immune responses in microenvironments of secondary lymphoid tissues and organs (Randall et al., 2008). Cell trafficking to secondary lymphoid organs like the spleen and lymph nodes occurs via both blood and lymphatic vessels, with white blood cells having exclusive access to the lymphatic system.

These lymphatic vessels connect to and drain many tissues (which could be sites of infection or inflammation) to provide a travelling road for antigens and activated immune cells. Most secondary lymphoid tissues connect to lymphatic vessels. However, the spleen, as an exception, is served primarily by blood vessels (Neely & Flajnik, 2016). Travel of immune cells through both lymph and blood vessels is aided by gradients of chemokines secreted by multiple cell types including pAPCs, lymphocytes, stromal cells, and epithelial cells.

1.2.3a The lymph nodes

Lymph nodes (LNs) are tissues completely dedicated to regulation of immune responses. LNs contain networks of stromal cells for support, macrophages, dendritic cells, and many lymphocytes (Bajénoff et al., 2007; Johnson, 2021). Structurally, lymph nodes are composed of three concentric distinct microenvironments: the cortex, the paracortex, and the medulla. The outermost layer, the cortex, contains organized follicles mostly comprised of B cells, macrophages, and follicular dendritic cells. The paracortex or T-cell zone, found within the cortex, is largely populated by T cells and dendritic cells that migrated from nearby tissues. Finally, the medulla is the innermost layer and represents the exit point for all lymphocytes. Populations of lymphocytes are scarce in this last microenvironment, with the exception of antibody secreting plasma cells.

T cells in the lymph nodes

As soon as naïve T lymphocytes enter lymph nodes, they start browsing MHC-peptide antigen complexes presented by APCs in the paracortex. TCRs that successfully bind MHC-peptide

complexes stop migrating and take residence in the current lymph node for several days while they proliferate. Depending on cues from APCs, T cells will differentiate into effector cells like $CD8^+$ or $CD4^+$ lymphocytes. T cells that do not find their MHC-peptide match exit the lymph node and continue circulating through the body (Bajénoff et al., 2007).

B cells in the lymph nodes

High-affinity and antibody-secreting plasma cells are products of differentiated B cells originally activated in lymph node follicles, or B-cell follicles. BCR engagement with antigen and contact with activated $CD4^+$ T_H cells are necessary for optimal activation of B cells (von Andrian & Mempel, 2003; Kuka & Iannacone, 2018). Small soluble antigens are capable of entering B-cell follicles on their own, while larger antigens are presented by follicular dendritic cells. B cells that successfully bind antigen with their BCRs become partially activated and also engulf their bound antigen for processing. Once this occurs, B cells migrate into T-cell zones to proliferate and finish differentiation into either antibody-producing plasma cells or B cells that will create a germinal center in another follicle. Secondary follicles or germinal centers are where B cells proliferate and undergo clonal selection to produce B cells with high-affinity neutralizing antibodies (Kuka & Iannacone, 2018).

Memory T and B cells in the lymph nodes

Interactions between APCs and activated T_H cells will eventually produces memory T and B cells. Memory effector cells exit lymph nodes and circulate to other tissues or to the site of pathogen encounter. Memory cells can take residence in lymph nodes and become central memory cells,

which are distinct in phenotype and function from those that exit lymph nodes. Some memory cells become tissue-resident memory cells that settle long-term in peripheral tissues (von Andrian & Mempel, 2003).

1.2.3b The spleen

This secondary lymphoid organ plays a major role in organizing immune responses against blood-borne pathogens. While lymph nodes drain antigens from local tissues, the spleen helps fight systemic infections by responding to blood-borne antigens (Neely & Flajnik, 2016). Since spleens are not supplied with lymphatic vessels, antigens and lymphocytes are trafficked into and out spleens through splenic arteries, and splenic veins, respectively (Lewis et al., 2019). Spleens are compartmentalized into two main microenvironments, the red pulp and the white pulp, with the marginal zones in between them. While red pulps include circulating and dying red blood cells, white pulps contain B-cell follicles and a network of T lymphocytes, known as the periarteriolar lymphoid sheath (PALS) (Lewis et al., 2019).

Initiation of adaptive immune responses within spleens involve circulating naïve B cells that encounter antigen in their B cell follicles. Naïve $CD4^+$ and $CD8^+$ T cells encounter MHC-peptide complexes on surfaces from APCs in the PALS or T-cell zones. Activated $CD4^+$ T_H cells also provide help to B cells and T cells that have similarly recognized their cognate antigens. Afterwards, $CD8^+$ T cells exit into circulation and some $CD4^+$ T cells and B cells are left behind to form germinal center follicles, allowing for generation of memory B cells and plasma cells (Lewis et al., 2019).

1.2.4 Tertiary lymphoid tissues

Sites of active infection and immune activity are considered tertiary lymphoid tissues. Activated effector lymphocytes and tissue-resident memory cells that return to areas of insult and inflammation create new microenvironments for organized immune responses, like B-cell follicles and T cell areas. Tertiary lymphoid tissues are ectopic sites where immune responses can develop independently of secondary lymphoid organ activity, although there remains a fair number of uncharacterized properties regarding how immune responses are sustained in these structures (Carragher et al., 2008; Gago da Graça et al., 2021).

1.3 Innate immunity

Innate immune responses are quickly engaged following exposure to foreign antigens. Both cellular activity and anatomical barriers – physical and chemical barriers – are involved in innate immune responses. However, for the purpose of this discussion, I will focus on reviewing the former. Cellular innate immune responses are quickly activated as soon as pathogens overcome physical and chemical barriers. Engagement of cell surface or intracellular receptors (PRRs) that recognize conserved molecular components from pathogens (PAMPs) is the initial step in a sequence of events to eliminate such pathogens. Engaged receptors prompt immune cells to phagocytose extracellular threats, while other receptors stimulate production of an array of proteins and substances. These produced factors have a range of effects from direct anti-pathogenic activity, to recruitment and activation of additional immune cells (Kimbrell & Beutler, 2001). Despite the proficiency of the innate arm of immunity, some pathogens overcome these

innate immunity effector mechanisms, which is why innate immunity also helps promote a successful adaptive immune response (Gasteiger & Rudensky, 2014).

1.3.1 Innate response receptors and signaling

To combat infections, the innate immune system has developed several families of pattern recognitions receptors with essential roles in kickstarting desired immune responses. The presence of numerous PRRs guarantees that cells can recognize not only PAMPs from almost any pathogen but also damage-associated molecular patterns (DAMPs) released by damaged cells and tissues – including stress signals released by intestines after chemotherapy. Some PRRs are expressed on cellular plasma membranes to detect extracellular PAMPs or DAMPs, while others are found inside cells and recognize endocytosed or cytosolic antigens like nucleic acids from replicating viruses (Hato & Dagher, 2015).

Toll-like receptors

Included in the families of PRRs are 13 different membrane proteins called toll-like receptors (TLRs) that upon antigen binding are induced to homo- or heterodimerize and activate a varied set of immune responses. To respond as efficiently as possible to multiple potential ligands or antigens, TLRs exist in both plasma membranes and endosomal and lysosomal membranes. TLRs expressed on plasma membranes recognize PAMPs like lipopolysaccharides, peptidoglycans, and other components of fungi, bacteria, or parasites (Chaplin, 2010).

Intracellular TLRs recognize ligands like DNA and RNA, which are released during endosomal and lysosomal degradation of pathogens like bacteria and viruses. There are several distinct signaling responses that can be activated after TLRs bind their ligand. Responses initiated by TLRs will depend on the TLR that is engaged and adapter proteins (MyD88 or TRIF) that associate with their cytoplasmic portions. Overall, signaling pathways activated by TLRs involve transcription factors like NF-kB, AP-1, and interferon regulatory factors (IRF) (Kawai & Akira, 2010).

C-type lectin receptors

Another family of cell surface PRRs is the C-type lectin receptors (CLRs). This family of receptors recognizes carbohydrate components like mannose, glucans, and fucose derived from all sorts of pathogens like fungi, bacteria, and parasites. Signaling pathways of most CLRs depend on cytoplasmic protein kinase-mediated phosphorylation for activation of transcription factors. Subsequently, gene expression changes caused by these transcription factors includes expression of genes that code for proinflammatory cytokines like IL-1β, IL-23, and TNF (Iwasaki & Medzhitov, 2015; Brown et al., 2018).

NOD-like receptors

Nucleotide oligomerization domain or NOD-like receptors (NLRs) are a large family of cytosolic proteins activated by intracellular PAMPs or DAMPs. Many NLRs have poorly characterized functions, and they are divided into three major groups based mostly on their structural features (Kawai & Akira, 2010). These PRRs bind intracellular PAMPs, like cell wall components, and can initiate signaling pathways upstream of IRF, MAPK, and NF-kB (Rathinam et al., 2012).

AIM2-like receptors

ALRs or AIM2-like receptors bind double-stranded DNA (dsDNA) molecules from cytosolic bacteria and viruses (Kawai & Akira, 2010). Upon binding, they form inflammasome structures – large complexes of PRRs protein domains with caspases and other proteins – that promote interferon production and inflammation. These PRRs play an important role in defending hosts from intracellular bacterial infections.

RIG-I-like receptors

RIG-I-like receptors (RLRs) cytosolic members, RIG-I and MDA5, bind viral dsRNA. On binding, RLRs undergo conformational changes and form tetramers. These tetramers are arranged into larger assemblies of PRRs that also bind to dsRNA. As with other PRRs, RIG-I-like receptors trigger signaling of transcription factors like NF-kB and IRF, which induces expression of potent antiviral interferons and cytokines (Iwasaki & Medzhitov, 2015; Yoneyama et al., 2015).

cGAS and STING receptors

Cyclic GMP-AMP synthase (cGAS) is a nucleotidyltransferase that recognizes cytosolic DNA. After binding dsDNA, this transferase synthesizes cGAMP – 2', 5'-cyclic GMP-AMP dinucleotide – from GTP and ATP. cGAMP then acts as a messenger and binds STING (stimulator of interferon genes) proteins that are associated to endoplasmic reticulum membranes. STING, also a cytosolic PRR, undergoes conformational changes upon binding of cyclic dinucleotides. These conformational changes reposition STING inside Golgi-complex membranes, where they recruit and activate the

kinase TBK1. In turn, TBK1 phosphorylates and activates IRF3, and NF-kB to produce type-I interferons and cytokines (Jiang et al., 2020).

1.3.2 Effector mechanisms of innate immunity

Innate immune responses protect their hosts by using effector mechanisms that include molecules with direct antipathogenic activity and cellular responses to eliminate pathogens or infected cells. To mount these protective responses, the PRR signaling pathways described above activate transcription factors that in turn encode an arsenal of proteins with the necessary antipathogenic properties. Ultimately, the variety of these proteins is determined by diverse pathogen-derived PAMPs in combination with different immune cell types.

Antimicrobial peptides

Some cells and tissues, particularly epithelial cells, constitutively express defensin and cathelicidin peptides, which are important protective physical barriers (De Smet & Contreras, 2005). Neutrophils are another example of cells that constitutively synthesize and package defensins and cathelicidins into granules, which they use to kill phagocytosed bacteria, viruses or parasites (Borregaard, 2010). Many other cell types express these peptides after stimulation of their PRRs, particularly of TLRs and NLRs.

Cytokines and chemokines

Several key cytokines are transcriptionally induced by PRR activation. Cytokines have roles in activating and regulating a wide array of inflammatory, innate, and adaptive immune responses.

These roles can range from recruiting and activating lymphocytes, permeabilizing vascular vessels for cell trafficking, and creating systemic effects – like inducing and enhancing hematopoiesis. Some of the most important cytokines include IL-1, TNFα, and IL-6 – a potent proinflammatory cytokine which will be the topic of extensive discussion in Part III of this Chapter, and in Chapter 2 (Iwasaki & Medzhitov, 2015; Brown et al., 2018). Briefly, IL-6 is a class 1 cytokine that activates several signaling pathways upon binding to its receptor, mainly JAK/STAT signaling. IL-6 is produced by monocytes/macrophages, dendritic cells, NK cells, lymphocytes, epithelial cells, and vascular endothelial cells. Similarly, it can stimulate and act on bone marrow cells, lymphocytes, and vascular endothelial cells. Immune and inflammatory effects stimulated by IL-6 include promotion of hematopoiesis, increased vascular permeability, and induction of the acute-phase response – a cluster of physiological processes that occurs soon after infections or some malignant conditions (Baumann & Gauldie, 1994).

Chemokines are small proteins that help to effectively recruit cells into, within, and out of tissues. These proteins create a gradient of concentration that induce cells to move towards higher concentrations of protein. Some chemokines are responsible for homeostatic and constitutive migration of white blood cells throughout the body (Hughes & Nibbs, 2018). Other chemokines are induced by PRR activation and have roles in all stages of immune responses like attracting cells to clear infections or to further amplify current immune activities.

Type-I interferons

Type-I interferons are a major class of antimicrobial cytokines induced by PRRs. When infected with a virus and following binding of cytosolic PRRs like ALRs, RLRs, and cGAS, many cell types are stimulated to produce IFNα and IFNβ via activation of IRF transcription factors. IFNα and IFNβ exert their antiviral effects by binding to IFNα receptors (IFNAR) expressed by most cell types. IFNAR bound to ligands will dimerize and induce activation of the JAK/STAT signaling pathway, which allows for STAT1 and STAT2 dimers to enter the nucleus and initiate transcription of specific genes. Genes turned on by IFN signaling are regarded as interferon-signaling genes (ISGs) and include: protein kinase R (PKR) that is activated by dsDNA binding, 2', 5'-Oligoadenylate A synthetase (OAS) which is activated by dsRNA biding, and IFN-induced proteins with tetratricopeptide repeats (IFIT), also activated by dsRNA binding (Yan & Chen, 2012; McNab et al., 2015). Additionally, type-I interferons increase expression of MHC class-I molecules in target cells, allowing them to be more easily recognized by effector T_C cells (Musella et al., 2021). These cytokines are also important for regulating cancer immunosurveillance and the activities of macrophages and NK cells (Zitvogel et al., 2015).

Phagocytosis

Cellular uptake and destruction of particulates is an important line of defense against pathogens. Phagocytosis is mainly carried out by monocytes, macrophages, neutrophils and dendritic cells. Phagocytic activity is engaged through various cell surface receptors which induce polymerization of actin microfilaments and extension of the phagocyte's plasma membrane to engulf and internalize pathogens. Phagosomes – the vesicles with engulfed pathogens – are then fused with

lysosomes, which deliver hydrolytic enzymes and prompt changes in pH to kill and degrade entrapped pathogens (Rosales & Uribe-Querol, 2017).

Aging cells or cells that have died from damage (necrosis) or apoptosis (programmed cell death) are also eliminated by phagocytic cells. Dead or dying cells attract phagocytes by releasing DAMPs and engaging their PRRs (Gordon, 2016). Apoptotic cells induce phagocytic activity by releasing or expressing “eat-me” signals like lipids, proteins like annexin-1, and carbohydrates on their surface. In contrast, healthy cells express a larger amount of “do not eat-me” signals like the surface protein CD47, which transmits phagocytosis inhibitory signals through SIRPα receptors on macrophages (Gordon, 2016).

1.3.3 Innate and adaptive immune system interactions

Many pathogens evolve or have evolving features that allow them to evade innate immune responses. Therefore, antigen-specific responses generated by the adaptive immune system are, in many cases, necessary to successfully and completely eliminate pathogens. To achieve these necessary cell-mediated and antibody responses, the innate immune system provides important initiating and regulating signals (Gasteiger & Rudensky, 2014).

To properly generate adaptive immune responses, pathogens or antigens have to be delivered into lymphoid tissues and exposed to B and T lymphocytes. Once pathogens or antigens arrive to nearby draining lymph nodes, dendritic cells recognize them with their PRRs and are stimulated to begin maturation. After maturation, dendritic cells process antigens and present them bound

to MHC class-I molecules, or on MHC class-II. Also, these mature pAPCs upregulate expression of costimulatory molecules like CD80 or CD86. These costimulatory molecules are necessary for proper activation of naïve T cells, which eventually become antigen-specific effector cytotoxic and helper T cells (Iwasaki & Medzhitov, 2015; Sun et al., 2020).

Activated dendritic cells are stimulated to secrete specific cytokines essential for regulating T cell differentiation. Induction and production of various cytokines prompt naïve T cells to differentiate into several types of helper cell subsets like T_H1, T_H2, T_H17 and T_{REGs}, among others. For example, some extracellular bacteria and internalized viruses activate DCs and stimulate them to secrete IL-12, which skews T cell differentiation into a T_H1 subtype. In turn, T_H1 helper cells secrete IFNγ, which promotes macrophage and NK-mediated elimination of infected cells (Sun et al., 2020).

1.4 Adaptive immunity

As discussed above, once naïve B and T lymphocytes exit the bone marrow and thymus, they enter the bloodstream and circulate between secondary and tertiary lymphoid tissues browsing for antigens. Attracted by chemokines to B-cell follicles and T-cell zones, naïve B and T cells, respectively, spend many hours searching for antigens on the surface of APCs. Specifically, processed antigens in the form of MHC-peptide complexes are presented to T cells in the T-cell zones, while unprocessed antigens are presented to B cells in the B-cell zones (Lewis et al., 2019).

1.4.1 Encounter of antigens by lymphocytes

Naïve B cells become partially activated after finding and binding to antigens in B-cell follicles. Subsequently, engagement with activated helper T cells provide B cells with ligands like CD40L, which stimulate CD40 signaling and release of cytokines that promote B cell differentiation. Different helper T cells can provide distinct types of stimulation to B cells. For example, T_H2 cells secrete IL-4 and prompt differentiating B cells to form germinal centers and undergo class switching to produce IgE antibody isotypes (Kuka & Iannacone, 2018).

Naïve $CD8^+$ T cells, precursors of cytotoxic T lymphocytes, are major participants in cellular immune response, as they kill infected cells by inducing apoptosis. Like B cells, these $CD8^+$ T cells require assistance from helper T cells to become fully activated and to generate memory cells (Kok et al., 2021). Three signals are necessary to produce fully activated T cells. One is triggered by engaging their TCR with MHC-peptide complexes on antigen-presenting cells, while the second is triggered by engagement of costimulatory molecules like CD28. The third signal is provided by local cytokines secreted by both APCs and helper T cells in contact with the naïve T cell. These helper T cells also stimulate DCs to increase both antigen presentation and costimulatory molecule expression. Therefore, DCs licensed by $CD4^+$ T cells, deliver their aide to would-be CTLs by forming stable tricellular complexes where T cells engage APCs through MHC-peptide complexes and costimulatory receptors (Borst et al., 2018).

1.5 Immune tolerance

The process and mechanism by which a host is protected from anti self-immune attack is referred to as immune tolerance or self-tolerance. Homeostatic immunologic processes are constantly at work to safely recognize and protect self-components and beneficial commensals like gut bacteria, while stimulating immunity against foreign invaders. Tolerance is achieved by multiple mechanisms, which include protecting immune system components from being exposed to specific antigens (evasion). Also, there are mechanisms in place to remove self-reactive lymphocytes that could cause harm. These lymphocytes are either completely removed by elimination, or they are extrinsically regulated to protect self-structures (engagement) (Goodnow et al., 2005; Gregersen & Behrens, 2006). These processes are delicately balanced to help create self-tolerance while maintaining an immune system that is ready to act against any pathogens.

Evasion

Sequestration, partitioning, or evasion of antigens away from immune cells is an effective way to reduce or completely avoid self-reactivity. For example, the eyes are considered sequestered sites with little or no lymphatic drainage. These features prevent tissue-specific antigens from the eyes from reaching many cells of the immune system (Taylor, 2016). Therefore, in microenvironments where inflammation can be highly destructive, there is bias towards active suppression of anti-self-responses (Forrester et al., 2008).

Elimination and engagement

Central tolerance is a phenomenon that occurs in primary lymphoid tissues by which self-reactive lymphocytes are destroyed or created (Nemazee, 2017; Yap et al., 2018). This elimination occurs first during lymphocyte development, soon after both B- and T-lymphocytes undergo DNA rearrangement to generate diversity on their antigen-specific receptors. At this stage, variable gene regions on the antigen-specific lymphocyte receptors that react against self will be generated. For example, between 20% and 50% of TCRs and BCRs, respectively, generated by DNA recombination bind with too much affinity to self-antigens (Goodnow et al., 2005). However, most of these developing lymphocytes with receptors that recognize self are eliminated by apoptosis through negative selection.

During development, some T cells in the thymus with high affinity against self are positively selected and engaged for survival. These self-reactive cells, known as natural or thymic regulatory T cells (n/tT_{REG} cells), are responsible for suppressing and regulating autoimmune responses against self-antigens in peripheral tissues (Josefowicz & Rudensky, 2012). The grand majority of T_{REG} cells will express CD4 and CD25 molecules on their surface and will also be characterized by expression of the master regulator transcription factor FOXP3 (Lu et al., 2017). These cells dampen immune responses by secreting suppressive cytokines like IL-10 and TGF-β, by expressing inhibitory costimulatory molecules like CTLA-4, or even by killing other immune cells. While T_{REGs} are the best characterized cells with immune-inhibitory functions, other cells like subsets of B cells, dendritic cells, and macrophages can also participate in inhibiting anti-self-responses (Devaud et al., 2014; Vadasz et al., 2015; Lu et al., 2017).

Finally, peripheral tolerance is generated and necessary when anti-self lymphocytes manage to escape the process of negative selection in primary lymphoid organs (ElTanbouly & Noelle, 2021). Anti-self lymphocyte escape occurs because not all self-antigens are expressed during the negative selection process and because some weakly self-reactive clones can go unnoticed and survive elimination. What typically occurs next is that naïve T lymphocytes that recognize self-antigen are engaged without proper costimulatory signals and either become apoptotic, unresponsive or anergic, or stimulated to express FOXP3 and become peripheral or induced T_{REGs} (p/iT_{REGs}) (Hasegawa & Matsumoto, 2018; ElTanbouly & Noelle, 2021).

1.5.1 Cancer and the immune system

Cancer occurs when host cells undergo aberrant and uncontrolled cell division that cripples tissue and organ function, eventually leading to death. Since these neoplasms are self in origin, self-tolerance mechanisms can block the detection and elimination of cancerous rogue cells. Although many tumor cells express unique or developmentally inappropriate antigens that could make them potential targets for immune responses, the increased genetic instability of these cells can give them an advantage to eventually evade immune detection and elimination. In fact, we know that the immune system is constantly detecting and controlling cancerous cells in the body and that even under this pressure, unregulated cell proliferation and transformation can lead to tumor growth. Ultimately, what is cancer if not the immune system behaving overly-tolerant and failing at its job of eliminating a threat?

1.5.1a An overview of cancer biology

Benign tumors are not capable of indefinite growth and do not disseminate nor invade healthy surrounding tissues. However, malignant tumors or cancers continue to grow and become more invasive with time. If left unchecked by the immune system, most malignant tumors will eventually exhibit metastasis – when cancerous cells dislodge from the original tumor and seed distant sites and continue to proliferate (Fares et al., 2020). Embryonic tissues of origin are used to classify cancers, with most being carcinomas – tumors that develop from epithelial origins like skin and linings of internal organs. Less frequent tumors are sarcomas, which arise from mesodermal connective tissues like bone, cartilage, and fat. Finally, there are cancers derived from blood cells, which can develop at different stages of hematopoiesis.

Cell transformations that lead to unregulated proliferation occur via random DNA damaging events, DNA repair defects, exposure to carcinogens, some viral infections, and chromosomal alterations (Blackadar, 2016). Contrary to normal tissues that tightly control cell proliferation and apoptotic events, malignant tumors tend to suffer from imbalances in genes that control these homeostatic processes. Normal cellular genes that are associated with either promoting or blocking cellular proliferation and survival fall into three major categories: oncogenes, tumor-suppressor genes, and apoptotic genes (Hanahan & Weinberg, 2011).

Oncogenes

Genes involved in aspects of promoting cell growth and proliferation are known as proto-oncogenes. Cancer and uncontrolled proliferation may arise from mutated proto-oncogenes –

known as oncogenes – that undergo changes in expression. Proto-oncogenes are encoded by growth factors and their receptors, which can result in uncontrolled proliferation if abnormally expressed. Signal transduction pathways, like those containing the *src* and *abl* genes, are another category of proto-oncogenes (Lee & Muller, 2010). The products of these genes are tyrosine kinases that transduce signals upstream of transcription factors that control cell proliferation and cell cycle progression – *abl* and its gene product will be discussed in further detail in Part II of this Chapter.

Tumor-suppressor genes

These genes encode proteins that inhibit cell proliferation. Normally, tumor-suppressor genes prevent inappropriate progression of cells through the cell cycle and perform DNA repair. Unlike oncogenes, a single allele alteration is typically not sufficient to allow for cell transformation. It is when both allele copies are damaged that these tumor-suppressor genes fail to function properly (Knudson, 1971; Lee & Muller, 2010).

Apoptosis genes

Genes involved with programmed cell death or apoptosis can be either inhibitors or promoters of controlled cell death. Anti-apoptotic genes behave like oncogenes, promoting cell survival, while pro-apoptotic genes behave like tumor-suppressor genes, inhibiting cell survival. Therefore, overactivity or failure, respectively, of genes involved in apoptosis can result in cell transformation and cancer generation (Hanahan & Weinberg, 2011).

However, most cancers do not arise from a single insult to any of these major gene categories. Instead, a series of somatic mutations take cells closer to uncontrolled proliferation, growth, and survival. “Driver” mutations are the early genetic changes that initiate cellular transformative processes and subsequent tumor clonal evolution. While “passenger” mutations arise stochastically as byproducts of genome instability and may or may not have a role in cancer progression (Martínez-Jiménez et al., 2020). Ultimately, as few as one or two genes may be sufficient to serve as driver mutations for any given malignancy.

There are several unifying characteristics, known as cancer hallmarks, supported from studies in animals and humans, that define cancer. These hallmarks represent a series of progressively evolving alterations in normal cells that ultimately enable them to become cancerous (Hanahan & Weinberg, 2011). These hallmarks include: genome instability, altered metabolomic pathways, sustaining proliferative signaling, evading growth suppressors, resisting cell death, activating angiogenesis, activating invasion and metastasis, and having unlimited replicative potential (Hanahan & Weinberg, 2011). However, it has become increasingly clearer that malignant cells do not arise only from cell-intrinsic alterations, but that microenvironmental conditions and normal cell types that form tumor-associated stroma also play a role in tumorigenesis. The roles and contributions from these stromal cells include allowing cancers to avoid immunological destruction, and enabling inflammation that paradoxically can result in enhanced tumorigenesis (Hanahan & Weinberg, 2011).

Additionally, the complexity and heterogeneity of cancer is not only restricted to diverse stromal components. Indeed, there is also heterogeneity within cancer cells, creating vast tumor heterogeneity across cancer types (McGranahan & Swanton, 2017). Consequently, tumor and stroma heterogeneity underscore the challenges in understanding the complex biology of cancer and its treatment. However, advances in cancer genetics and cancer immunotherapy have allowed for recent clinical breakthroughs in the fight against cancer. While there is still no "one-size fits all" approach to treat cancer, our ability to manipulate tumor-specific immune responses has been added to current standards of care to treat cancer patients, promoting better prognoses for many (Hoteit et al., 2021).

1.5.1b Tumor antigens

Cancer cells are self-cells and most of their produced antigens are subject to the same tolerance-inducing processes that other normal non-cancerous cells are subject to. However, cancer cells also produce unique antigens that are identified by the immune system, known as tumor derived antigens. These tumor derived antigens are divided into two groups. First, tumor-specific antigens (TSAs) include antigens encoded from genes solely expressed in tumor cells. The most common types of TSAs are derived from single-nucleotide polymorphisms, which cause non-synonymous mutations that translate to gene products that trigger antigen-specific responses (Smith et al., 2019). Second, tumor-associated antigens (TAAs) include antigens from genes expressed at inappropriate stages of development, overexpression of certain genes, and proteins post-translationally modified (Gubin et al., 2015).

1.5.1c Immune responses to cancer

Cancer immunosurveillance is the mechanism by which the immune system controls or inhibits cancer growth. However, immune involvement in cancer regulation has the potential to both inhibit and enhance tumor progression. The process by which the immune system positively (antitumor) and negatively (protumor) influences which cancerous cells are eliminated or not, is referred as cancer immunoediting (Dunn et al., 2002). This process is divided into three phases: elimination, equilibrium, and escape. During elimination, cancer cells are identified and destroyed by the immune system until a balance of cell destruction and survival is created, attaining an equilibrium state. After an undefined period of equilibrium, the most aggressive and least immunogenic cancer clones are selected to thrive, spread, and to eventually escape the immune system (Dunn et al., 2002).

Cancer inhibition by innate immunity

Macrophages have a significant role in immune responses to cancer. Macrophages express Fc receptors that recognize antibodies bound to antigens and use them to mediate ADCC. Many tumor-targeting therapeutic antibodies have a human IgG1 portion, which is recognized by Fcγ receptors expressed by macrophages. For example, the anti-CD20 monoclonal antibody rituximab destroys malignant B lymphocytes by engaging Fcγ receptors from monocytes and macrophages (Uchida et al., 2004). Additionally, macrophages typically cluster with tumor cells, and the presence of proinflammatory macrophage subtypes, like M1 macrophages, have been correlated with tumor regression (Oshi et al., 2020).

Another type of innate immune cells involved in antitumor immunity are NK cells. These cells have inherent abilities to destroy tumor cells by using a series of surface receptors that recognize activating and inhibiting signals. NK cells can identify and kill tumor cells by detecting lack of self-signals, like absence of MHC molecules on target cells (Vivier et al., 2011). Additionally, changes or alterations in DAMP expression e.g., due to DNA damage in target cells, can also engage NK cell activity. Once activated, NK cells use cytosolic granules with perforin to kill target cells and also contribute to DC infiltration into tumors by releasing cytokines like IFNγ, TNFα, CCL5 and XCL1 (Bottcher et al., 2018).

Cancer inhibition by adaptive immunity

Cancer cells express tumor antigens that engage BCRs and TCRs, provoking humoral and cell-mediated immune responses, respectively. Cytotoxic T lymphocytes recognize tumor antigens presented with MHC class-I molecules on target tumor cells. Meanwhile, B cells respond to tumor-specific antigens by generating antibodies that can promote tumor cell detection and killing.

Ultimately, to unleash potent T cell antitumor immune responses, several stepwise events that engage both innate and adaptive components of the immune system must occur (Demaria et al., 2019). These events are referred to as the Cancer-Immunity Cycle and consist of 7 discrete steps (Chen & Mellman, 2013). First, uptake of tumor antigens must occur by APCs. Second, these antigens are presented through endogenous and exogenous antigen processing pathways to T cells. Third, naïve T cells are primed and activated by recognizing such antigens within LNs.

Fourth, effector T cell trafficking begins through blood vessels. Fifth, effector T cells infiltrate into tumor microenvironments. Sixth, T cells bind to cancer cells using their antigen-specific receptors. Seventh and final, target cancer cells are destroyed by effector T cells. (Chen & Mellman, 2013; Chang & Beatty, 2020).

Cytokines and cancer immunity

Cytokines associated with CTL and T_H1 responses are also linked to antitumor immunity. For example, IFNγ can exert direct antitumor effects on target cells via upregulation of MHC class-I molecules. IFNγ also stimulates MHC class-II expression on APCs. IL-12 is another example, as this cytokine enables dendritic cells to appropriately prime necessary CTL and T_H1 responses (Lippitz, 2013). Other cytokines, like TNFα and IL-6, can have both anti- or pro-tumorigenic effects, highlighting the complex roles of cytokines in tumor immunity. IL-6's pro- and anti-inflammatory effects will be discussed in a portion of Chapter I, Part III.

Taken together, several adaptive and nonadaptive cells are involved in cancer identification and elimination. Although leukocyte infiltration of tumors is important and necessary for cancer control and eradication, prolonged inflammation can have the opposite effect and lead to generation of immune suppressive microenvironments. Consequently, learning more about how the immune system fabricates desired and undesired immune responses to tumors, will better poise us to design more effective preventative and therapeutic strategies for cancer patients.

Chapter 1, Part II: Lymphoid leukemias and chemotherapy induced immunity

2.1 Chronic and acute lymphoid leukemias

Leukemias arise from uncontrolled proliferation of immature and mature hematopoietic cells. As this disease progresses, it leads to disruption of normal hematopoiesis and bone marrow failure. Factors that place people at risk of increased development of leukemia include: genetic disorders, a history of familial leukemia, exposure to certain chemicals, and previous treatment for other cancers.

In this Part-II of the chapter, I will be summarizing and discussing lymphoid leukemias, which are some of the many malignancies that can arise from cells of the immune system. As the name suggests, lymphocytic leukemias affect lymphoid cells. Several forms of lymphoid leukemias exist, with some being more common in children and others in adults. Similarly, the type of lymphoid leukemia and extrinsic factors like the tumor microenvironment determine the complexity of treatment and prognosis of patients. Depending on how fast the disease progresses, leukemias are classified into chronic or acute leukemias, known as chronic lymphocytic leukemia (CLL) and acute lymphocytic leukemia (ALL), respectively. Since the body of work presented in Chapter 2 of this thesis focuses on the study and treatment of acute lymphoblastic leukemia, the main discussion of this section will be dedicated to ALL and the scientific and clinical progresses made in understanding this disease.

2.1.1 Chronic lymphoid leukemia

B-cell chronic lymphocytic leukemia or CLL is one of the most common occurring leukemias in the United States, with an estimated 21,250 new cases for 2021 (American Cancer Society, 2021). The clinical evolution of CLL involves the precursor syndrome monoclonal B-cell lymphocytosis, intrinsic cell processes like enhanced proliferation and disrupted apoptosis, and extrinsic factors or microenvironmental dependencies (Kitada et al., 1998; Nishio et al., 2005; Strati & Shanafelt, 2015). CLL incidence increases with age, and some families have strong genetic predispositions to develop CLL without any single gene being commonly mutated or associated with the disease (Strati & Shanafelt, 2015). Immunophenotypically, CLL is classified as expressing traditional mature B-cell markers like CD19, CD23, surface immunoglobulins, and the pan-T-cell marker CD5 (Keating et al., 2003).

A central characteristic of CLL is tumor immune resistance early in the progression of disease. Studies showing that allogeneic T cells were unable to generate cytotoxic reactions against CLL target cells are evidence of the potential immunosuppressive features of this cancer (Krackhardt et al., 2002). Others have also documented the early appearance of these immune system defects. For example, overall survival (OS) of CLL patients is predicted at diagnosis by the absolute number of existing T cells and NK cells, along with the lack of some serum immunoglobulins (Rozman et al., 1988; Palmer et al., 2008). Additionally, from disease diagnosis to time of treatment, CLL survival is sustained by microenvironmental changes that include the expansion of suppressive T_{REG} cells and T_H2 anti-inflammatory cytokine secretion profiles (Podhorecka et al., 2002; Giannopoulos et al., 2008). Work in human and mouse models of CLL have demonstrated

that these immunosuppressive effects are largely dependent on the antigen expression profile of CLL (Ramsay et al., 2008; Ramsay et al., 2012). Therefore, reversing immune deficiencies in CLL patients would greatly improve the long-term morbidities associated with this disease.

2.1.1a Treatment of CLL

Despite improved survival of symptomatic CLL with chemo-immunotherapies, CLL is still not cured in a subset of patients. It is estimated that 4,320 Americans will succumb to this disease in 2021 alone (American Cancer Society, 2021). Complete responses (CR) – no detectable cancer after treatment – and OS are only achieved in few patients. Additionally, traditional standard of care agents against this disease are associated with substantial toxicity, which provides even more challenges for the care of patients by physicians. Fortunately, in the past decade, improved characterization of the disease based on molecular features and the use of new molecular-targeted therapies have resulted in more targeted and effective treatments.

Initial treatment of CLL

Treatment for CLL is initiated when patients present with cytopenia, lymphadenopathy, hepatosplenomegaly, and thrombocytopenia (Cheson et al., 1996; Hallek et al., 2008). While different therapies are outlined below, the alkylating agent chlorambucil has served as frontline therapy for CLL for decades and remains in use today, with the most favorable collateral toxicity profile. Multiple schedules and doses of chlorambucil are administered orally to patients as tolerated.

Purine analogues like fludarabine have also become a mainstay therapy with clinical activity in CLL (Grever et al., 1988; Rai et al., 2000). Unfortunately, increases in OS are typically not observed with these agents, while higher toxicity rates compared to chlorambucil are seen in elderly patients. However, high overall response rates (ORR), patients whose tumors are significantly eliminated or completely eliminated, for CLL patients treated with fludarabine prompted several additional studies to compare alkylating agents in combination with fludarabine (Keating et al., 1991; Flinn et al., 2007; Eichhorst et al., 2009). Preclinical studies have demonstrated that DNA-repair mechanisms initiated after treatment with alkylating agents are inhibited by administration of fludarabine (Yamauchi et al., 2001). Later, clinical trials showed that combining fludarabine with chlorambucil was not as efficient as combining fludarabine with another alkylating agent, cyclophosphamide (Elias et al., 1993; Eichhorst et al., 2006). Overall, these large studies demonstrated that therapy with the FC regimen (fludarabine and cyclophosphamide) produces better and longer progression-free survival than single agents alone.

Rituximab, an agent that targets the CD20 antigen on the surface of normal and cancerous B lymphocytes, is also clinically active against CLL (Byrd et al., 2001; Jaglowski et al., 2010). Some of rituximab's mechanisms of action that likely contribute to apoptosis in CLL include the induction of complement-dependent cytotoxicity and ADCC. In general, this drug also has a favorable toxicity profile which has led to its combination with several other chemotherapies like the ones previously mentioned, thus creating the FC-rituximab (FCR) regimen (Byrd et al., 2003; Keating et al., 2005).

Most patients younger than 65 years of age are considered for FCR schedules as initial therapy. Another option available for patients of all demographics and aged 65 or older is ibrutinib, a Bruton tyrosine kinase (Btk) inhibitor that targets BCR signaling (Honigberg et al., 2010). Recent clinical trials have shown significant improvements in PFS and OS for patients treated with ibrutinib over chlorambucil, which resulted in the approval of this inhibitor as frontline treatment for CLL patients (Burger et al., 2015).

Treatment of relapsed CLL

Patients that experience relapse with CLL are treated with similar regimens than those experiencing an initial diagnosis of CLL. However, as the CLL could potentially become more advanced on relapse, patients also undergo cytogenetic analysis and assessment of mutational status on genes of interest. Nonetheless, for patients younger than 65 years, a repeat of FCR regimen or ibrutinib is normally administered. For many older patients, the use of chemo-immunotherapy is not tolerated and targeted inhibitors like rituximab and ibrutinib are preferred. Finally, for patients that progress after kinase inhibitor treatments, schedules with venetoclax or idelalisib and rituximab are favored (Chantry et al., 1997; Roberts et al., 2016).

2.1.2 Acute lymphoid leukemia

There are 5,690 estimated new cases of acute lymphocytic leukemia or ALL in the United States for 2021 (American Cancer Society, 2021). Children and teenagers account for half or more of these cases, which makes ALL the most frequent cause of death from cancer in ages 20 or lower, with the highest incidence occurring at three to five years of age (Ries et al., 1999; Linabery &

Ross, 2008). Fortunately, the 5-year overall survival rate is about 90% for children and 80% in adolescents, although survival following relapse remains poor (Smith et al., 2010; Santiago et al., 2017). The other half of cases occur largely in patients of 60 years of age or older, revealing a bimodal pattern of incidence for this disease (Dores et al., 2012). However, the overall survival rate in adults over the age of 60 is under 30% (Faderl et al., 2010; Gökbuget et al., 2012). Additionally, all forms of ALL, which can originate from B- and T-lymphocyte precursors, have higher occurrence rates in males than in females. More than 80% of all cases are leukemias derived from B-cell precursors over T-cell precursors (Hunger & Mullighan, 2015a). All following subsections and discussions will specifically focus in B-cell acute lymphoblastic leukemia (B-ALL) since this disease was the major target malignancy used for the body of work presented in Chapter 2.

Most patients have no inherited risk factors that lead to ALL development, although Down's syndrome is significantly associated with significantly increased risk (Buitenkamp et al., 2014). However, there are polymorphic variants in several genes that lead to specific ALL subtypes and germline mutations that can be linked with familial ALL (Treviño et al., 2009; Perez-Andreu et al., 2013; Shah et al., 2013). Also, there are few environmental and prior-therapy risk factors, with some cases being linked to carcinogens, radiation, and prior exposure to chemotherapy (Kendall et al., 2011).

2.1.2a Genetic and mutational landscape of B-ALL

Cytogenetic and molecular features are important prognostic factors in ALL that are commonly used to stratify patients into favorable or adverse disease outcomes after treatment. Conventional cytogenetics typically involve staining of bone marrow cells with fluorescent *in situ* hybridization (FISH) techniques. Other conventional techniques used in the initial assessment of disease include amplification of selected DNA regions with the polymerase chain reaction method (PCR) and whole-exome high-throughput sequencing to perform mutational analysis.

Roughly 10% alterations in chromosome numbers are found in ALL cases (Harrison, 2009; Hunger & Mullighan, 2015b). Overall, distinct types of cytogenetic abnormalities are present in about 75% of ALL cases, which means that chromosomal alterations are a recurrent phenomenon in this disease. The most common translocation in adult ALL – which is detected in 20-30% of adult cases and will be discussed in further detail below – is the Philadelphia (Ph) chromosome, named after the city where it was first identified (Nowell & Hungerford, 1960). Other common translocations seen in B-ALL include *MLL*-rearrangements, and *ETV6-RUNX1*, with the prevalence of these and other subtypes varying with age, as shown in adapted (**Figure 1.3**). Mutational analysis has also revealed that more than 40% B-ALL subtypes harbor distinct sequence mutations in signaling pathways involving tumor suppression, cell cycle activity, epigenetic control and regulation of lymphoid development (Mullighan et al., 2007). These include alterations in transcription factors like *IKZF1* and *PAX5* in about 30% and 25% of cases, respectively, and cell cycle regulation genes like *CDKN2A/2B*.

Figure 1.3

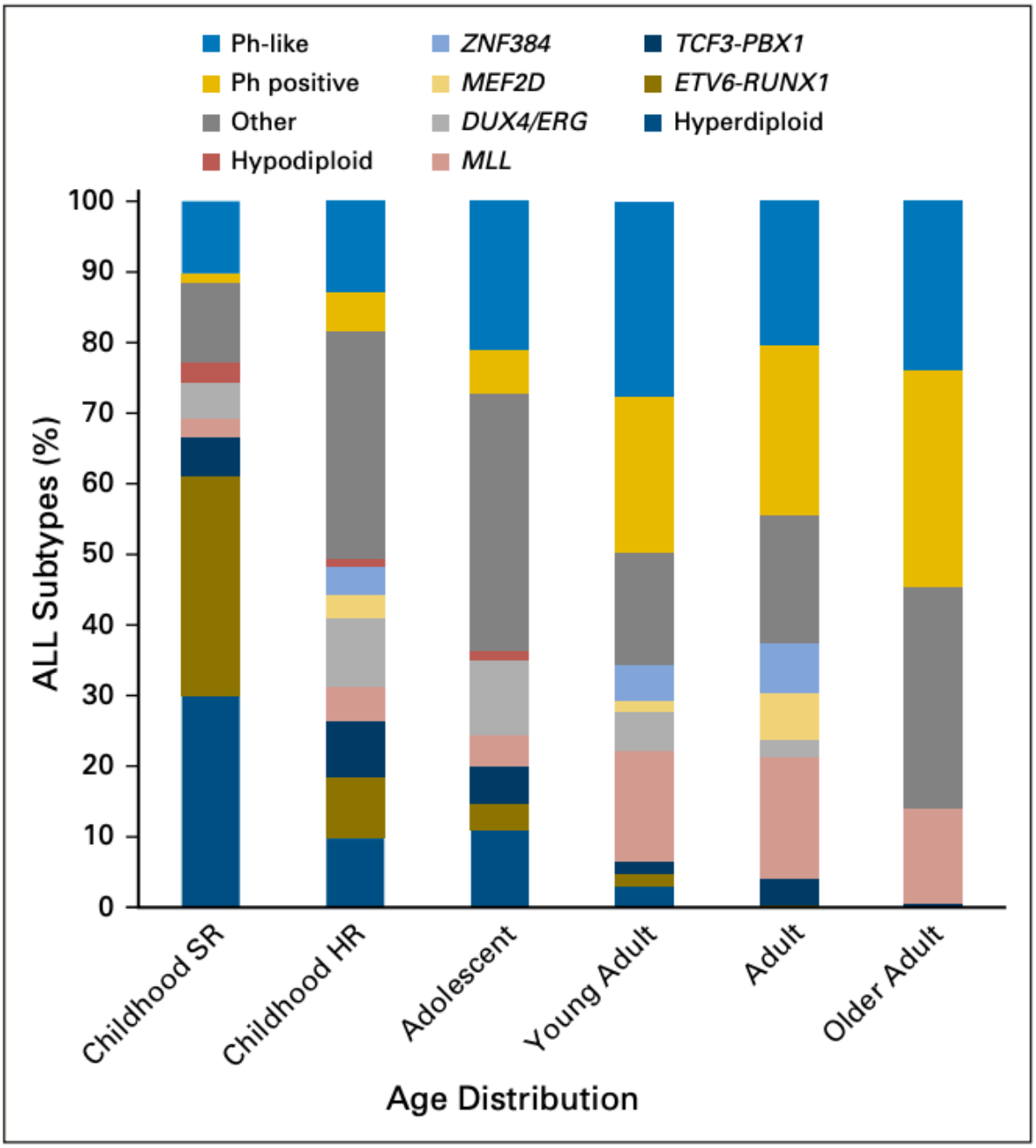
Ph-like
Ph positive
Other
Hypodiploid
ZNF384
MEF2D
DUX4/ERG
MLL
TCF3-PBX1
ETV6-RUNX1
Hyperdiploid
ALL Subtypes (%)
100
90
80
70
60
50
40
30
20
10
0
Childhood SR
Childhood HR
Adolescent
Young Adult
Adult
Older Adult
Age Distribution

Figure 1.3 | Acute lymphoblastic leukemia (ALL) subtypes distributed by age. Standard-risk (SR) encompasses ages 1-9, high-risk (HR) encompasses ages 10-15, adolescents encompass ages 16-20, young adults encompass ages 21-39, adults encompass ages 40-59, and older adults encompasses ages 60 and older. "Other" category includes ALLs without recurrent abnormalities. Figure adapted as is from (Iacobucci & Mullighan, 2017).

2.1.3 Treatment of B-ALL

Frontline therapy against B-ALL is given over the course of 3 years and is divided into three phases: remission or induction, consolidation, and maintenance (Hunger & Mullighan, 2015a). The goal of remission therapy, which can last from 4-6 weeks, is to debulk the tumor and to allow for the recovery of normal hematopoiesis. Drug regimens that elicit CRs of 75-90% include, combinations of chemotherapeutics like vincristine, glucocorticoids, asparaginase, and an anthracycline (Pui et al., 2008). Some regimens also include the use of cyclophosphamide and intrathecal chemotherapy, with the latter intended to eliminate disease present in the central nervous system (CNS) (Aur et al., 1971; Kantarjian et al., 2004).

Post-remission or consolidation therapy is typically administered for 6 to 8 months to circumvent what might otherwise be a short remission. Intensive combination chemotherapy is once again administered to avoid development of CNS leukemia and eliminate residual disease. Diverse post-remission regimens have been developed, with many of them including the same drugs administered for initial induction, plus the addition of high-dose methotrexate, cytarabine, and asparaginase (Pui et al., 2008). Finally, maintenance or continuation treatment is an

antimetabolite low-intensity therapy regimen that typically lasts for 18-30 months. The backbone of drugs used at this stage is composed of daily administration of mercaptopurine and methotrexate, with some regimens adding the use of vincristine and glucocorticoids.

Relapse occurs in about 20% of childhood ALL cases and it occurs more frequently with increasing age (Nguyen et al., 2008). Relapsed disease has much lower cure rates and adverse prognostic factors include: a shorter time-to-relapse, and bone marrow relapse over extramedullary relapse (Raetz & Bhatla, 2012). Unfortunately, many of the relapsed patients will develop resistance to common therapeutic drugs (Mullighan et al., 2011; Meyer et al., 2013).

Antibody-targeted and cellular immunotherapies

Acute lymphoid leukemias can be categorized based on the cell surface markers they express. The antigens expressed on their cell surface provide information regarding the differentiation state of the cell and prognostic information. In general, the immunophenotype presented by B-ALL cells include the expression of CD19, CD20, CD22, and CD52, among others (Campos-Sanchez et al., 2011; Terwilliger & Abdul-Hay, 2017). Consequently, several immunotherapies have been developed to target specific markers on B-cell lymphoblasts.

CD20 is expressed on the surface of 30-50% B-cell leukemic blasts, and can be targeted by a handful of monoclonal antibodies like rituximab, ofatumumab, and obinutuzumab (Jabbour et al., 2015; Man et al., 2017). Binding of rituximab to CD20 promotes the removal of B cells from the circulation through apoptosis induction, ADCC, and complement-mediated cytotoxicity.

Patients with Ph^-, $CD20^+$ B-ALL have shown improved 2-year PFS and OS when treated with a combination of chemotherapy and rituximab (Jabbour & Kantarjian, 2016; Maury et al., 2016; Salvaris & Fedele, 2021).

CD22 is expressed on the surface of roughly 90% B-cell lineage blasts, and is targeted with inotuzumab ozogamicin, moxetumomab pasudotox, coltuximab ravtansine, and epratuzumab (Sullivan-Chang et al., 2013; Jabbour et al., 2015). Additionally, another antigen expressed in 36-66% of leukemic blasts is CD52 (Hu et al., 2009). The recombinant monoclonal antibody alemtuzumab targets $CD52^+$ cells and causes ADCC-mediated lysis, although the use of this drug has been linked with severe and toxic immunosuppression (Jabbour et al., 2015; Mohseni et al., 2018).

With the exception of fully differentiated transformed plasma cells, CD19 is the most widely expressed B-lineage specific antigen, with more than 90% B-ALL blasts having this surface marker present during all stages of maturation. CD19 is the target of blinatumomab and denintuzumab mafodotin (Man et al., 2017). Briefly, blinatumomab is a bispecific T-cell engager (BiTE) antibody that binds CD3 on the surface of T cells and CD19 on B cells. Treatment with blinatumomab results in the release of inflammatory cytokines, proliferation of T cells and $CD19^+$ cell lysis. As a single agent, this antibody produces CRs of 40-45% in patients with relapsed or treatment refractory B-ALL (Schlegel et al., 2014; Topp et al., 2015).

A recently FDA approved and exciting approach to target leukemias is the genetic engineering of T cells that express an anti-CD19 antibody fragment coupled with intracellular signaling domains of the TCR. These chimeric antigen receptor-modified T cells (CAR T-cells) recognize and kill $CD19^+$ cells in an MHC-independent manner (Kochenderfer & Rosenberg, 2013; Singh & McGuirk, 2020). Even second and third generations of CAR T-cells have been developed, in which one or two extra costimulatory domains are included in the engineered T cells to enhance its replicative, and cytotoxic properties (Imai et al., 2004; Savoldo et al., 2011; Maude et al., 2015). Therapy with CAR T-cells have produced outstanding CR rates (70-90%) in patients with relapsed and refractory B-ALL (Davila et al., 2014; Lee et al., 2015).

CAR T therapy failure can occur via different mechanisms. These include the limited longevity of CAR T-cells in patients, an issue that can be circumvented with reinfusion of new CAR T-cells, cost permitting (Xu et al., 2020). Additionally, relapsed $CD19^-$ disease has been shown to be a major form of CAR T-cell therapy escape (Lacey et al., 2016). These challenges have prompted the development of CAR T-cells that target additional antigens on the surface of B-ALL cells (Haso et al., 2013; Shah et al., 2016). Furthermore, CAR T-cell therapy is associated with adverse effects and toxicities to patients that limit therapy effectiveness, and these include hypotension, fevers, neurologic complications, and severe cytokine release syndrome (CRS) (Kochenderfer et al., 2012; Maude et al., 2014). Although these effects can be mitigated by therapy with anti-IL6 monoclonal antibodies, the timing and method of administration could still be optimized and standardized (Neelapu et al., 2018; Chen et al., 2019). Interestingly, treatment with IL-6 signaling inhibitors could also reduce the need for adjuvant concomitant therapy. This could prove

particularly beneficial in reducing the overall comorbidities induced by high-intensity treatment schedules for B-ALL patients, a concept supported by the results presented in Chapter 2 of my thesis.

2.1.4 BCR-ABL$^+$ B-ALL

Although most common in adults, the *BCR-ABL* translocation has a frequency of 2-5% in pediatric ALL cases, and of 20% for young adults (Gleißner et al., 2002). The Ph$^+$ chromosome results from the t(9;22)(q34.1;q11.2) translocation, and encodes for a constitutively active tyrosine kinase. This reciprocal translocation places most of the *ABL1* proto-oncogene (exons 2-11) on chromosome 9 end-to-end with the 5' region of the *BCR* gene on chromosome 22 (Iacobucci & Mullighan, 2017). The genetic product of this translocation can lead to the formation of two isoforms, depending on the site of translocation or breakpoint. The major breakpoint creates a 210 kDa protein that is detected in 24-50% of adult BCR-ABL$^+$ cases, while much rarer in childhood cases (Westbrook et al., 1992; Chissoe et al., 1995; Rieder et al., 1996). Alternatively, the minor breakpoint generates a protein of 190 kDa which can be identified in 55-77% of adult cases and in more than 90% of pediatric patients (Jain et al., 2017).

Mechanistically, multiple signaling pathways are downstream of the BCR-ABL protein. These include pathways such as MAPK, Ras, NF-kB, c-Myc, PI-3K, and JAK-STAT (Sattler & Griffin, 2001). In addition, BCR-ABL activity can alter pro- and anti-apoptotic proteins and lead to uncontrolled proliferation (Man et al., 2017). Therefore, BCR-ABL$^+$ B-ALL is a high-risk subset of ALL that is associated with adverse and poor prognosis. Chemotherapy alone produced long-term survival

rates in less than 20% of these patients (Bloomfield et al., 1986; Faderl et al., 2000). Consequently, for patients with suitable donors, allogeneic stem cell transplant (ASCT) in the first remission became the only curative option (Fielding et al., 2009). Fortunately, the development of tyrosine kinase inhibitors (TKIs) against this chimeric protein ultimately allowed for the significant improvement of response rates and overall survival of these patients (Liu-Dumlao et al., 2012). The use of TKIs as frontline therapy has even improved patient outcomes to the point where ASCT may not be necessary for many patients (Thomas et al., 2004).

BCR-ABL translocations are associated with two different hematological malignancies, inducing either chronic myelogenous leukemia (CML) or B-ALL. Seminal studies from David Baltimore, Owen Witte, and colleagues, demonstrated the cancer driving properties of the BCR-ABL fusion protein in CML, paving the road towards the development of targeted inhibitors against this oncogene (Daley et al., 1990; Lugo et al., 1990). The first TKI drug developed, imatinib (commercially known as Gleevec), was pioneered by Brian Druker and colleagues (Druker et al., 1996). This drug obtained FDA approval in the year 2000, and due to its success in the treatment of CML, other groups developed second and third generation TKIs against BCR-ABL (Druker et al., 2001a; Druker et al., 2001b; O'Brien et al., 2003; Puttini et al., 2006; Saglio et al., 2010; Cortes et al., 2013).

Early studies with imatinib and other first-generation inhibitors failed to show long-term effectiveness in patients with Ph^+ B-ALL (Bernt et al., 2014). Instead, it was soon after found that integrating the use of TKIs with chemotherapy regimens drastically improved the OS of $BCR\text{-}ABL^+$

B-ALL patients (Schultz et al., 2009; Boulos et al., 2011; Abou Dalle et al., 2019). As discussed above, effective treatment of B-ALL requires intensive chemotherapy combinations. Specifically, for the management of Ph^+ B-ALL two possible strategies exist: intensive chemotherapy with hyper-CVAD (cyclophosphamide, vincristine, Adriamycin, and dexamethasone) and a TKI, or less intensive chemotherapy regimens with a TKI (Daver et al., 2015).

However, while complete responses have improved, relapses of BCR-ABL^+ B-ALL remains a problem, even when combining TKIs with chemotherapy regimens (El Fakih et al., 2018). The survival of Ph^+ B-ALL patients remains minimal relative to other subtypes of B-ALL (Geyer et al., 2017). These observations highlight the need to further understand the biology of Ph^+ B-ALL, particularly its response to treatment and how to improve therapy combinations that lead to enhanced overall survival and eliminate the resurgence of therapy-refractory disease – ideally through the development of immunologic memory. Chapter 2 of this thesis will present and discuss rational combination-therapy approaches to treat Ph^+ B-ALL that generate long-term immune responses.

2.1.4a BCR-ABL^+ B-ALL mouse model

For the experiments presented in Chapter 2, I used a C57BL/6 transplantable mouse model of Ph^+ B-ALL developed in the research group of Charles Sherr (Williams et al., 2006; Williams et al., 2007). Briefly, bone marrow derived cells from *Arf*$^{-/-}$ donor mice were retrovirally infected with p190 BCR-ABL containing vectors and pre-B cells were later selected for and enriched. These

resulting transformed cells generate a highly penetrant disease that resembles human B-ALL when transplanted into immunocompetent syngeneic mice.

2.2 Immunogenic cell death

Cancer develops within and interacts with a complex cellular environment that can stimulate tumor growth, angiogenesis, invasion and metastasis, and fuel resistance or immune suppressive effects against many anti-cancer agents (Hanahan & Coussens, 2012). Fortunately, this tumor microenvironment is dynamic, meaning it can be remodeled or affected through therapies that modify the interactions between tumor cells and stromal cells, creating even more therapeutic opportunities. Specifically, several cytotoxic and cytostatic chemotherapeutics have the potential to increase the immunogenicity of tumor cells by inciting immunogenic cell death (ICD) – a form of regulated cell death that stimulates the activation of innate and adaptive immune responses (Galluzzi et al., 2020b). However, chemotherapeutic agents may also promote immunosuppressive adverse effects like myelo- and lympho-depletion, which can limit the desired immunostimulatory outcomes (Zitvogel et al., 2011). In this section, I review and summarize the studies on conventional cytotoxic chemotherapeutic induced immunogenicity, focusing on the responses from the host immune system.

2.2.1 Cell death and immunogenicity

Programmed cell death or apoptosis occurs as a part of normal development and maturation of cells, and is necessary for maintaining normal physiologic processes, like removing individual cells without damaging surrounding tissues. Cells undergoing this type of cell death are typically

phagocytosed by APCs and normally fail to elicit any sort of immune response – leading to immune-silent or tolerogenic cell death. However, preclinical data challenging this notion has accumulated over the last decades, and now it has become widely accepted that there are context-specific instances of immunogenic-apoptosis or stress-induced regulated cell death that can be inflammatory. Other forms of cell death, like necrosis, autophagy, necroptosis, and ferroptosis have also been reported to be immunogenic since release of intracellular inflammatory contents – adjuvants – usually accompany these processes (Green et al., 2009; Galluzzi et al., 2018; Galluzzi et al., 2020b). Ultimately, immune-silent death is differentiated from immunogenic-death based on a series of certain activated molecular pathways that can trigger antigen-specific immune responses.

ICD relies on three major processes to drive innate and adaptive immune responses: initiating stimulus, antigenicity, and adjuvanticity. Antigenicity refers to the target cells' capacity of expressing and presenting antigens that will activate the host's antigen-specific naïve T cell clones (Palucka & Coussens, 2016). As discussed above in the first Part of this Chapter, healthy cells will very rarely express antigens that fail to induce clonal deletion of T cells and central tolerance, therefore limiting their ability to stimulate ICD. Adjuvanticity refers to the spatiotemporal release of DAMPs that are necessary for the recruitment and maturation of APCs (Garg et al., 2015; Bloy et al., 2017). Therefore, adjuvanticity largely depends on dying cells and their ability to initiate danger signals, while antigenicity relies on both the dying cells' repertoire of antigens, and the host's TCR and BCR stock.

2.2.1a Types of ICD by initiating stimulus

Pathogen-induced ICD

Defense mechanisms against viruses and bacteria require the detection of PAMPs through PRRs. Once these warning signals are activated, infected cells can undergo autophagy and subsequently release immune activating cytokines like type-I IFNs. These pro-inflammatory cytokines activate APCs, which present antigen to $CD8^+$ and $CD4^+$ T cells to generate an adaptive immune response (Galluzzi et al., 2017).

Necroptotic ICD

Necroptosis is an "accidental" programmed cell death that produces irreversible plasma membrane permeation and dissolution (Wang et al., 2014). Necroptosis is normally provoked by the receptor-interacting serine/threonine kinase 3 (RIPK3)-catalyzed phosphorylation of the pseudokinase mixed lineage kinase domain-like (MLKL), which is responsible for membrane permeabilization (Dondelinger et al., 2014). This form of cell death is highly inflammatory and engages antigen-specific immune responses (Galluzzi et al., 2017).

ICD from physical cues

Irradiation, hypericin-based photodynamic therapy (PDT), and high hydrostatic pressure have all been shown to trigger ICD in mouse cancer models (Obeid et al., 2007a; Garg et al., 2012; Fucikova et al., 2014). The molecular pathways that account for the immunogenicity of cells exposed to these cues have been characterized less extensively compared to other inducers of

ICD. Overall, these pathways seem to be more heterogenous since not all danger signals are necessary for the engagement of immune responses (Galluzzi et al., 2017).

Therapy-induced ICD

Exposure to anti-tumor therapy elicits physical signals that activate cellular immune responses. Antigen presenting cells like DCs that are exposed to chemotherapy-killed or dying tumor cells become stimulated to upregulate co-stimulatory molecules and release pro-inflammatory cytokines to activate T lymphocytes (Galluzzi et al., 2017). This type of ICD will be further discussed below since the chemotherapeutic agent used in the body of work presented in Chapter 2 of this thesis is proposed to induce immunogenic cell death.

2.2.2 Immunogenic chemotherapy

Chemotherapy treated cancer cells emit signals as they succumb to immunogenic cell death. These signals or events include tumor cell surface exposure of the endoplasmic reticulum chaperone protein calreticulin (CRT), which generates an “eat-me” signal that promotes tumor antigen engulfment (Obeid et al., 2007a; Obeid et al., 2007b). Another indicator of ICD is the release of ATP from lysosomes, which can induce NK cell proliferation and production of IFNγ, plus recruitment and maturation of macrophages (Elliott et al., 2009; Beavis et al., 2012). Additionally, certain ICD inducing drugs may also stimulate type-I IFN signaling pathways, which contribute to activation of host antitumor immunity (Sistigu et al., 2014). Post-apoptotic release of the nuclear chromatin binding protein HMGB1 is another well characterized signal that leads to activation of multiple toll-like receptors (Yabai et al., 2009; Li et al., 2013). Studies using cells

with loss-of-function alleles for TLRs highlight the importance of these chemotherapy-induced ICD responses (Apetoh et al., 2007). Ultimately, the release of HMGB1 stimulates and enables antigen presentation by DCs, which leads to the activation of CD8+ T cells (Zitvogel et al., 2008).

Other molecular chaperones that appear on tumor cell surfaces include proteins such as HSP90, which help in enhancing DC maturation (Spisek et al., 2007). ICD also allows for the downregulation of "do not eat-me" signals presented on the surface of tumor cells. Once proteins like CD31, CD46, and CD47 are no longer presented on the surface of tumor cells, tumor phagocytosis and DC-tumor cell adhesion are enhanced (Chao et al., 2010; Martins et al., 2010). Chemotherapy may also intensify tumor cell immunogenicity by prompting expression of MHC-I proteins and the presentation of tumor-specific antigens (Zitvogel et al., 2006). NK cells can also be prompted to increase and decrease the expression of stimulatory and inhibitory ligands, respectively (Khallouf et al., 2012). Conversely, factors that inhibit ICD include: extracellular hydrolysis of ATP, conversion of AMP into the highly immunosuppressive nucleoside adenosine, and depletion of myeloid and lymphoid compartments (Feng et al., 2011; Beavis et al., 2012). Interestingly, ablation of immune components can also target immunosuppressive cell types like T_{REGs} and myeloid-derived suppressor cells (MDSCs), resulting in desired anti-cancer drug responses (Sanchez-Perez et al., 2014; Ahlmann & Hempel, 2016).

Several cancer chemotherapeutic drugs have the potential to induce ICD. These include cyclophosphamide, mitoxantrone, oxaliplatin, epirubicin, idarubicin, and doxorubicin (Pol et al., 2015; Hernández et al., 2021). ICD drugs are not only proposed to induce innate and adaptive

immune responses after treatment, but are also believed to have prophylactic benefits. Thus, one of the approaches to determine whether cell death may be immunogenic is to perform vaccination experiments (Kepp et al., 2014; Galluzzi et al., 2020b). Essentially, dying tumor cells treated *ex vivo* are transplanted into immunocompetent syngeneic mice with the hope of immunizing the transplant host against further challenges with the same tumor – serving, in principle, as an anti-cancer vaccine. However, not all immunogenic therapy agents are capable of showing vaccine activity in mouse models (Obeid et al., 2007b).

Cell-based assays for the characterization of ICD features (release of HMGB1, CRT surface exposure, etc.) are necessary to determine the ability of a drug to induce ICD. While some drugs are capable of triggering multiple of these molecular pathways, not all drugs are equally successful. Therefore, cancer chemotherapeutics characterized as non-immunogenic in one setting or drug-tumor combination, may still stimulate immune responses in another context. As an example, the drug cisplatin is unable to stimulate CRT surface expression but can induce HMGB1 release in colon cancers, and it can also be used to eliminate MDSCs in a model of mouse melanoma (Tesniere et al., 2010; Chen et al., 2012). These effects could also be confounded by the dose and treatment schedule, and drug combinations chosen for concomitant administration (Wu & Waxman, 2018; Deutsch et al., 2019).

2.2.2a Anthracyclines

This class of antibiotic drugs has been used as anti-tumor agents for numerous cancer types since the 1960s, and ranks amongst the most effective cancer therapies ever developed (Weiss, 1992).

Daunorubicin (DNR) and doxorubicin (DOX) were the first anthracyclines to be discovered and characterized. The only structural difference between these compounds is the presence of a primary alcohol in DOX, which is substituted for methyl in DNR. Although a minor change, this alcohol substitution has important implications for the activity of both anthracyclines. Whereas DOX is essential for the treatment of breast cancer, childhood solid tumors, sarcomas, and lymphomas, DNR is routinely used for treating acute lymphoblastic and myeloblastic leukemias (Minotti et al., 2004). However, these drugs are used somewhat interchangeably, as Adriamycin is the brand name for DOX, which is used as part of the hyper-CVAD regimen for management of Ph^+ B-ALL, as mentioned above (Daver et al., 2015).

With the widespread use of anthracyclines in clinical patients, several mechanisms of action have been proposed to be involved in tumor control. These mechanisms of action include: intercalation between the base pairs of DNA, which leads to macromolecule synthesis inhibition; generation of free radicals, that also leads to DNA damage and lipid degradation or peroxidation; DNA binding and alkylation – alkyl groups added to DNA bases can promote DNA mutations; DNA cross-linking, which can halt DNA replication and transcription; and interference with DNA unwinding and strand separation via inhibition of topoisomerase-II activity, which results in DNA damage and/or apoptosis (Gewirtz, 1999; Tacar et al., 2013). Nonetheless, the concentrations at which DOX and other anthracyclines are clinically administered could ultimately determine if one or multiple modes of action are exhibited over others.

Anthracyclines were one of the first classes of agents to be identified as promoting immunogenic responses after treatment. Early studies performed by Guido Kroemer and colleagues showed that DOX-induced apoptosis of *in vitro*, *ex vivo*, and *in vivo* treated cells is capable of inducing ICD and subsequent activation of cellular immune responses (Casares et al., 2005; Obeid et al., 2007b). Others have also shown that anthracyclines play important roles in eliciting immune responses (Fucikova et al., 2011; Inoue et al., 2014). Indeed, humoral and cellular immune responses are associated with anthracycline treatments. For example, DOX treatment of different cancer types led to the production of IL-17, which in turn allowed for the activation of IFNγ producing $CD8^+$ T cells (Ma et al., 2011). Additionally, starvation-induced autophagy and mitoxantrone-induced ICD has been shown to provoke tumor regression while reducing the toxicity associated with this anthracycline (Castoldi et al., 2019).

Despite these preclinical studies that attest to the potential of various ICD-inducing drugs, anti-tumor responses in the clinic are frequently followed by tumor regrowth. Possible reasons why clinicians see little immunogenicity from these agents in the clinic include that many of these chemotherapeutics have been largely characterized as ICD-inducing in immunodeficient settings (e.g., human xenograft models), which can produce misleading or incomplete conclusions (Gardai et al., 2005). Additionally, cytotoxic chemotherapeutic drugs are historically administered on a maximum tolerated dose (MTD) schedule. This approach can lead to high toxicity, myelo- and lympho-depletion, and selection of drug-resistant clones, and in most cases, this is accompanied by the absence of immune-monitoring (Holohan et al., 2013; Pol et al., 2015). Therefore, many,

if not all, ICD inducers are currently administered to ensure maximal cytotoxicity at the potential expense of activating host immune responses.

Appealing approaches to overcome these issues include the use of combination chemotherapy with immunotherapies, and employing ICD inducers at low-metronomic doses (Chen et al., 2017; Kerbel & Shaked, 2017). Immunotherapy combination treatments could range from the use of anti-tumor vaccines, treatment with TLR agonists, and inhibitors of immunosuppressors – like the use of immune checkpoint inhibitors, and targeting suppressive cytokines. In fact, the work presented in Chapter 2 of this thesis speaks to the advantages of combining conventional cytotoxic chemotherapeutics with immunotherapies. Therefore, it is evident that optimally designed chemotherapeutic regimens have real promise to improve patient care via the immune activating properties of ICD, as evidenced by the abundance of active clinical trials involving DOX and other anthracyclines in combinations with other immunotherapies (Vanmeerbeek et al., 2020).

2.2.3 Immune checkpoint inhibitors

Immunotherapies are currently the most rapidly expanding cancer drug class. The oncology and anticancer therapeutic field have been completely revolutionized since the advent of cancer immunotherapies, particularly with the development of immune checkpoint-inhibitors or -blockade (ICIs or ICB) (Sharma & Allison, 2015). The objective of this class of drugs is to engage immune pathways against cancer by ultimately harnessing and unleashing T cell responses. Initially, efforts for developing cancer immunotherapies were focused in amplifying immune

activation mechanisms like enhancing antigen uptake by APCs, and promoting T cell priming and proliferation (Sanmamed & Chen, 2018; Tang et al., 2018). However, these immune enhancing strategies often result in scarce objective responses in patients due to frequent immune-related adverse events, or to homeostatic signaling programs that aim to stop immune responses before they become too harmful for the host. As an example, and as discussed in the first Part of this Chapter, some TCRs may develop cross-reactivity with self-antigens during T cell maturation. To prevent these cytotoxic cells from attacking self, numerous immune checkpoint pathways regulate T cell activity during an immune response – part of the process of peripheral tolerance. Therefore, based on these results, much of the recent efforts to develop immunotherapies has shifted to the development of inhibitors against these immune checkpoint pathways and to restoring anti-tumor T cell responses that were already initiated.

Blocking inhibitory checkpoints with ICIs has been demonstrated to successfully extend survival in patients with a variety of tumor types (van den Eertwegh et al., 2012; Sanmamed & Chen, 2018). Two immune checkpoint pathways central to the process of peripheral tolerance have gained significant traction as targets for ICIs over the years: the programmed death-1 (PD-1), and the cytotoxic T-lymphocyte antigen-4 (CTLA-4) proteins. In fact, the 2018 Nobel Prize in Physiology or Medicine was jointly awarded to Tasuku Honjo and James Allison for their discoveries and contributions to the molecular understating of these checkpoint pathways (Huang & Chang, 2019). The PD-1 and CTLA-4 pathways are now known to operate at different stages of an immune response. CTLA-4 effects are typically seen at the initial stages of T-cell

activation in the lymph nodes, while the PD-1 pathway regulates previously activated T cells at later stages of the immune response, within peripheral tissues (Ribas & Wolchok, 2018).

CTLA-4 pathway

CTLA-4 or CD152 is expressed on T_C cells upon TCR activation and co-stimulatory engagement through CD28. Once T cell activation occurs, intracellular CTLA-4 translocates to the cell surface where it outcompetes CD28 for binding with CD80 or CD86 surface proteins expressed on APCs. This, in turn, results in T cell-intrinsic suppression that leads to proliferation and activation arrest (Brunner et al., 1999; Chambers et al., 2001). Additionally, CTLA-4 can be expressed on T_{REG} cells where it can play a role in T_{REG}-mediated immune tolerance by stimulating the release of indoleamine 2,3-dioxygenase (IDO), an immunosuppressive mediator (Wing et al., 2008).

Clinical trials published in the year 2000 introduced the use of two fully-humanized CTLA-4 inhibitors, the antibodies ipilimumab and tremelimumab; with ipilimumab (Yervoy) receiving FDA approval by 2011. These antibodies were generating durable tumor regressions, particularly in patients with advanced metastatic melanoma, where a 15% objective response rate that has even lasted for more than 10 years after therapy stop in a fraction of patients was recorded (Hodi et al., 2010; Eroglu et al., 2015; Schadendorf et al., 2015). Further studies revealed that patients who respond better to this therapy tend to have tumors with higher mutational burden (Van Allen et al., 2015).

PD-1 pathway

PD-1 or CD279, upon engagement of its ligands PD-L1 or PD-L2 (CD274 and CD273, respectively), recruits the tyrosine phosphatase SHP2 and inhibits the signaling cascade of the TCR (Ishida et al., 1992; Hui et al., 2017). PD-1 can be expressed by activated T cells, B cells, and myeloid cells. The ligands for PD-1 are normally expressed on the surface of cells within a tumor, with PD-L1 being more broadly expressed by many somatic cells upon stimulation with inflammatory cytokines (Baumeister et al., 2016). PD-L2 expression is mainly restricted to APCs. PD-1 engagement with its ligands induces T cell exhaustion and restrains antitumor cytotoxic T cell responses (Wherry, 2011; Ribas, 2015).

PD-1 is a negative regulator of pre-existing immune responses, and its blockade results in preferential activity stimulation of already existing antitumor T cells. Therapeutic blockade of the PD-1 pathway leads to durable response rates in multiple cancer types, including melanoma, renal cell carcinoma, and non-small cell lung cancer. A handful of anti-PD-1 or anti-PD-L1 antibodies are already approved by the FDA, with nivolumab being the first inhibitor used in patients in 2006 (Sznol et al., 2010). Similar to CTLA-4 inhibitory therapy, patients with tumors that have high mutational burden develop the highest antitumor activity when treated with anti-PD-1 or anti-PD-L1 therapy (Ribas & Wolchok, 2018).

2.2.3a CTLA-4 and PD-1 blockade combination therapy

Syngeneic mouse model studies found evidence of synergy when combining inhibitory treatment of these two pathways, since the two have non-redundant co-inhibitory roles (Postow et al.,

2015; Wei et al., 2017). By 2009, the first patients began treatment with combination checkpoint blockade using ipilimumab and nivolumab to block CTLA-4 and PD-1, respectively. In the initial phase 1 clinical trial, objective responses higher than 50% were observed in patients with metastatic melanoma treated with combination immunotherapy. While phase 2 and 3 studies confirmed a response rate of approximately 60% with higher 3-year survival compared to patients receiving single therapy alone (Wolchok et al., 2017).

2.2.3b Immunogenic chemotherapy and immune checkpoint inhibitors

Single agent PD-1 pathway blockade has relatively less unfavorable toxicities compared to single agent CTLA-4 therapies (Boutros et al., 2016; Postow et al., 2018). However, these toxicities can often lead to moderate-to-severe immunological adverse events that will require immunosuppressive drug administration in some patients, particularly when these agents are used in combination. Thus, a substantial number of research efforts have focused on combining other standard of care chemotherapeutics with ICIs, with the hope to achieve both early- and long-term disease control with limited overlapping toxicities (Galluzzi et al., 2020a).

In theory, ICD-inducing therapies can initiate or restore immune responses by converting 'cold' tumors into abundantly immune cell-infiltrated 'hot' tumors that become poised to better respond to ICIs (Galon & Bruni, 2019). For example, neoadjuvant chemotherapy – administered before main treatments like surgery removal – has been shown to aide in the reduction of T_{REG} cells from breast cancer patient tumors (Ladoire et al., 2008). Additionally, numerous induction-chemotherapies like oxaliplatin, cisplatin, docetaxel and 5-fluoroacil have been shown to induce

PD-L1 expression owing to excessive tumor-infiltrating myeloid cells and T cells, constituting a barrier to the effectiveness of these agents (Ding et al., 2014; Leduc et al., 2018). Thus, blockade with ICIs could prove useful in enhancing the immune responses prompted by the use of ICD-inducers. However, much work remains to be performed to understand the optimal dosing and administration schedules necessary to determine the most effective combination of chemotherapeutics with ICI immunotherapies.

Chapter 1, Part III: The tumor microenvironment and IL-6 in cancer inflammation

3.1 The importance of the tumor microenvironment

The TME is the complex and rich multicellular ecosystem in which a tumor develops. Typically, the TME is constituted of the following components: immune cells like T and B lymphocytes, tumor-associated macrophages (TAMs), MDSCs, DCs, NK cells, and neutrophils; other stromal cell types such as cancer-associated fibroblasts (CAFs), pericytes, and mesenchymal stromal cells; the extracellular matrix (ECM), secreted molecules like growth factors, cytokines, chemokines, and extracellular vesicles; and the blood and lymphatic vascular networks (Balkwill et al., 2012; Marar et al., 2021). This environment dynamically regulates cancer establishment and progression, and can influence therapeutic outcome. The non-malignant cells of the TME often have pro-tumorigenic qualities, while malignant cells eventually can invade and spread to healthy tissues through the circulatory or lymphatic system (Hanahan & Weinberg, 2011).

Over the past decades, our understating of the complexity of the TME has evolved. Importantly, it has become abundantly clear that depending on the site of tumorigenesis and the stage of cancer progression, cells in the TME can be either tumor-suppressive or tumor-supportive (Salmon et al., 2019). Given these opposing functions, multiple strategies to therapeutically target the TME have been developed. Therapeutically targeting the TME was initially believed to be fairly straight forward and to represent a "one size fits all" approach (Joyce, 2005). However, the TME is now recognized to regulate therapeutic interventions by manifesting intrinsic resistance, revealing resistance that existed prior to treatment, or by acquiring resistance during the course of treatment (Binnewies et al., 2018; Bejarano et al., 2021). For example, both radiotherapy and chemotherapy can aide in the recruitment of immunosuppressive TAMs to tumors, which results in inhibition of therapy-induced cancer cell death (Seifert et al., 2016; Olson et al., 2017). Importantly, certain interventions that target the TME can also have anti-tumorigenic synergistic effects. These include the depletion or recruitment of pro- and anti-tumorigenic cell sub types, respectively, promoting immunogenic cell death, and encouraging T cell-dependent antitumor immunity. Some of these approaches were discussed in the previous Part of this Chapter.

3.1.1 Major stromal components of the tumor microenvironment

Normal cells, initially present or recruited to the tumor, are pivotal participants in tumorigenesis. These stromal cells contribute to the development and expression of many cancer hallmarks by producing and releasing growth and homeostatic promoting signals (Hanahan & Weinberg,

2011). Therefore, the biology of a tumor can only be fully elucidated if the individualized cell types within the TME and their complexity are also understood.

Macrophages

Tissue homeostasis and protection against infection is largely carried out by phagocytic cells that engulf dying cells and clear cellular debris. In cancer, these homeostatic functions are normally suppressed since many myeloid cells within tumors are incompletely differentiated – e.g., monocyte-derived cells (DeNardo & Ruffell, 2019). Fully differentiated cells, like tissue-resident macrophages, and circulating monocytes that fail to fully differentiate, collectively represent the TAMs and MDSCs. Evidence now shows that these cell types play important roles in modulating immune responses by secreting cytokines and chemokines (Gabrilovich & Nagaraj, 2009; Cassetta & Pollard, 2018).

T lymphocytes

As discussed previously, T cells can be classified into $CD4^+$ and $CD8^+$ T cells. APCs load antigen onto MHC-I and -II molecules and present them to $CD8^+$ and $CD4^+$ T cells, respectively. Importantly, $CD8^+$ T cells are also capable of detecting cross-presented antigens loaded onto MHC class-I molecules, which can lead to potent cytotoxic reactions that cause tumor cell death (Joffre et al., 2012; Freidrich et al., 2017).

Dendritic cells

Dendritic cells are professional APCs, and are critically needed for an effective anticancer immune response. After TAA phagocytosis, DCs process antigens for MHC loading either through the cytosolic pathway or the vacuolar pathway. Mature DCs express greater levels of co-stimulatory molecules to properly prime T cell antitumor immunity. DCs can be classified according to their function and cytokine secretion profiles into three main subtypes: monocyte-derived DCs, plasmacytoid DCs, and conventional DCs type 1 or 2 (Salah et al., 2021).

Cancer-associated fibroblasts

In many solid malignancies, CAFs are the predominant cell population of the tumor stroma and are typically very heterogenous in both origin and functionality (Sahai et al., 2020). These cells have diverse functions like matrix deposition and remodeling, production of growth factors, and they participate in extensive signaling interactions with both cancer cells and infiltrating leukocytes. Importantly, these cells also modulate and influence angiogenesis, drug access, and therapy responses, making them potential targets for therapeutic strategies against cancer.

Extracellular matrix

The ECM is the physical network of secreted proteins and glycans that provide structure to a tissue. In normal development and under homeostatic conditions, fibroblast are normally the major producers of tissue ECM. However, tumor cells and endothelial cells can also produce components of the ECM, like collagens, laminins, hyaluronic acid, and proteoglycans (Henke et al., 2020).

Blood vessels

Nutrients and oxygen are critical for the growth of both primary and metastatic cancers. Therefore, tumors have developed numerous mechanisms for promoting tumor vascularization. These include the formation of new vessels through angiogenesis stimulation, co-opting preexisting vessels, and vascular mimicry – when cancer cells differentiate into endothelial-like cells (Qian et al., 2016).

Lymphatic vessels

The lymphatic vessel's primary role is to remove interstitial fluids from tissues – e.g., removal of water to prevent edema. Additionally, while not a formal part of the immune system, lymphatic vessels are also excellent transport routes for antigens and immune cells to draining lymph nodes. Unfortunately, these vessels can also be co-opted by cancer cells to promote dissemination and colonization into other tissues (Randoplh et al., 2017).

3.1.2 Hematological tumor microenvironments

Similar to all solid malignancies, hematologic tumors were initially believed to be solely driven by genetic or epigenetic lesions within transformed cells. However, it is now widely appreciated that the composition and function of TMEs also have an important role in the pathogenesis and chemoresistance of hematological malignancies. Similar to normal HSCs, leukemia stem and initiating cells (LSC and LIC, respectively) rely and co-opt the local microenvironment interactions that facilitate their survival within bone marrow and/or lymphoid organ niches. Thus, the

leukemic niche microenvironment also represents a therapeutic target for patients with hematologic malignancies (Forte et al., 2019).

The potential role of the leukemic niche as an oncogenic driver or facilitator of hematologic malignancies was initially suggested in the late 1990s (Dührsen & Hossfeld, 1996). Over the years, the characterization of alterations in both cancer and stromal cells have led to the proposition that two non-mutually exclusive contributions from hematopoietic niches to leukemogenesis are in place. First, that the transformation of niche cells predisposes the transformation of the malignant cells. For example, age-related remodeling of bone marrow niches has been shown to promote myeloid cell proliferation (Ho et al., 2019). Second, that niche remodeling is a consequence of transformed hematopoietic cell activity. Several studies have shown the effects of malignant cells on the transcriptome, proteome, and function of niche stromal cells (Flores-Figueroa et al., 2002; Méndez-Ferrer et al., 2020).

3.1.3 Therapy resistance mediated by the tumor microenvironment

Tumor recurrence following anticancer therapy remains a significant challenge in patient care. Environment-mediated drug resistance is one of the major contributors to the survival of cells exposed to therapy (Meads et al., 2009). Some of the basic mechanisms by which microenvironments confer chemoprotection will be briefly discussed in the following section.

Fibroblast-mediated resistance

Early co-culture experiments with irradiated and non-irradiated fibroblasts demonstrated that damaged fibroblasts supported tumor cell growth better than non-damaged cells, implying that this cell type played a role in therapy responses (Ohuchida et al., 2004). Now, it is widely appreciated that CAFs not only deposit ECM but also remodel the protein matrix to promote tumor invasion, metastasis, and resistance to therapies (Bhome et al., 2017). Additionally, secretion of growth factors and several cytokines like VEGF (vascular endothelial growth factor), IL-6, CXCL9, and TGF-β from CAFs have been shown to modulate angiogenesis and T cell responses, respectively (Fukumura et al., 1998; Fearon et al., 2014).

Vascular-mediated resistance

Compared to healthy tissues, the vasculature of tumors is dysfunctional and exhibits heterogenous permeability, which can result in a barrier against optimal drug delivery (Jain, 2005). Increased ECM deposition, reduced cellular tight junctions, and different rates of endothelial cell proliferation can all result in severe tumor vasculature abnormalities (De Palma et al., 2017). In many cases, this aberrant vasculature eventually leads to the creation of hypoxic environments which are also associated with increase tumor aggressiveness (Schito & Rey, 2020). Another consequence of dysfunctional vessels is the selective infiltration blockade of immune cell types like T_C lymphocytes (Schaaf et al., 2018). Thus, most efforts at therapeutically targeting the vasculature aim at either depleting dysfunctional vascular vessels or at normalizing these vessels to improve the delivery of drugs and immune cells.

Immune-mediated resistance

As proposed with the immunoediting theory, the immune system is an active component of the tumor microenvironment that can affect clinical responses and resistance. For example, tumors treated with antiangiogenic therapy may develop resistance by the intervention of immature myeloid cells and TAMs that secrete pro-inflammatory factors to compensate for lost angiogenic activity (Shojaei et al., 2007; Phan et al., 2013). Melanoma patients treated with targeted inhibitors developed an increase in tumor antigen expression that led to higher infiltration of $CD8^+$ cells. However, at time of progression, biopsies showed that $CD8^+$ infiltrates had decreased and there was emergence of immune cell exhaustion markers and ligands that promote T-cell inhibitory signals (Frederick et al., 2013). Therefore, even in cases with initial immune responses, many immune suppressive mechanisms may eventually interfere and manifest as inherent or acquired resistance to therapy (Bejarano et al., 2021).

3.2 Prognostic role of TME immune infiltrates

Apart from CAFs, immune cells are the most abundant non-cancerous cells found in the TME of many tumors. At the time of diagnosis, most cancers have evaded immunosurveillance and/or immune control. With the advent of ICIs for the treatment of numerous cancers, an enormous interest in the local immune context of tumors also emerged. This includes understanding the interactions between the immune system and the cancer cells before, during, and after treatment (Palucka & Coussens, 2016). If successful, anticancer immunotherapies like cytotoxic agents and targeted inhibitors, should be able to have profound effects on the local immune infiltrate and promote the destruction of cancerous cells. In fact, residual signs of pre-existing or

active anticancer immune responses can be detected and are normally indicative of more favorable prognoses (Galon et al., 2006; Mlecnik et al., 2011). These tumor-immune system interactions have already set the foundation for rationally stratifying risk and therapeutic strategies for patients.

3.2.1 Immune hot, altered-excluded, altered-immunosuppressed, and cold tumors

The Immunoscore is a recently designed consensus and standardized scoring system based on the quantification of two lymphocyte populations (CD3 and CD8) at the invasive margin and central regions of tumors (Angell & Galon, 2013; Galon et al., 2014). The Immunoscore ranges from 0 to 4, with 0 indicating low regional densities of both cell types, while a score of 4 indicates high immune cell presence at both locations. Highly infiltrated tumors are also widely referred as 'hot' tumors, and non-infiltrated tumors as 'cold' tumors. This immune-based classification of tumors has proven particularly successful for stratifying patients with colon cancers, and has also been validated in melanoma tumors (Gajewski et al., 2017; Pages et al., 2018).

Immune-classified tumors can also show altered phenotypes that resemble intermediates between highly- or non-infiltrated tumors. Camus and colleagues were the first to describe these altered phenotypes that manifest as "excluded" or "immunosuppressed" patterns (Camus et al., 2009). The excluded phenotype reflects the intrinsic ability of the host's immune system to engage a T-cell mediated immune response, which is then curtailed by the tumor and normally displayed as T cells localized around the tumor margins. On the other hand, immunosuppressed

phenotypes suggest the absence of physical barriers and is displayed as a low degree of immune infiltrates.

In general, tumors that have favorable prognoses have a dense T lymphocyte infiltrate. The main tools for immune-based classification of these tumors are the haematoxylin and eosin (H&E) staining and immunohistochemistry techniques. Both of these approaches achieve the spatial resolution and quantification necessary to classify tumor-infiltrating immune cells. Bulk tissue gene expression techniques are also used to document immune-infiltration criteria (Newman et al., 2015). Apart from the presence of tumor-infiltrating lymphocytes (TILs), the expression of PD-L1 on other immune or cancer cells is also another characteristic of hot tumors (Hegde et al., 2016). The opposite is true of cold tumors, which will typically lack PD-L1 and MHC-I expression as a sign of immunologically ignorant tissues. However, it is important to appreciate that the Immunoscore is not meant to replace other parameters of patient stratification, but to aide in the guidance of this practice.

3.3 Cytokines in cancer therapy

A recurring theme in this thesis has been how secreted factors like cytokines modulate growth and maturation of healthy cells, while helping coordinate all sorts of immune responses. Since these small molecular messengers are often released during defined periods of time, they enable the communication of cells in paracrine and autocrine ways over short distances. Following cytokine binding, target cells expressing the appropriate cytokine receptor will undergo intracellular signal amplification that results in gene transcription changes. Notably, cytokines

participate in pro- and anti-inflammatory events that range from tumor control to mediating the development of therapy resistance. These opposing consequences are typically the result of different cytokine/receptor concentrations, the timing of exposure, and whether one or more cytokines are participating in the induction of said cellular function or fate.

Numerous cytokines interfere with tumor cell growth either directly by provoking anti-proliferative and pro-apoptotic effects, or indirectly by stimulating immune cell cytotoxic activities (Conlon et al., 2019). These observations led to preclinical and clinical testing of recombinant cytokines as monotherapies or in combination therapies for tumor control. One example is the cytokine IL-2, which has been demonstrated to be clinically active against advanced renal cell carcinoma, and metastatic melanoma (Fyfe et al., 1995; Atkins et al., 1999). Another example is the use of IFNα in the clinic, which is approved for the treatment of melanoma, follicular non-Hodgkin lymphoma, and AIDS-related Kaposi's sarcomas (Groopman et al., 1984; Solal-Celigny et al., 1993; Kirkwood et al., 1996). Although the clinical use of these cytokines marked a milestone for cancer immunotherapy, the low response rates and high toxicity associated with high-dose systemic administrations highlighted the limitations of these approaches. This is especially true when compared to other immunotherapies like targeted and ICI therapies (Waldmann, 2018).

The next generation of cytokine drugs are already being tested clinically, and their examination are mainly based on the following three approaches (Berraondo et al., 2019). First, improving the pharmacokinetics of systemically administered recombinant cytokines. Optimized

pharmacokinetics should increase the half-life of the drugs in circulation, avoid complete kidney filtration, and potentially lead to higher concentrations of drugs in TMEs. Second, improving local administration of drugs. This is an alternative approach to achieve higher local concentrations in TMEs, which can be accomplished by direct injection of recombinant proteins or by intratumoral gene therapy (Jackaman et al., 2003; Hu et al., 2018). Third, using already approved immunotherapies in combination with cytokine drugs.

3.3.1 Inhibiting immunosuppressive cytokines

Numerous cytokines improve the process of antigen priming and presentation, increase the amount of effector immune cells in the TME, and enhance cytotoxic effects. Conversely, what about the cytokines that have the opposite effects and are considered immunosuppressive? Pro-tumorigenic cytokines released by tumor or stromal cells in the TME, and their effects, can be neutralized by the use of antagonistic antibodies, and small molecule inhibitors. However, many of these cytokines have context-dependent roles and may exert pro- and anti-tumor activities depending on other elements also found in the TME. In fact, the next Chapter of my thesis presents data that supports the neutralization of IL-6, a canonically pro-inflammatory cytokine, to enhance the effect of other immunotherapies. The final portion of this Chapter will be dedicated to discussing the biology of IL-6 and its role in the treatment of cancer.

Other examples of typically immunosuppressive cytokines include tumor necrosis factor-alpha (TNFα) and transforming growth factor-beta (TGF-β). TNFα is a pro-inflammatory cytokine mainly produced by myeloid cell lineages, although lymphocytes, endothelial cells, fibroblasts and

adipocytes can also produce and secrete this molecule. TNFα antagonists were first developed to treat autoimmune diseases such as rheumatoid arthritis and Crohn's disease, among others (Palladino et al., 2003). More recently, infliximab, one TNFα antagonist, became included in the treatment regimens for corticosteroid-refractory adverse immune effects associated with ICI therapy (Haanen et al., 2017). Staying true to its contextual roles, TNFα depletion can hamper antitumor immune responses when combined with other immunotherapies, while it can also help overcome resistance to anti-PD-1 therapy in melanoma (van Horssen et al., 2006; Bertrand et al., 2017).

During the initial stages of tumorigenesis, TGF-β can inhibit cell-cycle progression and limit the growth of transformed cells. However, in later stages of disease there are also instances when tumor cells develop resistance to these anti-proliferative activities (Akhurst & Hata, 2012). TGF-β can also have impact on other TME cells like decreasing the activity of $CD8^+$ lymphocytes and NK cells. Similar to TNFα, the activity of TGF-β inhibitors in combination with other immunotherapies is being clinically pursued (Knudson et al., 2018; Tauriello et al., 2018).

3.4 IL-6 biology: from discovery to drug development of a complex cytokine

In 1973, a group of researchers led by Tadamitsu Kishimoto reported that a soluble factor secreted by T cells was involved in the production of antibodies from B cells (Schimpl & Wecker, 1972; Kishimoto & Ishizaka, 1973). This soluble factor was later cloned as the human B-cell differentiation factor (BCDF) or B-cell stimulatory factor-2 (BSF-2) (Hirano et al., 1986). It was not until 1989 that molecules with different names and functions studied by various groups were

found to be identical to BSF-2, resulting in the consolidation of these identities into what is currently known to be IL-6 (Kishimoto, 1989; Tanaka et al., 2014). IL-6 activity is involved in several autoimmune diseases like rheumatoid arthritis, which prompted researchers and clinicians to better understand the biological role of IL-6 in these diseases and to develop IL-6 inhibitors as treatment options (Garbers et al., 2018; Kang et al., 2019).

In the late 90's, rheumatoid arthritis (RA) changed from a severely debilitating disease to a more manageable condition thanks to the advent of neutralizing antibodies against TNFα (Elliot et al., 1994). Around the same time, Kishimoto's group and others made interesting observations that *IL-6* knockout (*IL-6* KO) mice were completely protected from arthritis in specific disease models (Alonzi et al., 1998; Ohshima et al., 1998). These findings led to the development of the first humanized IL-6 receptor (IL-6R)-blocking antibody – tocilizumab – as an alternative strategy to treat RA (Sato et al., 1993; Tanaka et al., 2012). Approaches to design inhibitors that target the IL-6R were based on the knowledge that IL-6 signal transduction depended on the formation of hexameric complexes of IL-6, the IL-6R, and the glycoprotein 130 (gp130). Thus, it initially proved challenging to design inhibitors to target all the binding regions of this complex and impede signal transduction (Yawata et al., 1993). Targeting just the IL-6R was simpler since the concentrations of the receptor were shown to have less interpatient variability than concentrations of IL-6, which in theory could also help with selecting doses and timings of administration (Meyers et al., 1991). About 20 years later, tocilizumab (TCZ) is now approved for the treatment of RA and other conditions in more than 100 countries, and a handful of antibodies targeting IL-6 have also been developed since (Kishimoto, 2010; Choy et al., 2020).

3.4.1 IL-6 signaling

IL-6 is a 26 kDa protein made up of 184 amino acids that make four-helical turns. This cytokine is produced and secreted by many cell types which include activated immune cells like T- and B-lymphocytes, monocytes and macrophages, endothelial cells, fibroblasts, skeletal muscle cells, and hepatocytes (Choy & Rose-John, 2017). As suggested by its range of producers, IL-6 is a pleiotropic cytokine with functions in numerous organs and tissues, during homeostatic conditions and during infection, inflammation, and cancer (Heinrich et al., 1990; Scheller et al., 2011). For example, upon appropriate stimulation, like recognition of pathogens through TLRs, macrophages and dendritic cells can promptly synthesize and secrete IL-6. Additionally, lymphocytes not only secrete IL-6 but they also sense and respond to IL-6 presence. B cells stimulated by IL-6 differentiate into antibody producing plasma-cells, while it can also stimulate T-cell development into different subtypes of $CD4^+$ T cells (Tanaka et al., 2014).

IL-6 binds to the IL-6R, an 80 kDa protein that is completely devoid of signaling ability. The IL-6 signaling cascade is only initiated when the IL-6–IL-6R complex binds to the membrane and signal transducer protein gp130 – also known as IL-6R subunit beta (Schaper & Rose-John, 2015). While IL-6R expression is restricted to hepatocytes, epithelial cells, and some immune cells, expression of gp130 is ubiquitous, which explains the pleiotropic functions of IL-6. To add to the signaling complexity of this cytokine, the IL-6R can also exist in a soluble form (sIL-6R). In both human and mice, sIL-6R is generated via cleavage of membrane-bound IL-6R (mIL-6R) by the proteases ADAM10 and ADAM17. In humans only, sIL-6R can also be generated by the translation of alternatively spliced *IL6R* mRNA (Lust et al., 1992; Riethmueller et al., 2017). Overall, it is believed

that hepatocytes and hematopoietic cells are the main sources of sIL-6R in mice (McFarland-Mancini et al., 2010).

IL-6 binds to both forms of the receptor with similar affinity and several modes of gp130 activation can be initiated after complex formation with either receptor. First, classic IL-6R signaling is mediated by IL-6–mIL-6R and gp130 complexes. Second, trans-signaling occurs when IL-6–sIL-6R complexes bind the membrane-bound gp130. Importantly, this mode of signaling can stimulate cells that lack expression of mIL-6R, since gp130 is universally expressed (Rose-John & Heinrich, 1994). Last and more recently described, IL-6 trans-presentation is the third mode of gp130 activation by IL-6. To date, this process is restricted to specialized DCs that present complexed IL-6–mIL-6R to gp130 expressing T cells, which results in priming of pathogenic T helper 17 cells (Heink et al., 2017).

Neutralization of IL-6 with antibodies inhibits both classic and trans-signaling, while anti-IL-6R antibodies can block all three modes of signaling. IL-6 trans-signaling can be selectively blocked by the soluble form of gp130 (sgp130), which binds to IL-6–sIL-6R complexes (Jostock et al., 2001). sgp130 can also bind IL-6–mIL-6R complexes, but in doing so, it only blocks IL-6 trans-presentation and not classic signaling (Lamertz et al., 2018).

Once IL-6R signaling is initiated, two main pathways downstream of IL-6 are activated: the Janus kinase (JAK) signal transducer and activator of transcription 3 (STAT3) pathway, and the JAK-SH2 domain tyrosine phosphatase 2 (SHP2)-mitogen-activated protein kinase (MAPK) pathway

(Schaper & Rose-John, 2015). In the cytoplasm JAK is constitutively bound to gp130, and when IL-6 binds to its receptor, JAK phosphorylates STAT3, which stimulates the formation of homodimers. Then, STAT3 homodimers translocate into the nucleus where they act as a transcription factor of genes such as *BCL2*, *BIRC5*, *MYC*, *NOTCH1*, cyclins and several matrix proteases (Kang et al., 2019). STAT3-dependent IL-6R signaling also generates a negative feedback loop that terminates JAK activation. Briefly, STAT3 induces suppressor of cytokine signaling 1 (SOCS1) and SOCS3, which bind to activated JAK and phosphorylated gp130, respectively, and end their signaling activities (Babon et al., 2012).

3.4.2 IL-6 signaling inhibition in oncological indications

IL-6 has been shown to directly affect the proliferation of cancer cells through the engagement of several cellular processes. These include cell-cycle progression pathways in melanoma, where STAT3 upregulates the expression of cyclins like D1, D2, B1, and MYC, along with downregulation of CDK (Niu et al., 2002). Also, IL-6 can increase the expression of several survival proteins in breast cancer cells, such as Bcl-2, Bcl-XL, Mcl-1, survivin or BIRC5, and XIAP (Gritsko et al., 2006). Since STAT3 is activated in many cancer types, specific inhibitors have been developed as anticancer agents for different parts of the STAT3 signaling pathway (Gu et al., 2020). STAT3 has several conserved functional domains that include an N-terminal, a coiled-domain, a DNA-binding domain (DBD), Src-homology-2 (SH2) domain, and a transactivation domain (TA). The DBD allows STAT3 to bind downstream of the target gene-promoters to induce expression of such target genes. The SH2 domain facilitates protein-protein interactions with tyrosine phosphorylated proteins, which makes this domain critical for the formation of STAT3 dimers

(Abroun et al., 2015). Both STAT3 antagonists OPB-31121 and OPB-51602, target the SH2 domain of STAT3 and clinical trials of these drugs were conducted in patients with hepatocellular carcinoma and with non-small cell lung cancer, respectively; although harmful side effects have been associated with both compounds (Okusaka et al., 2015; Wong et al., 2015). Additionally, despite the molecular understating of STAT3 and the vast evidence implicating STAT3 in cancer progression, there is a lack of clinically available drugs that directly target this transcription factor (Gu et al., 2020).

Under most homeostatic conditions, the concentration of IL-6 in the circulation is around 1-5 pg/mL, while some pathologies can induce an increase to the ng/mL range (Calabrese & Rose-John, 2014). The levels of IL-6 can range from 100-500 pg/mL in numerous cancer types, which prompted the use of monoclonal anti-IL-6 antibodies in patients with prostate cancer and renal cell carcinoma (Rossi et al., 2010; Fizazi et al., 2012). These observations, the lack of clinically approved specific-downstream signaling inhibitors, and the advent of ICIs have generated many new hypotheses to broadly target IL-6 inhibition. Specifically, combining the limited efficacy of ICIs in some cancer types with general blockade of IL-6. For example, there are also accounts of increased IL-6 secretion from primary stellate cells in pancreatic ductal adenocarcinoma (PDAC) patients (Mace et al., 2018). In this same study, the authors report that IL-6 blockade combined with PD-L1 blockade promote neoplastic control through increased T cell infiltration into the tumors of PDAC bearing mice. This and a handful of other studies in solid malignancies, serve as pre-clinical evidence that blockade of IL-6 signaling may enhance the efficacy of other immunotherapies (Li et al., 2018; Tsukamoto et al., 2018).

Over the last decade, our research group has expanded the knowledge of IL-6 in disease, particularly its role in hematologic malignancies and therapeutic targeting in this context. For example, using a model of Burkitt's lymphoma, Gilbert and Hemann were able to show that TME derived IL-6 aided in lymphoma cell survival following treatment with doxorubicin (Gilbert & Hemann, 2010). It was also reported that IL-6 could either promote or inhibit lymphoma progression depending on the developmental stage of this malignancy (Gilbert & Hemann, 2012). These and other results highlight the impact of the TME on therapeutic outcome, particularly how paracrine secreted signals like IL-6 allow for the survival and persistence of tumor cells that eventually fuel relapse (Bent et al., 2016).

Inspired by these observations, and considering how cytotoxic chemotherapy rarely generates durable anti-tumor immune responses in patients, I embarked into studying the role of IL-6 in anticancer immunity stimulated by immunotherapies. The next chapter of my thesis presents the body of work on how production of IL-6 by the TME regulates the therapeutic efficacy of doxorubicin against a mouse model of Ph^+ B-ALL. Importantly, we show that resistance against doxorubicin promoted by IL-6 is due to suppression of $CD4^+$ and $CD8^+$ T cell immune responses.

Thesis summary

Overall, the studies described above suggest that unleashing immune responses against tumors is one of the best approaches for clinical management of cancer patients. Genetic and cellular alterations of transformed cells provide the immune system with delicate cues to recognize non-self and generate T-cell responses that eradicate cancer cells, ensuing in a dance of tumor control and immune escape. Many immunomodulatory strategies have been recently developed to capitalize on and potentiate these pre-existing anticancer immune responses. However, even when T-cell responses occur and are reinvigorated by treatment, they rarely provide long-lasting protective immunity. These dynamics and a better understanding of T-cell inhibitory signals have spotlighted tumor microenvironments as culprits and perpetrators of many mechanisms that confer resistance against immune responses. In the following Chapter, I will outline our efforts to uncover and eliminate tumor microenvironment derived elements that mediate protection of cancer cells against therapy. These studies add to the growing bodies of work that attempt to manipulate and enhance antitumor immunity to generate long-lasting immune responses in cancer patients.

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Chapter 2

Microenvironmental IL-6 inhibits anti-cancer immune responses generated by cytotoxic chemotherapy

Chapter 2: Microenvironmental IL-6 inhibits anti-cancer immune responses generated by cytotoxic chemotherapy

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Author contributions

E.H.B., and M.T.H. conceptualized the study. E.H.B., L.R.M.B., and M.T.H. designed the study. E.H.B., L.R.M.B., I.Z., D.R.G., and J.F. acquired the data. E.H.B., L.R.M.B., I.Z., D.R.G., and J.F., and M.T.H. interpreted and analyzed the data. E.H.B., L.R.M.B., and M.T.H. drafted the manuscript and figures. All authors revised, edited, and approved the final version of the manuscript and agree to be held accountable for personal contributions.

Abstract

Cytotoxic chemotherapeutics primarily function through DNA damage-induced tumor cell apoptosis, although the inflammation provoked by these agents can stimulate anti-cancer immune responses. The mechanisms that control these distinct effects and limit immunogenic responses to DNA-damage mediated cell death *in vivo* are currently unclear. Using a mouse model of BCR-ABL$^+$ B-cell acute lymphoblastic leukemia, we show that chemotherapy-induced anti-cancer immunity is suppressed by the tumor microenvironment through production of the cytokine IL-6. The chemotherapeutic doxorubicin is curative in IL-6-deficient mice through the induction of CD8$^+$ T-cell-mediated anti-cancer responses, while moderately extending lifespan in wild type tumor-bearing mice. We also show that IL-6 suppresses the effectiveness of immune-checkpoint inhibition with anti-PD-L1 blockade. Our results suggest that IL-6 is a key regulator of anti-cancer immune responses induced by genotoxic stress and that its inhibition can switch cancer cell clearance from primarily apoptotic to immunogenic, promoting and maintaining durable anti-tumor immune responses.

Introduction

Most conventional chemotherapeutics exert their cytotoxic mechanism of action by interfering with diverse proteins that affect DNA synthesis and replication. These cellular disruptions lead to the induction of genotoxic stress which results in DNA damage and ultimately in cell death (Longley & Johnston, 2005). Most cancers are initially treated with conventional chemotherapeutics, but complete tumor eradication is difficult to achieve with either targeted or cytotoxic agents. Persistent disease, frequently termed minimal residual disease, fuels eventual tumor relapse and treatment failure in many patients, underscoring a need to find ways to enhance the long-term efficacy of our front-line arsenal of cancer therapeutics.

Some of the most widely used chemotherapeutics, such as doxorubicin, have been suggested to induce anti-tumor immunity through the stimulation of immunogenic cell death (ICD) (Kroemer et al., 2013). The generation of anti-cancer immunity is a promising approach to target residual disease in cancer, and can result in durable tumor responses (Sharma & Allison, 2015; Palucka & Coussens, 2016). Lasting anti-cancer immune responses require both antigen recognition and adjuvant signals, such as those that result from cell stress or death (Galluzzi et al., 2017; Demaria et al., 2019). Immune-stimulating chemotherapies incite the release of pro-inflammatory signals, including damage-associated molecular patterns (DAMPs), that indicate danger and act as immunologic adjuvants, provoking anti-tumor immunity. However, even in settings where tumor antigens are present, cytotoxic chemotherapy rarely generates durable anti-cancer immune responses. This suggests that any immune stimulus from genotoxic therapy is insufficient or ultimately suppressed. The mechanisms by which this occurs are not well understood.

BCR-ABL$^+$ B-cell acute lymphoblastic leukemia (B-ALL) is a treatment-refractory subtype of B-ALL with a ~50% 3-year overall survival following the use of cytotoxic chemotherapeutics in combination with targeted BCR-ABL kinase inhibitors (Pui et al., 2008; Terwilliger & Abdul-Hay, 2017). Most chemotherapy regimens for ALL include the anthracycline doxorubicin, which can promote ICD and has the potential to induce anti-tumor immunity (Pui et al., 2008; Kroemer et al., 2013; Terwilliger & Abdul-Hay, 2017). However, patients with BCR-ABL$^+$ B-ALL rarely experience immune-mediated cures after doxorubicin therapy. Immune evasion is a hallmark of cancer development (Hanahan & Weinberg, 2011; Vinay et al., 2015), and occurs through tumor-intrinsic changes and alterations in the diverse immune and non-immune cell types that make up the tumor microenvironment (TME) (Hanahan & Coussens, 2012; Junttila & de Sauvage, 2013; Klemm & Joyce, 2015; Medler et al., 2015; Spranger et al., 2015). Which of these are essential for repressing immune responses to cytotoxic chemotherapy is of significant interest, and has both pre-clinical and clinical relevance.

Chemotherapy has the potential to overcome some of the barriers against an effective anti-tumor immune response by stimulating the production of cytokines, chemokines and other damage signals that recruit immune cells into the TME and prime innate and adaptive immune responses. However, immunogenic chemotherapy only disables some immune-evasive mechanisms. A promising therapeutic strategy is combining chemotherapy with blockade of immune checkpoint proteins and immunosuppressive cytokines and metabolites, such as IL-10, IDO and other chemokines that influence the activity of immune cells present in the

microenvironment (Smyth et al., 2016). Simultaneously targeting multiple TME evasive mechanisms may potentially improve the treatment of cancer.

IL-6 is a pleiotropic cytokine frequently found in diverse TMEs. IL-6 is involved in the regulation of tissue repair, the acute-phase response (Kopf et al., 1994), and is indispensable for the initiation of both innate and adaptive immune responses in many contexts (Taniguchi & Karin, 2014; Hunter & Jones, 2015). IL-6 has been implicated in tumor development and resistance to therapy in diverse cancer types, including through its effects on the immune system (Gilbert & Hemann, 2010; Gilbert & Hemann, 2012; Tanaka et al., 2014; Bent et al., 2016; Flint et al., 2016; Li et al., 2018; Mace et al., 2018; Tsukamoto et al., 2018). In addition to its well-established pro-inflammatory effects, IL-6 has also been suggested to have pro-resolving and anti-inflammatory properties, and chronic IL-6 activity can stimulate immune suppressive signals and impair the generation of a robust immune response (Tanaka & Kishimoto, 2014; Taniguchi & Karin, 2014; Hunter & Jones, 2015; Kang et al., 2019). Consequently, the ultimate impact of IL-6 on the generation of anti-tumor immune responses *in vivo* remains unclear.

Our previous studies have identified that TME-derived IL-6 is acutely induced following chemotherapy treatment, activating cancer cell anti-apoptotic signaling and shielding lymphoma cells from cell death (Gilbert & Hemann, 2010; Bent et al., 2016). Here, we find that production of IL-6 by the TME regulates chemotherapy efficacy in ALL by inhibiting anti-leukemia immunity. Using a syngeneic mouse model of ALL, we show that the wild-type tumor microenvironment is immunosuppressive. The immunogenic-chemotherapeutic doxorubicin extends survival through

the direct induction of tumor cell death in wild-type mice, similar to its impact in patients with B-ALL (Pui et al., 2008; Terwilliger & Abdul-Hay, 2017), but fails to promote durable anti-cancer immunity. Interestingly, we find that IL-6 knock-out (KO) mice treated with doxorubicin completely clear leukemic cells, with the majority of these mice undergoing T-cell-dependent anti-leukemia immune responses and developing lasting immunologic memory. Thus, the presence or absence of IL-6 dictates doxorubicin efficacy by shifting its mechanism of anti-cancer clearance, which becomes primarily immunogenic in the absence of IL-6. Our results suggest that the inhibition of IL-6 may be a broadly effective therapeutic strategy to promote durable responses to standard of care genotoxic drug regimens.

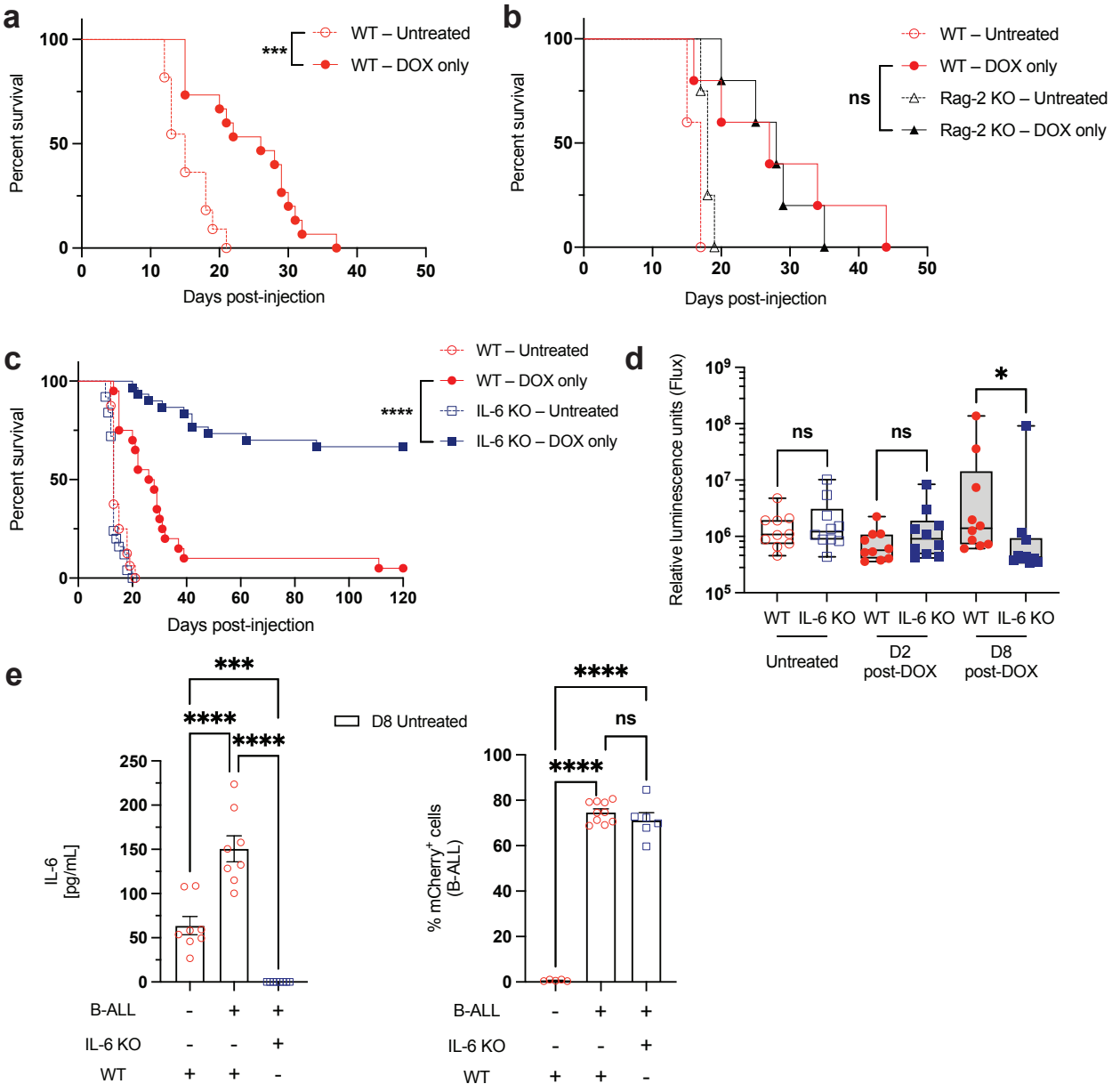
Results

Wild type B-ALL bearing mice are resistant to doxorubicin treatment

To explore the mediators of immune suppression and resistance to immunogenic chemotherapy, we used a transplantable syngeneic mouse model of BCR-ABL$^+$ B-ALL (Williams et al., 2006). Transplanted leukemia cells are found primarily in the bone marrow (BM), blood, and spleen, recapitulating the relevant tissue microenvironments in the human disease (Williams et al., 2007; Boulos et al., 2011). To investigate the response of this leukemia to immunogenic chemotherapy, wild type (WT) leukemia bearing recipients were treated with doxorubicin (DOX) and monitored for survival. Overall survival was extended in tumor bearing mice treated with doxorubicin, but all mice ultimately relapsed with chemoresistant disease (**Figure 2.1a**) – a phenotype that parallels treatment failure in the clinical setting (Williams et al., 2007; Boulos et al., 2011). To further investigate the immunogenicity of this model in its native tumor microenvironment, we

transplanted B-ALL tumor cells into Rag-2 KO recipient mice, which lack functional T- and B-cells. We found that lack of T- and B-cells did not significantly affect survival (**Figure 2.1b**). $CD8^+$ T-cell depletion in WT mice failed to yield a statistically significant impairment in doxorubicin response but did show a small numerical difference in survival (**Supplementary Fig. 2.1a,b**). Taken together, these data suggest that the improved survival following treatment is primarily a direct cytotoxic effect of chemotherapy and is largely independent of sustained anti-leukemia immune responses.

Figure 2.1


a
Percent survival
Days post-injection
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WT – Untreated
WT – DOX only
b
Percent survival
Days post-injection
ns
WT – Untreated
WT – DOX only
Rag-2 KO – Untreated
Rag-2 KO – DOX only
c
Percent survival
Days post-injection
****
WT – Untreated
WT – DOX only
IL-6 KO – Untreated
IL-6 KO – DOX only
d
Relative luminescence units (Flux)
ns
ns
*
WT IL-6 KO WT IL-6 KO WT IL-6 KO
Untreated
D2 post-DOX
D8 post-DOX
e
IL-6 [pg/mL]
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****
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D8 Untreated
B-ALL - + +
IL-6 KO - - +
WT + + -
% mCherry+ cells (B-ALL)
****
ns
****
B-ALL - + +
IL-6 KO - - +
WT + + -

Figure 2.1 | Doxorubicin extends survival of WT leukemic mice but elicits long-term disease elimination in the absence of IL-6. a, A Kaplan-Meier survival curve showing leukemic WT mice, either treated with doxorubicin (DOX) or untreated. n = 11 for WT-untreated, n = 15 for WT-treated. ***p = 0.0001. **b,** A Kaplan-Meier survival curve showing leukemic WT or immunodeficient Rag-2 KO mice, either treated with doxorubicin or untreated. n = 5 per cohort, except n = 4 for Rag-2 KO-untreated. **c,** A Kaplan-Meier survival curve showing leukemic WT or IL-6 KO mice, either treated with doxorubicin or untreated. n = 16 for WT-untreated, n = 20 for WT-treated, n = 25 for IL-6 KO-untreated, n = 30 for IL-6 KO-treated. Data from 4 independent experiments is shown. ****p<0.0001. **d,** A graph showing leukemia burden *in vivo* monitored by bioluminescence imaging. Leukemia burden in WT and IL-6 KO mice at various times before and after treatment. n = 10 per cohort. *p = 0.0115 by two-tailed Mann-Whitney test. **e,** Left, a graph showing the concentration of IL-6 present in the bone marrow of tumor-free or B-ALL bearing mice. n = 8 per cohort. Right, a graph showing $mCherry^+$ B-ALL percentages in the bone marrow of tumor-free or B-ALL bearing mice, quantified by flow cytometry. n = 10 for WT-ALL-untreated, n = 5 for WT-PBS-untreated, n = 6 for IL-6 KO-ALL-untreated. Data is represented as mean $\pm$ SEM. Shown are individual biological replicates. ***p = 0.0008, ****p<0.0001 by Ordinary one-way ANOVA test. Log-rank (Mantel-Cox) tests were used to compare Kaplan-Meier survival curves. Boxplots show the median as the center lines, upper and lower quartiles as box limits, and whiskers represent maximum and minimum values. D2 = Day 2, D8 = Day 8.

IL-6 promotes resistance to cytotoxic therapy

We have previously shown that TME-derived IL-6 modulates resistance to genotoxic chemotherapy in a mouse model of Burkitt's lymphoma (Gilbert & Hemann, 2010; Bent et al., 2016). To understand the effect that loss of IL-6 in the tumor microenvironment has on leukemia response to chemotherapy in B-ALL, we transplanted leukemia cells into syngeneic IL-6 KO mice (Kopf et al., 1994) and treated these mice with doxorubicin. Surprisingly, we found that doxorubicin-treated mice lacking IL-6 in the tumor microenvironment live significantly longer than WT treated mice, with a majority of mice appearing to be cured of their disease (**Figure 2.1c**). In the absence of treatment and shortly after treatment, we see no difference in leukemia tumor burden between WT or IL-6 KO mice. However, leukemic cell burden is significantly reduced in doxorubicin-treated IL-6 KO mice by 8 days after treatment (**Figure 2.1d, and Supplementary Fig. 2.2a**). Chemotherapy induction regimens against human B-ALL include corticosteroids, which have anti-inflammatory properties that could interfere with anti-tumor immune responses. Therefore, we administered doxorubicin and dexamethasone treatment on tumor bearing IL-6 deficient mice but failed to see any significant negative effect on anti-tumor immunity (**Supplementary Fig. 2.1c**). To determine how IL-6 is regulated by the presence of leukemic cells and whether leukemic cells or other microenvironmental cells are the primary source of IL-6 production, we transplanted B-ALL cells into WT and IL-6 KO hosts and harvested bone marrow samples to quantify the levels of IL-6 by ELISA. While B-ALL cells do not produce IL-6 themselves, they cause an upregulation in IL-6 production by the tumor microenvironment, regardless of comparable tumor burdens in both WT & IL-6 KO mice (**Figure 2.1e**).

Anthracyclines like doxorubicin are reported to induce cancer cell death programs that are immunogenic and prompt anti-tumorigenic host responses. To further characterize the dependence of our phenotype on ICD, we explored how doxorubicin regulates immunogenic DAMPs associated with ICD (Kroemer et al., 2013; Garg et al., 2015; Galluzzi et al., 2017; Garg & Agostinis, 2017). Analysis of CRT signal on the surface of treated leukemia cells shows that doxorubicin, but not imatinib, a BCR-ABL inhibitor which is not known to induce ICD, induces CRT surface exposure (**Supplementary Fig. 2.2b,d**). Unexpectedly, other classic hallmarks of ICD are not significantly induced after doxorubicin treatment in this model (**Supplementary Fig. 2.2c**).

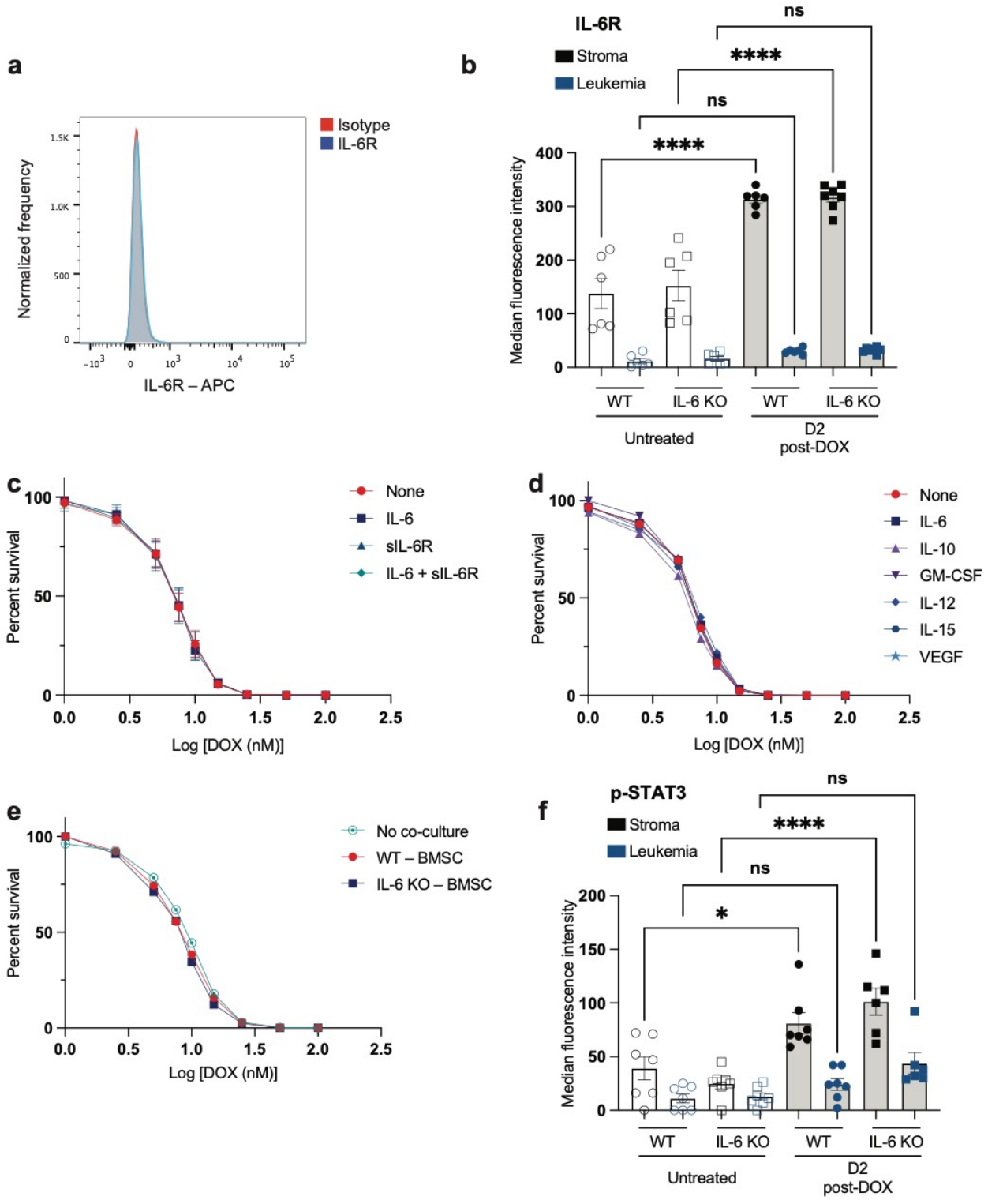
IL-6 does not signal directly to leukemia cells to affect survival

We next sought to understand whether IL-6 could directly promote therapeutic resistance in B-ALL cells. IL-6 signals through a receptor complex composed of the membrane-embedded signal transducer gp130 and either transmembrane or soluble forms of the IL-6 receptor (Tanaka et al., 2014; Kang et al., 2019). Cells do not have to express the IL-6 receptor (IL-6R) to engage in IL-6-mediated signaling but can activate signaling from binding of soluble IL-6R (sIL-6R)-IL-6 complexes to gp130. The IL-6R was not detected on leukemia cells either *in vitro* (**Figure 2.2a**) or *in vivo* (**Figure 2.2b**), but is expressed on many stromal cells in the BM microenvironment (**Figure 2.2b**). To test whether IL-6 can directly mediate resistance to doxorubicin, we cultured leukemia cells in the presence of IL-6, sIL-6R, or both IL-6 and sIL-6R to simulate signaling through sIL-6R-IL-6 complexes. Surprisingly, none of these conditions altered the sensitivity of leukemic cells to doxorubicin (**Figure 2.2c**), suggesting that IL-6 does not directly promote resistance to doxorubicin in this system.

We have previously shown that IL-6 regulates the production of a number of other cytokines and growth factors in the bone marrow (Gilbert & Hemann, 2012), leading to elevated levels of IL-10, IL-12, IL-15 and GM-CSF. To determine if these cytokines and growth factors can directly mediate resistance to doxorubicin, we cultured leukemia cells in the presence of these other cytokines. Interestingly, growth of leukemia cells in the presence of these cytokines or growth factors also had no impact on the cells' sensitivity to doxorubicin *in vitro* (**Figure 2.2d**). Co-culture of leukemia cells with bone marrow stromal cells from WT or IL-6 KO mice (**Figure 2.2e**) also did not have an effect on the cells' sensitivity to doxorubicin. These results suggest that the resistance conferred by IL-6 does not result from direct regulation of any soluble factor downstream of IL-6 signaling. We also do not observe significant levels of phosphorylated STAT3 (p-STAT3), a major signaling pathway downstream of the IL-6R, in leukemia cells *in vivo* before treatment (**Figure 2.2f**) or significant differences in gene expression downstream of STAT3 in leukemic cells grown in WT and IL-6 KO mice (see below). Interestingly, while stromal p-STAT3 levels increase in response to doxorubicin treatment, there are no differences in p-STAT3 levels between WT and IL-6 KO mice at the times tested (**Figure 2.2f**), although some change in pathway gene expression is noted (see below). To further understand the molecular mechanisms that mediate treatment resistance by IL-6, we performed immunoblot analysis of various IL-6 effectors from B-ALL bearing bone marrow lysates. Activation of S6 kinase (S6K), a target of PI3K/mTOR signaling, was not significantly changed by the absence of IL-6 nor exposure to doxorubicin treatment. Similarly, activation of ERK1/2, a target of Ras/MAPK signaling, remained unchanged regardless of treatment conditions (**Supplementary Fig. 2.3a-d**). Additionally, we were not able to detect release of the sIL-6R in co-culture of leukemia cells with bone-marrow stromal cells from WT or

IL-6 KO mice (**Supplementary Fig. 2.3e**). Thus, the therapeutic benefit we see *in vivo* appears to be independent of IL-6 activity directly on the cancer cells and most likely mediated by its impact on the stroma.

Figure 2.2


a
Isotype
IL-6R
Normalized frequency
IL-6R – APC
b
IL-6R
Stroma
Leukemia
ns
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Median fluorescence intensity
WT
IL-6 KO
WT
IL-6 KO
Untreated
D2 post-DOX
c
None
IL-6
sIL-6R
IL-6 + sIL-6R
Percent survival
Log [DOX (nM)]
d
None
IL-6
IL-10
GM-CSF
IL-12
IL-15
VEGF
Percent survival
Log [DOX (nM)]
e
No co-culture
WT – BMSC
IL-6 KO – BMSC
Percent survival
Log [DOX (nM)]
f
p-STAT3
Stroma
Leukemia
ns
****
ns
*
Median fluorescence intensity
WT
IL-6 KO
WT
IL-6 KO
Untreated
D2 post-DOX

Figure 2.2 | IL-6 does not promote intrinsic B-ALL chemoresistance. a, A flow cytometry plot showing IL-6R expression on the surface of leukemic cells *in vitro*. Histograms for IL-6R and isotype control-stained cells are overlaid. **b,** A graph showing IL-6R expression in the bone marrow of leukemia-bearing mice pre- and post-doxorubicin (DOX) treatment. Data was quantified by flow cytometry. $mCherry^+$ leukemia cells were used to distinguish stromal and leukemia cells. Median APC intensity from IL-6R stained cells minus isotype control-stained cells was calculated. Data from 2 independent experiments is shown, and represented as mean ± SEM. n = 6 per cohort, except n = 7 for both IL-6 KO-D2 post-doxorubicin stroma and IL-6 KO-D2 post-doxorubicin leukemia samples. ****p<0.0001 by Ordinary one-way ANOVA test. **c,** A dose-response curve showing leukemic cell viability in response to doxorubicin treatment in the presence or absence of IL-6 and/or sIL-6R. Viable cells were counted by flow cytometry 48 hours after the addition of doxorubicin. Data from 4 independent experiments is shown, and represented as mean ± SEM. **d,** A dose-response curve showing leukemic cell viability in the presence or absence of cytokines previously observed to be regulated by IL-6. Cells were treated as in (**c**) with the indicated cytokines. **e,** A dose-response curve showing leukemic cell viability of cells co-cultured with bone marrow stromal cells (BMSC) from WT or IL-6 KO mice in response to doxorubicin treatment as in (**c**). **f,** A graph showing p-STAT3 levels in the bone marrow of leukemia-bearing WT and IL-6 KO mice. There were no significant statistical comparisons between corresponding WT and IL-6 KO samples. Data is shown as in (**b**), from 2 independent experiments, and represented as mean ± SEM. n = 7 per cohort, except n = 6 for both IL-6 KO-D2 post-doxorubicin stroma and IL-6 KO-D2 post-doxorubicin leukemia samples. *p = 0.011, ****p<0.0001 by Ordinary one-way ANOVA test. D2 = Day 2.

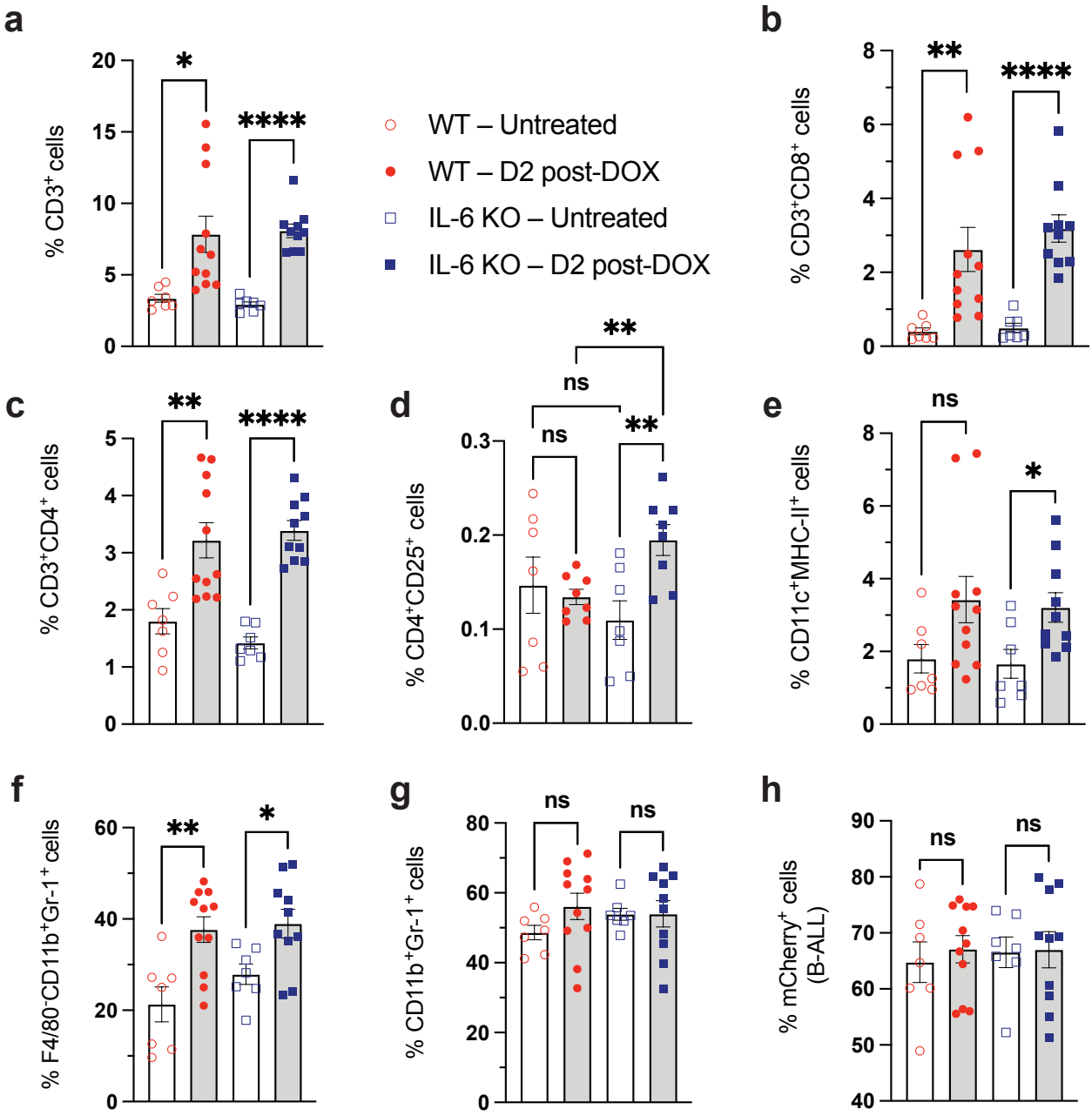
Doxorubicin induces immune cell infiltration into the leukemic bone marrow

ICD released immune-activating factors serve to recruit immune cells to sites of damage and activate downstream inflammatory signaling that can further recruit additional immune-cell subsets to the inflamed tissue, spurring anti-cancer immunity (Kroemer et al., 2013). To understand the role that doxorubicin has on immune cell recruitment to major sites of leukemia burden like the bone marrow and spleen, we profiled immune cell composition in leukemia-bearing mice before and after doxorubicin treatment. Before treatment, T-cells make up a small portion of cells in the bone marrow (**Figure 2.3a, Supplementary Fig. 2.3f, and Supplementary Table 2.1**) but are much more prevalent in the spleen (**Supplementary Table 2.2**). This suggests that the bone marrow, which is the primary site of residual leukemia after treatment, may be a T-cell exclusionary microenvironment (Spranger, 2016). Interestingly, doxorubicin treatment selectively promotes T-cell influx into the bone marrow, but not the spleen, with increased cytotoxic and helper T-cell subsets observed in both WT and IL-6 KO mice (**Figure 2.3b,c, and Supplementary Table 2.1**). We find relatively low levels of $CD3^+$–$CD4^+$–$CD25^+$ cells in the leukemic bone marrow (**Figure 2.3d**), a subset that includes T-regulatory (T-Reg) cells. These cell populations were subtly changed after doxorubicin treatment, suggesting that the T-cell recruitment promoted by doxorubicin is cell-type specific and that doxorubicin may increase the CTL/T-reg ratio in the bone marrow, a ratio that is positively associated with survival in multiple cancer types (Sato et al., 2005; Fridman et al., 2012).

Additionally, doxorubicin promotes increased $CD11c^+$–MHC-II^+ dendritic cell (**Figure 2.3e**) and $F480^-$–$CD11b^+$–Gr-1^+ neutrophil infiltration in the bone marrow (**Figure 2.3f**). There are no major

changes in the overall percentages of $CD11b^{+}$–$Gr\text{-}1^{+}$ cells (**Figure 2.3g**). This latter population includes multiple mature and immature myeloid cell subsets which make up a major portion of the cells in the bone marrow. At these early timepoints after doxorubicin treatment, there is no significant difference in leukemic cell burden in the BM of IL-6 KO and WT mice (**Figure 2.3h**), suggesting that the DNA damage induced by this agent may not account for its entire antitumor activity. Collectively, these data indicate that the bone marrow is an exclusionary environment for leukemia-reactive T-cells. Doxorubicin treatment leads to increased dendritic and T-cell infiltration, potentially contributing to leukemia recognition and clearance in the right environmental context.

Figure 2.3


a
b
c
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e
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g
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% CD3+ cells
% CD3+CD8+ cells
% CD3+CD4+ cells
% CD4+CD25+ cells
% CD11c+MHC-II+ cells
% F4/80-CD11b+Gr-1+ cells
% CD11b+Gr-1+ cells
% mCherry+ cells (B-ALL)
WT – Untreated
WT – D2 post-DOX
IL-6 KO – Untreated
IL-6 KO – D2 post-DOX
ns
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**
****

Figure 2.3 | Doxorubicin induces immune cell recruitment to the TME. a, A graph showing the percentages of T-cells ($CD3^+$) in the bone marrow of leukemia-bearing mice pre- and post-doxorubicin (DOX) treatment. *p = 0.0142, ****p<0.0001. **b,** Graph showing the percentages of cytotoxic T-cells ($CD3^+$–$CD8^+$) as in (**a**). **p = 0.0099, ****p<0.0001. **c,** Graph showing the percentages of helper T-cells ($CD3^+$–$CD4^+$) as in (**a**). **p = 0.0044, ****p<0.0001. **d,** Graph showing the percentages of a subset of T-cells ($CD3^+$–$CD4^+$–$CD25^+$) as in (**a**). n = 7 for WT-untreated, n = 8 for WT-D2 post-doxorubicin, n = 7 for IL-6 KO-untreated, n = 8 IL-6 KO-D2 post-doxorubicin mice. Data from 3 independent experiments is shown. **p = 0.0059 between IL-6 KO samples, **p = 0.005 between treated WT and IL-6 KO samples. **e,** Graph showing the percentages of dendritic cells ($CD11c^+$–MHC-II^+) as in (**a**). *p = 0.019. **f,** Graph showing the percentages of neutrophils ($F480^-$–$CD11b^+$–$Gr\text{-}1^+$) as in (**a**). *p = 0.0189, **p = 0.0029. **g,** Graph showing the percentages of myeloid-derived suppressor cells/monocytes ($CD11b^+$–$Gr\text{-}1^+$) as in (**a**). **h,** A graph showing $mCherry^+$ B-ALL percentages in the bone marrow of leukemia-bearing mice pre- and post-doxorubicin treatment. All data was quantified by flow cytometry. Data is represented as a percent of DAPI-negative (live), mCherry-negative (non-leukemic) cells for immune populations. Data for all panels is represented as mean $\pm$ SEM. n = 7 for WT-untreated, n = 11 for WT-D2 post-doxorubicin, n = 7 for IL-6 KO-untreated, n = 10 IL-6 KO-D2 post-doxorubicin mice, and data from 4 independent experiments is shown; applies for all panels unless otherwise noted. Analyzed by two-tailed Student t-test. There were no significant statistical comparisons between 'untreated' and 'DOX treated' samples of different genetic backgrounds, unless shown. D2 = Day 2.

Leukemia clearance in IL-6 KO mice is dependent on T-cell mediated anti-tumor immune responses

The inability of IL-6 to directly promote doxorubicin resistance stands in contrast with the increased efficacy of doxorubicin chemotherapy in IL-6 KO mice. This increased efficacy, and the T-cell influx we see after doxorubicin treatment, led us to investigate whether IL-6 might affect therapeutic response through modulation of the immune system. To study the role of T-cells in the durable responses observed in IL-6 KO mice, we depleted T-cells through the injection of anti-CD4 and CD8 antibodies. While T-cell-depleted IL-6 KO mice exhibit similar initial responses to doxorubicin 2 days after treatment, these mice fail to fully clear their leukemic burden, rapidly relapse, and do not exhibit the long-term survival typically seen after doxorubicin treatment of IL-6 KO mice (**Figure 2.4a,b**). These results suggest that T-cell anti-tumor activities are essential for the profound responses to doxorubicin seen in IL-6 KO mice. Depletion of $CD8^+$ or $CD4^+$ cells alone recapitulated the effect seen with combined CD4- and CD8-depletion (**Supplementary Fig. 2.1d,e**), suggesting that long-term survival of doxorubicin-treated IL-6 KO mice is dependent on both $CD8^+$ cytotoxic T-lymphocyte (CTL) and $CD4^+$ helper activity. These data indicate that doxorubicin has the potential to promote an anti-tumor immune response, likely in part through the recruitment of T-cells into the BM, but that this response is suppressed in WT mice through the production of IL-6. Next, to evaluate whether IL-6 KO mice develop lasting immunologic memory after doxorubicin treatment, we re-transplanted leukemia cells into previously cured IL-6 KO mice or naïve controls (**Figure 2.4c**) and monitored leukemia progression. Strikingly, previously cured mice were completely resistant to leukemia initiation upon tumor re-

transplantation (**Figure 2.4d,e**). These results suggest that IL-6 absence allows for the generation of lasting anti-cancer immunity that is mainly mediated by T-lymphocyte responses.

Figure 2.4

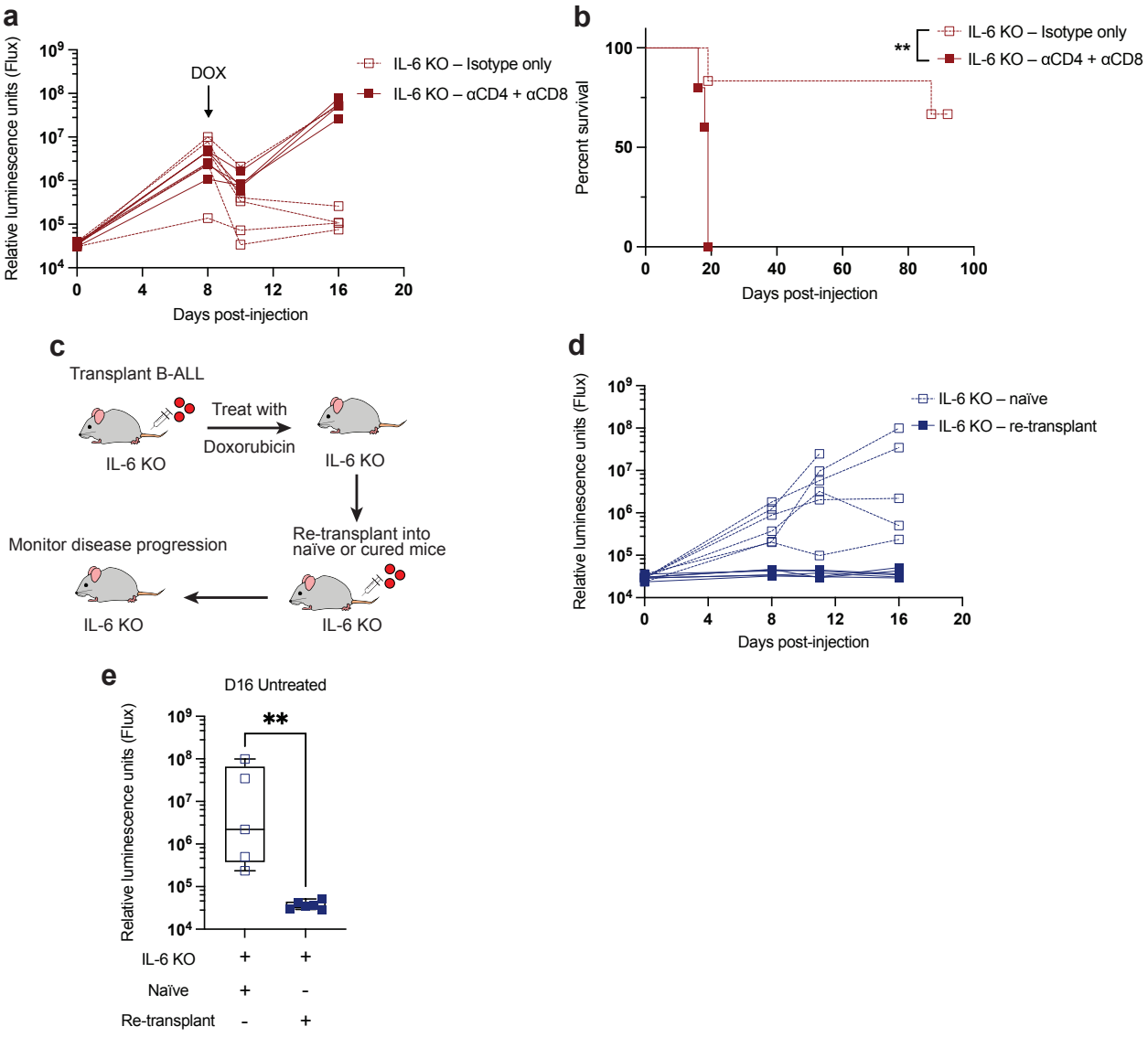
a
Relative luminescence units (Flux)
DOX
IL-6 KO – Isotype only
IL-6 KO – αCD4 + αCD8
Days post-injection
b
Percent survival
**
IL-6 KO – Isotype only
IL-6 KO – αCD4 + αCD8
Days post-injection
c
Transplant B-ALL
Treat with
Doxorubicin
IL-6 KO
IL-6 KO
Re-transplant into
naïve or cured mice
Monitor disease progression
IL-6 KO
IL-6 KO
d
Relative luminescence units (Flux)
IL-6 KO – naïve
IL-6 KO – re-transplant
Days post-injection
e
D16 Untreated
Relative luminescence units (Flux)
**
IL-6 KO + +
Naïve + -
Re-transplant - +

Figure 2.4 | T-cell dependent anti-tumor immunity develops after doxorubicin treatment of IL-6 KO leukemic mice. a, A graph showing leukemia burden *in vivo* monitored by bioluminescence imaging. CD4 and CD8 cells were depleted with antibodies and response to doxorubicin (DOX) treatment was followed. n = 5 per cohort. **b,** A Kaplan-Meier survival curve showing leukemic IL-6 KO mice treated as in (**a**). n = 6 for IL-6 KO-isotype, n = 5 for IL-6 KO-depleted. **p = 0.0059 by Log-rank (Mantel-Cox) test. **c,** IL-6 KO mice previously cured (living >80 days) by doxorubicin treatment were re-transplanted with leukemia cells and disease progression monitored by bioluminescence imaging in the absence of further treatment. **d,** A graph showing leukemia burden *in vivo* in control and re-transplanted leukemia bearing mice. n = 6 per cohort. **e,** A graph showing leukemic IL-6 KO mice as in (**e**), D16 after disease transplant in the absence of treatment. At this time point, n = 5 for IL-6 KO-naïve, n = 6 for IL-6 KO-re-transplant. **p = 0.0043 by two-tailed Mann-Whitney test. Boxplots show the median as the center lines, upper and lower quartiles as box limits, and whiskers represent maximum and minimum values. D8 = Day 8, D16 = Day 16.

IL-6 absence impacts diverse immune-modulatory pathways

To further investigate the differences between WT and IL-6 KO mice, B-ALL and stromal cells were sorted from the bone marrow and RNA-sequencing was performed (**Supplementary Fig. 2.4a**). DESeq2 was used to identify differentially expressed genes in the tumor and stroma of IL-6 KO mice relative to wild-type, and rank list genes by t-statistic. GSEA analysis of the pre-ranked list using the cancer 'Hallmarks' collection from MSigDB identified few differentially regulated sets, but showed the gain of inflammatory response genesets in IL-6 KO samples, suggesting a global

difference in immune states between WT and IL-6 KO mice (**Supplementary Fig. 2.4b**). This elevated immune signature in tumor stroma seems to include increased expression of genes that are pathway components or recognized targets of IL-6 signaling, implying a potential compensatory response to decreased IL-6 pathway flux. The directionality of gene expression changes in these samples indicate that IL-6 KO leukemic mice are poised to generate an enhanced immune response, fitting our experimental data. Next, we analyzed underlying expression of enriched genesets identified by GSEA to determine if these inflammatory responses are more prominent in tumor or stroma samples. We found the most variance between IL-6 KO and WT mice pertained to the stroma samples, for both global normalized gene expression (**Supplementary Fig. 2.4c**) and for the genesets within the GSEA Hallmarks collection (**Supplementary Fig. 2.4d-g**). These results support our prior data indicating that the primary differences in IL-6 KO mice relative to WT arise from the bone marrow stroma. Rather than acting directly on tumor cells, IL-6 deficiency appears to alter the bone marrow stroma to broadly create a permissive immune microenvironment.

IL-6 deficiency synergizes with anti-PD-L1 therapy to treat leukemia

PD-1 and other immune-checkpoint proteins that play key roles in the suppression of anti-cancer immune responses are induced during T-cell activation (Pardoll, 2012). It is thought that these proteins exist to restore normal homeostasis after an immune stimulus, preventing hyperactive immune responses and autoimmunity (Wherry & Kurachi, 2015). Cancer cells often express high levels of inhibitory checkpoint ligands and exploit the presence of these proteins on T-cells to inhibit their activity. T-cells in the bone marrow of IL-6 KO mice have reduced surface expression

of PD-1 (**Figure 2.5a**). Combination treatment of WT B-ALL-bearing mice with doxorubicin and PD-L1 antibody-blocking therapy reduced leukemia burden in a subset of mice (**Figure 2.5b**) and increased their survival (**Figure 2.5c**). These observations suggest that higher expression of PD-1 inhibitory signals present in IL-6 proficient microenvironments might contribute to the failure of immunogenic therapy. This model of B-ALL expresses high levels of the checkpoint ligand PD-L1 (**Figure 2.5d**), and PD-L1 expression has previously been implicated in B-ALL resistance to immune-stimulating therapy (Köhnke et al., 2015). To determine whether IL-6 loss could also enhance the efficacy of PD-L1 blockade and promote anti-leukemia immune responses, we treated WT and IL-6 KO leukemic mice with PD-L1 inhibitors and monitored disease progression and survival. While PD-L1 blockade exhibits modest efficacy in only a subset of WT mice, IL-6 KO mice undergo nearly complete leukemia eradication by 9 days after the initiation of PD-L1 blockade (**Figure 2.5e**). Almost all of the PD-L1-treated IL-6 KO mice underwent durable remissions and 80% remained alive without apparent disease more than 60 days after injection (**Figure 2.5f**). These data further suggest that production of IL-6 is a major barrier to the efficacy of immune-stimulating therapy in leukemia and that some, but not all, of its impact occurs through the regulation of T-cell PD-1 expression.

Figure 2.5

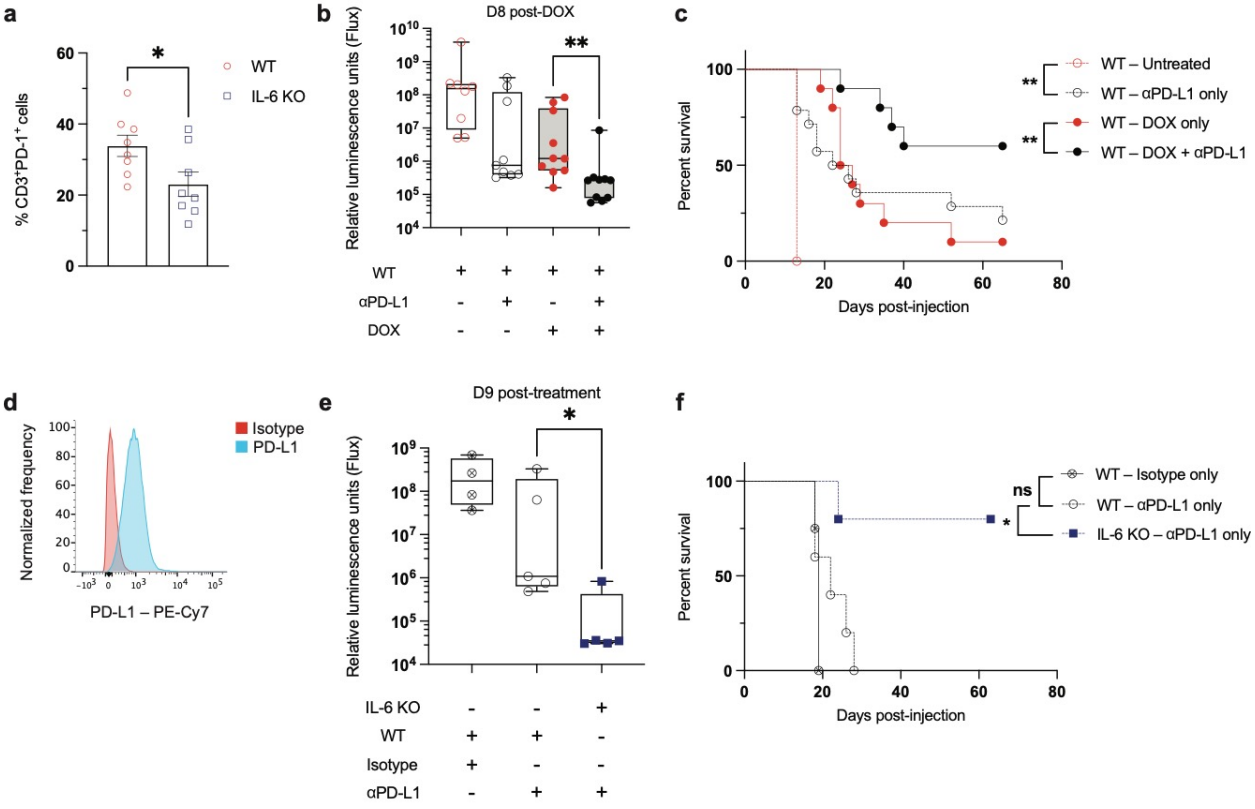
a
% CD3+PD-1+ cells
WT
IL-6 KO
b
D8 post-DOX
Relative luminescence units (Flux)
WT
αPD-L1
DOX
c
Percent survival
Days post-injection
WT – Untreated
WT – αPD-L1 only
WT – DOX only
WT – DOX + αPD-L1
d
Normalized frequency
Isotype
PD-L1
PD-L1 – PE-Cy7
e
D9 post-treatment
Relative luminescence units (Flux)
IL-6 KO
WT
Isotype
αPD-L1
f
Percent survival
Days post-injection
WT – Isotype only
WT – αPD-L1 only
IL-6 KO – αPD-L1 only
ns

Figure 2.5 | Combination treatment with doxorubicin and PD-L1 inhibition increases mouse survival, while IL-6 inhibits the efficacy of PD-L1 inhibition. a, A graph showing $CD3^+$ T-cells–$PD\text{-}1^+$ percentages in the bone marrow of tumor-free WT mice in the absence of treatment. *p = 0.032 by two-tailed Student t-test. **b,** A graph showing leukemia burden *in vivo* monitored by bioluminescence imaging. n = 10 per cohort, except n = 9 for both WT-untreated and WT-αPD-L1. **p = 0.0029 by two-tailed Mann-Whitney test. **c,** A Kaplan-Meier survival curve showing leukemic WT mice. n = 10 per cohort, except n = 5 for WT-untreated, n = 14 for WT-αPD-L1. **p = 0.0029 between WT-untreated and WT-αPD-L1 only, **p = 0.0071 between WT-doxorubicin only and WT-doxorubicin + αPD-L1, by Log-rank (Mantel-Cox) test. **d,** A flow cytometry plot showing PD-L1 expression on the surface of leukemic cells *in vitro*. **e,** A graph showing leukemia burden *in vivo* monitored by bioluminescence imaging. n = 5 per cohort, except n = 4 for WT-isotype. *p = 0.0317 by two-tailed Mann-Whitney test. **f,** A Kaplan-Meier survival curve showing leukemic WT and IL-6 KO mice. n = 5 per cohort, except n = 4 for WT-isotype. *p = 0.0126 by Log-rank (Mantel-Cox) test. Boxplots show the median as the center lines, upper and lower quartiles as box limits, and whiskers represent maximum and minimum values. D8 = Day 8, D9 = Day 9.

<u>Doxorubicin induced-immunity extends survival in tumor bearing mice treated with IL-6 receptor blockade</u>

To determine the potential clinical relevance of our observations in IL-6 KO mice, we next examined the efficacy of doxorubicin treatment when combined with IL-6R blockade in WT animals bearing B-ALL. After optimizing the dosage and administration schedule of the IL-6R inhibitor (**Supplementary Fig. 2.5a**), we observed that combination treatment with doxorubicin

and inhibition of IL-6 signaling with therapeutic antibodies significantly extended the survival of WT mice (**Figure 2.6a**). Notably, 48 hours after doxorubicin treatment, leukemic cell death has started to occur in both 'anti-Isotype' and 'anti-IL-6R' treatment combination groups. However, a week after doxorubicin administration there is significantly more leukemic cell clearance in mice treated with IL-6 receptor blockade (**Figure 2.6b**). In contrast, MC38-bearing mice were refractory to combination therapy (**Supplementary Fig. 2.5b**).

Others have shown that signaling downstream of IL-6 is important for the development, progression, and therapy response of many cancers (Kang et al., 2019), including pancreatic ductal adenocarcinoma (PDAC) (Lesina et al., 2011; Zhang et al., 2013; Mace et al., 2018). Therefore, we assessed if the combination of cytotoxic chemotherapy and IL-6R blockade might also promote tumor control in a preclinical model of PDAC subcutaneously injected into WT mice. Once tumors were established, mice received combination treatment with doxorubicin and IL-6R therapeutic antibodies. Expression of IL-6R was detected in the stromal cells *in vivo* (**Supplementary Fig. 2.5c**). Consistent with our observations in B-ALL, combination treatment with doxorubicin and αIL-6R had significant inhibition of PDAC tumor growth (**Figure 2.6c,d**). Similarly, doxorubicin treatment had significant inhibition of PDAC tumor growth in IL-6 KO mice (**Figure 2.6e,f**). Intriguingly, p-STAT3 levels from bulk PDAC tumor samples do not significantly change between WT and IL-6 KO mice, nor in response to doxorubicin treatment at the times examined (**Supplementary Fig. 2.5d**).

Finally, we re-transplanted PDAC cells, 5 days after doxorubicin treatment, into the opposite flanks of PDAC-bearing IL-6 KO mice (**Supplementary Fig. 2.5e**). The PDAC tumors transplanted into previously doxorubicin-treated IL-6 KO animals showed reduced growth compared to the tumors transplanted into untreated IL-6 KO hosts (**Figure 2.6g,h**). These results suggest a role for IL-6 deficiency in maintaining an active and long-lasting anti-tumor immunity. Thus, antibody-mediated inhibition of IL-6 signaling can promote durable responses to genotoxic chemotherapy in both hematopoietic and solid malignancies.

Figure 2.6

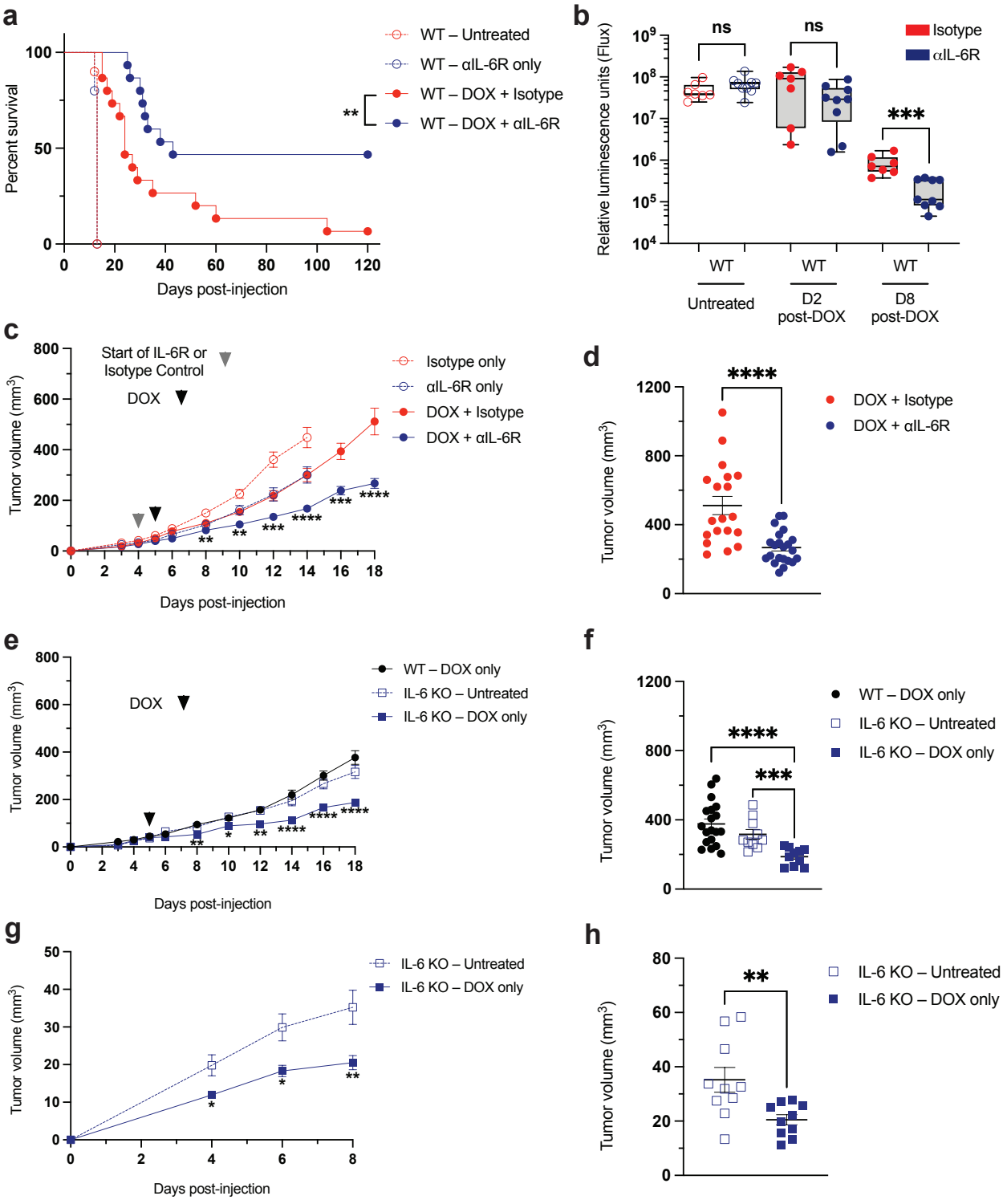
a
Percent survival
Days post-injection
WT – Untreated
WT – αIL-6R only
WT – DOX + Isotype
WT – DOX + αIL-6R
**
b
Relative luminescence units (Flux)
ns
ns
***
Isotype
αIL-6R
WT
WT
WT
Untreated
D2 post-DOX
D8 post-DOX
c
Tumor volume (mm3)
Start of IL-6R or Isotype Control
DOX
Isotype only
αIL-6R only
DOX + Isotype
DOX + αIL-6R
Days post-injection
d
Tumor volume (mm3)
****
DOX + Isotype
DOX + αIL-6R
e
Tumor volume (mm3)
DOX
WT – DOX only
IL-6 KO – Untreated
IL-6 KO – DOX only
Days post-injection
f
Tumor volume (mm3)
****
***
WT – DOX only
IL-6 KO – Untreated
IL-6 KO – DOX only
g
Tumor volume (mm3)
IL-6 KO – Untreated
IL-6 KO – DOX only
Days post-injection
h
Tumor volume (mm3)
**
IL-6 KO – Untreated
IL-6 KO – DOX only

Figure 2.6 | Therapeutic IL-6 inhibition enhances the efficacy of doxorubicin treatment. a, A Kaplan-Meier survival curve of B-ALL-bearing mice. n = 15 for both doxorubicin (DOX) + Isotype and doxorubicin + αIL-6R treated, n = 10 for untreated, and n = 5 for αIL-6R only treated mice. **p = 0.0062 by Log-rank (Mantel-Cox) test. **b,** A graph showing leukemia burden in WT mice treated with doxorubicin and either an IL-6R blocking antibody or isotype control. Tumor burden was monitored by bioluminescence imaging. n = 7 for doxorubicin + Isotype, n = 9 for doxorubicin + αIL-6R treated mice. ***p = 0.0002 by two-tailed Mann-Whitney test. **c,** A graph showing PDAC tumor burden in WT mice treated with doxorubicin and either an IL-6R blocking antibody or isotype control. n = 10 for Isotype only, n = 15 for αIL-6R only, n = 19 for doxorubicin + Isotype, and n = 22 for doxorubicin + αIL-6R. Data from 4 independent experiments. **p<0.035, ***p<0.005, ****p<0.0001. **d,** A graph showing PDAC tumor burden on day 18 for individual animals within the indicated treatment groups. Shown Ns are the individual biological replicates from (**c**). ****p<0.0001. **e,** A graph showing PDAC tumor burden in WT and IL-6 KO mice treated with doxorubicin or untreated. n = 10 per cohort, except n = 19 for WT-doxorubicin only. Data from 1 independent experiment. *p<0.05, **p<0.035, ****p<0.0001. **f,** A graph showing PDAC tumor burden on day 18 for individual animals within the indicated treatment groups. Shown Ns are the individual biological replicates from (**e**). ***p = 0.0003, ****p<0.0001. **g,** A graph showing PDAC tumor burden of secondary transplants (5 days after doxorubicin treatment) into IL-6 KO mice that had already received a primary PDAC transplant and were treated with doxorubicin or untreated. n = 10 per cohort. *p<0.05, **p<0.035. **h,** A graph showing PDAC tumor burden on day 8 for individual animals within the indicated treatment groups. Shown Ns are the individual biological replicates from (**g**). **p = 0.0039. Two-tailed Mann-Whitney tests were used to

compare groups in panels (**c-h**), and data is represented as mean ± SEM. Boxplots show the median as the center lines, upper and lower quartiles as box limits, and whiskers represent maximum and minimum values. D2 = Day 2, D8 = Day 8.

Discussion

Genotoxic chemotherapy primarily exerts its effects via DNA damage-induced cell death (Longley & Johnston, 2005). However, work from multiple labs has demonstrated that a subset of commonly used chemotherapeutics can also stimulate immunity in specific contexts (Kroemer et al., 2013; Galluzzi et al., 2017). Despite this, it remains unclear the extent to which immune responses contribute to the efficacy of cytotoxic chemotherapy and the contexts in which they do so. In both mice and humans, immunogenic chemotherapy rarely promotes lasting anti-tumor immune responses. While there are many examples of the TME regulating therapeutic efficacy *in vivo* (Klemm & Joyce, 2015), how the TME tunes the immune responses to immunogenic cell death (ICD) is poorly understood and of broad clinical relevance. Here, we show that IL-6 controls a mechanistic switch between primarily cytotoxic cell death and immune-mediated clearance of tumor cells after genotoxic chemotherapy treatment.

We used a mouse model of acute lymphoblastic leukemia that closely recapitulates the microenvironment and therapy responsiveness of the human disease (Williams et al., 2006) to investigate the mechanisms of immune suppression after treatment with immunogenic chemotherapy. We show that while doxorubicin modestly extends animal survival in WT mice, it does not generate robust anti-cancer immunity and mice ultimately fail to clear their leukemia

burden. In contrast, in the absence of IL-6, the majority of leukemic mice are cured after doxorubicin treatment in an immune-mediated fashion. This demonstrates that IL-6 is an important TME-derived paracrine factor that suppresses the generation of robust anti-tumor immunity. Consequently, we find that microenvironmental context not only impacts therapy responsiveness but alters the mechanism by which a commonly used clinical agent exerts its activity. This data indicates that the efficacy of conventional DNA-damaging therapies and their ability to induce anti-cancer immunity in human cancers may be limited by immunosuppressive factors in the TME, such as IL-6. These results highlight the role of the TME in the cancer cell's response to therapy and indicate how further study of the microenvironmental regulators of ICD could impact the clinical utility of cytotoxic chemotherapeutics.

We have previously shown that the bone marrow is a site of resistance to antibody-based therapy in double-hit lymphoma, where the immune-suppressive microenvironment impairs innate immune-mediated clearance of antibody bound cells (Pallasch et al., 2014). Interestingly, our findings here demonstrate that doxorubicin promotes T- and dendritic cell influx into the bone marrow, transforming it into a pro-immunogenic microenvironment. Major determinants of immunogenicity and ICD include the release of HMGB1 and surface exposure of CRT from dying cancer cells (Apetoh et al., 2007; Obeid et al., 2007). Doxorubicin treated leukemia cells induce CRT surface exposure and HMGB1 release in the TME is preserved although other mediators of immunogenicity in this system remain to be defined. While the pro-immunogenic conditions present after doxorubicin treatment are favorable for the clearance of leukemic cells, microenvironmental IL-6 production – which is increased by the presence of leukemic cells –

suppresses the expected anti-cancer immune responses. Thus, cancer-cell induced IL-6 release in designated microenvironments may help to disguise immunogenic cell death states.

Paracrine signals produced in the TME play a major role in defining the immune context of tumors and show great potential for therapeutic manipulation. IL-6 is a pleiotropic cytokine involved in the regulation of many processes including immune activation (Kang et al., 2019), but chronic IL-6 activity can also weaken the generation of an effective immune response (Hunter & Jones, 2015). For example, chronic STAT3 activity downstream of IL-6 can impair the generation of new adaptive immune responses (Kortylewski et al., 2005). In the context of ICD, IL-6 may impair anti-cancer immunity through the creation of a microenvironment in which an acute inflammatory stimulus from cell death is less likely to generate a productive immune response. Our data suggests multiple downstream effectors are likely active in mediating the profound regulation of anti-cancer immunity we see after cytotoxic therapy. While we detected no differences in p-STAT3 protein levels between IL-6 KO and WT leukemic and PDAC bearing mice at the times tested, our RNA-sequencing studies do show alterations in JAK/STAT pathway components in stroma from IL-6 KO mice. We also find higher T-cell PD-1 expression in the presence of IL-6 indicating a potentially more exhausted T-cell population. Future detailed interrogation of the effectors downstream of IL-6 will be necessary to elucidate the mechanisms underlying this significant clinical response.

While showing promising efficacy in a number of cancer types, immunotherapy can increase the activity of the immune system, causing a variety of inflammatory and auto-immune phenomena

that instigate significant morbidity. These immune related adverse events are commonly treated with steroids. However, there is concern that high-dose steroids may not fully help to alleviate the immune-related adverse events and additionally that they may blunt the anti-cancer effects of immunotherapies (Postow et al., 2018). This emphasizes the need to find alternatives for treating immune-related adverse consequences. The humanized monoclonal anti-IL-6R antibody, Tocilizumab, has been used to treat inflammatory toxicity associated with immune-checkpoint blockade and the cytokine release syndrome (CRS) associated with chimeric antigen receptor T-cell therapy (Le et al., 2018). Interestingly, our data suggest that IL-6 blockade may be able to decouple auto-immune and anti-cancer immune responses, potentially increasing anti-cancer immunity while treating auto-immune toxicity. This phenomenon has recently been reported for TNF-blockade as well (Perez-Ruiz et al., 2019).

Given the many mechanisms by which cancer can evade immune surveillance (Vinay et al., 2015; Palucka & Coussens, 2016), combination therapies that block multiple immune suppressive mechanisms will be essential to promote responses in the majority of tumors. Consistent with this idea, leukemic WT mice treated with doxorubicin and PD-L1 inhibitors are more readily able to clear their disease when compared to single-agent treated mice. Additionally, therapeutic IL-6R inhibitors can synergize with doxorubicin to eliminate leukemic cells from WT mice. Likewise, we show that this combination improves the response of PDAC tumors, suggesting that this phenomenon may extend to certain solid tumors. While IL-6 likely executes its immune-suppressive properties through the regulation of multiple immune processes, our data demonstrate that loss of IL-6 enhances the generation of anti-cancer immunity in response to

multiple immune-stimulating therapies. IL-6 inhibition could help sustain the limited anti-cancer immune responses normally induced by cytotoxic agents in the clinic.

Here, we establish that three interventions, IL-6 inhibition, doxorubicin treatment, and PD-L1 blockade, each of which alone fails to promote lasting anti-leukemia immunity, achieve much more durable responses in combination. Importantly, we show that the state of the TME profoundly impacts both the efficacy and the primary mechanism of action of a commonly used cytotoxic agent. Taken together, these data suggest that combination therapy with immunogenic chemotherapy, manipulation of the tumor microenvironment through IL-6 inhibition, and checkpoint blockade is a promising therapeutic approach for treating human cancer.

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Materials and methods

Cell culture and chemicals

B-ALL cells were grown at 37 ˚C, 5% CO_2, in 500 mL RPMI, 50 mL FBS, 10 mL glutamine, 5.5 mL β-ME (5 mM), and 5 mL Pen. Strep. Luciferase$^+$ BCR-ABL$^+$ B-ALL male cells were a gift from Richard Williams (Williams et al., 2006). To make mCherry$^+$ B-ALL cells, the MSCV-mCherry retroviral vector was transfected into Phoenix cells to produce retrovirus and B-ALL cells were infected in the presence of polybrene and sorted twice on a FACS-AriaIII (Becton Dickinson) to get a pure mCherry$^+$ population. PDAC and MC38 cells were grown at 37 ˚C, 5% CO_2, in 500 mL DMEM, 50 mL FBS, and 5 mL Pen. Strep. PDAC cells were a gift from Matthew Vander Heiden. MC38 or Colon 38 cells were acquired from the Developmental Therapeutics Program Tumor Repository at Frederick National Laboratory. All cell lines used regularly tested negative for *Mycoplasma* detection (MycoAlert Plus kit, Lonza).

Mice and transplantation

C57BL/6J (wild type) and C57BL/6J *Il-6*$^{-/-}$ mice, 6-8 week-old, were purchased from Jackson Laboratory (RRID: IMSR_JAX:000664, and IMSR_JAX:002650). 500,000 BCR-ABL$^+$ B-ALL cells (mCherry$^+$ or negative depending on the experiment) were injected via tail vein into C57BL6/J mice of the appropriate genotype. On day 8 post-injection, mice were treated via intraperitoneal injection with 10 mg/kg doxorubicin (LC Labs) dissolved in normal saline solution. Mice were sacrificed when moribund. When applicable, mice were treated for 7 days with 50 mg/kg imatinib by oral gavage and sacrificed when moribund. For re-transplantation experiments IL-6 KO mice previously cured of B-ALL by doxorubicin treatment were re-injected with 500,000 B-ALL cells

(>100 days after initial injection) and disease burden and survival were monitored. 500,000 MC38 or PDAC cells were injected via subcutaneous injection into the hind-flanks of C57BL6/J mice. 200,000 PDAC cells were used for re-transplantations into IL-6 KO mice previously treated with doxorubicin. Subcutaneous tumor burden was measured with electronic calipers using the following formula: 1/2 × D × d^2; where 'D' is the major measurable axis and 'd' is the minor axis. Maximal tumor burden/size allowed was no larger than 1 cm in any direction and no deep ulceration. On a case-by-case basis, veterinary technicians allowed exceptions of tumor sizes larger than 1 cm if no deep ulceration was present and if mice seemed alert and responsive. Mice were bred in the SPF-animal facility in the Koch Institute and the Massachusetts Institute of Technology Department of Comparative Medicine approved all procedures and animal handling in the work presented here. Animals were monitored carefully for fitness and sacrificed when moribund in accordance with institutional Committee on Animal Care (CAC) procedures. Both female and male sexes were used. Food (ProLab RMH 3000) and water were given *ad libitum*. Animals were housed at 68-72 ˚F, with a relative humidity of 30-70%, and a dark/light cycle of 12/12 hours.

Bioluminescence imaging

Leukemic mice were imaged 1 day before doxorubicin treatment, the day of treatment, 2 days post-treatment, and 8- or 9-days post-treatment depending on the experiment. 165 mg/kg luciferin was injected prior to imaging and mice were anaesthetized using isoflurane prior to imaging on the IVIS Spectrum-bioluminescence and fluorescence imaging system (Perkin Elmer), and analyzed with the Living Image software.

Immune profiling

Leukemic mice were sacrificed 8 days post-injection (untreated), 2 days after doxorubicin, or 7 days post-treatment for analysis of immune-cell infiltration in bone marrow and spleen. Bone-marrow cells from WT and IL-6 KO mice were extracted by crushing both femurs and tibias with mortar and pestle in RBC Lysing Buffer (Sigma-Aldrich, R7757) for 5 minutes and re-suspended in 3% FBS-PBS (FACS Stain buffer). Splenic cells were extracted by crushing the spleen between glass slides into RBC Lysing Buffer and following the same protocol as above. Cells were stained with combinations of the following conjugated antibodies: CD3–FITC (17A2, BioLegend #100204; 1:100), CD4–APC (RM4-5, BD Biosciences #561091; 1:100), CD4–APC-Cy7 (GK1.5, BioLegend #100414; 1:100), CD8–PE-Cy7 (53-6.7, BD Biosciences #552877; 1:100), CD25–APC-Cy7 (PC61, BioLegend #102026; 1:100), CD69–PerCP-Cy5.5 (H1.2F3, BioLegend #104522; 1:100), CD11c–FITC (HL3, BD Biosciences #553801; 1:100), CD103–PerCP-Cy5.5 (2E7, BioLegend #121416; 1:100), CD86–APC (GL-1, BioLegend #105012; 1:100), MHC-II–APC-Cy7 (M5/114.15.2, BioLegend #107628; 1:100), MHC-II–PerCP-Cy5.5 (M5/114.15.2, BioLegend #107626; 1:100), CD11b–PE-Cy7 (M1/70, BioLegend #101216; 1:100), F4/80–APC (BM8, BioLegend #123116; 1:100), Gr-1–FITC (RB6-8C5, eBioscience #50-991-9; 1:100), IL-6R–APC (D7715A7, BioLegend #115812; 1:100), PD-1–BV421 (29F.1A12, BioLegend #135217; 1:100), MHC-I–FITC (34-1-2S, Abcam #ab95572; 1:100), MHC-II–FITC (M5/114, Abcam #ab239229; 1:100), and PD-L1–PE-Cy7 (10F.9G2, BioLegend #124314; 1:100) for one hour at 4 ˚C. 3 μM DAPI was added to the last wash to determine live cells and samples were analyzed on LSR-II HTS flow cytometer (Becton Dickinson). For all flow cytometry experiments, FlowJo was used for analysis.

Cytokine dose response

B-ALL cells were plated at 10,000/well in a 96-well plate. Cells were treated with ≥10 ng/mL IL-10, GM-CSF, IL-12, IL-15, VEGF, IL-6, sIL-6R, or IL-6 + sIL-6R (PeproTech) and doxorubicin (LC Labs) at 100 nM, 50 nM, 25 nM, 15 nM, 10 nM, 7.5 nM, 5 nM, 2.5 nM, 1 nM, 0.5 nM, and 0 nM concentrations. Cell count was obtained via flow cytometry FACS Calibur HTS (Becton Dickinson) with propidium iodide used to exclude dead cells.

Bone marrow co-culture

Bone-marrow cells from WT and IL-6 KO mice were extracted as described above, without the use of RBC lysis buffer. Extracted cells were plated in leukemia cell medium. Washes were performed until adherent cells became confluent at which point, they were transferred to 96-well plates, adhered for 24 hours and used for co-culture dose-response experiments as described above.

PDAC tumor dissociation

PDAC tumors harvested from euthanized mice were placed in 2.5 mL's of 1% FBS-RPMI and manually minced with blades. 5 mL's of digestion buffer was then added and samples were incubated for 30 minutes in a 37 ˚C water bath inside gentleMACS C tubes (Miltenyi Biotec, 130-096-334). Digestion buffer was prepared as follows: 1% FBS, 0.8M HEPES pH~7.5 (Invitrogen, 15630080), 1 mg/mL collagenase (Millipore Sigma, C2674), 4 U/mL DNAaseI (New England Biolabs, M0303), in HBSS (Millipore Sigma, 55037C). MACS tubes were then agitated with a MACS dissociator (Miltenyi Biotec, 130-093-235) for 1 minute, and samples were quenched with 5 mL's

FBS. Samples were then filtered through 70 and 30 µm filters (Miltenyi Biotec, 130-110-916 and 130-110-915, respectively), and spun at 1,200 rpm for 10 minutes. Samples were washed once with PBS by repeating spinning cycle, and finally resuspended as single cell suspensions in PBS.

p-STAT3 stain

Bone marrow and splenic cells from WT and IL-6 KO mice were extracted as described above, fixed in 3-4% paraformaldehyde, stained with primary p-STAT3 (Tyr705, D3A7, Cell Signaling Technology #4323S; 1:25) or IgG-isotype control (DA1E, Cell Signaling Technology #2975S; 1:25) at the same concentration. Alternatively, cells were fixed and permeabilized with a nuclear staining buffer set (Thermo Scientific, 00-5523-00), following manufacturer's instructions. Prior to fixing and permeabilization, staining of cell surfaces markers was performed with CD3–BV605 (17A2, BioLegend #100237; 1:100), and Zombie Aqua Fixable Viability Dye (BioLegend, 423102; 1:100). PhosSTOP 1X (Sigma-Aldrich, 4906837001) was used in every buffer. Cells were analyzed by flow cytometry using an LSR-II (Becton Dickinson) or LSR-Fortessa, and p-STAT3 levels were measured. Median FITC channel of isotype controls were subtracted from p-STAT3-stained samples to get p-STAT3 levels in a given cell population.

Western blot assays

Bone marrow cells from WT and IL-6 KO mice, untreated or doxorubicin treated, were harvested by centrifugation of dissected femur and tibia. Red blood cells were depleted from the bone marrow by a 5-minute incubation in red blood cell lysis buffer (Sigma-Aldrich, R7757). Red cell lysis was quenched with PBS. PhosSTOP 1X (Sigma-Aldrich, 4906837001) was used in every

buffer. A column with CD19 magnetic beads (Miltenyi Biotec, 130-121-301) was used to enrich for B-ALL cells ($CD19^+$), and the $CD19^-$ flowthrough was regarded as the stromal cells from the leukemic bone marrow. Tissue samples were homogenized in standard RIPA buffer, with a cocktail of protease (Thermo Scientific, 87786) and phosphatase inhibitors (Sigma-Aldrich, 4906837001). Protein concentrations were measured using BCA (Fisher Scientific, 23225). Cell extracts with the same amount of protein were mixed with 6X reducing Laemmli buffer (Boston BioProducts, BP-111R), boiled at 95 °C for 5 min, and subjected to electrophoresis using 4-20% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad). Proteins were transferred to methanol-activated PVDF membranes (Millipore Sigma, IPFL00010) and blocked with TBST buffer (LI-COR Biosciences, 927-66003) for 1 hour at room temperature. Blots were incubated at 4 ˚C overnight with primary antibodies, followed by secondary antibodies conjugated with LI-COR fluorophores. Samples were scanned with an Odyssey CLx imaging system (LI-COR Biosciences). Anti-actin (13E5, Cell Signaling Technology #4970S; 1:1000), anti-S6K (R&D Systems #AF8964; 1:200), anti-p-S6K (Thr389, Cell Signaling Technology #9205S; 1:1000), anti-vinculin (E1E9V, Cell Signaling Technology #13901S; 1:1000), anti-ERK (W15133B, BioLegend #686902; 1:1000), and anti-p-ERK (Thr202/Tyr204, Cell Signaling Technology #9101S; 1:1000), anti-rat IRDye 680RD (LI-COR #926-68076; 1:5000), anti-rabbit IRDye 800CW (LI-COR #926-32211; 1:5000), anti-goat IRDye 680RD (LI-COR #926-68074; 1:5000), anti-rabbit IRDye 800CW (LI-COR #926-32213; 1:5000). Cropped and uncropped blot images are shown in (**Supplementary Fig. 3a-d**).

In vivo T-cell depletion and mouse antibody treatment

WT and IL-6 KO leukemic mice were IP-injected on days 3 and 4 post B-ALL transplantation and then every 3 days thereafter with 200 µg CD4 (GK1.5, BioXCell #BE0003-1) and 200 µg CD8 (2.43, BioXCell #BE0061) depletion antibodies dissolved in sterile PBS. IL-6R Ab (15A7, BioXCell #BE0047) was injected every other day (500 µg/mouse) starting 3 days after leukemia transplantation (unless noted otherwise). PD-L1 antibody, at 200 µg/mouse (10F.9G2, BioXCell #BE0101), was injected on Days 7, 10, and 13 after disease transplantation. Rat IgG2b (LTF-2, BioXCell #BE0090) was used as an isotype control. IL-6R Ab was injected every other day (200 µg/mouse) starting 4 days after PDAC or MC38 transplantation. Randomization of animal cohorts was performed before transplantation of disease and before start of any treatment. When able, the experimenter was blinded to the individual mice being examined, although this was not performed in all experiments. Cohorts of 5 mice per cage were used and key findings repeated in multiple independent experiments, as detailed in figure legends.

ELISA assays

B-ALL and bone marrow cells harvested from the same mouse, untreated or doxorubicin treated, were seeded in 6-well tissue culture plates for 24 hours. The cell culture plates were centrifuged, and the supernatants collected and stored at -80 ˚C until measuring of the cytokine levels. HMGB1 (Fisher Scientific, NBP262782), CXCL10 (Thermo Scientific, BMS6018), IL-6 (Thermo Fisher Scientific, 88-7064-88), sIL-6R (R&D Systems, MR600).

RNA isolation from tumor stroma

Cells were isolated from the bone marrow of wild-type and IL6-KO mice by centrifugation of dissected femur and tibia. Red blood cells were depleted from the bone marrow by a 5-minute incubation in red blood cell lysis buffer (Sigma-Aldrich, R7757). Red cell lysis was quenched with PBS. Cells were centrifuged at 500 x g for 5 minutes and supernatant was aspirated. Cells were resuspended in FACS buffer (PBS with 5 % FBS and 1 ug/mL DAPI). Cells were sorted on a FACS-AriaIII (Becton Dickinson) running BD FACS Diva software. Stromal cells were isolated from B-ALL by gating for mCherry-negative cells following isolation of live singlets. Cells were collected and centrifuged at 500 x g, then supernatant was aspirated. Cells were snap frozen in liquid nitrogen. Cells were thawed on ice and RNA was isolated using the RNeasy Isolation Kit (Qiagen, 74134) according to the manufacturer's instructions.

RNA sequencing, data processing, and analysis

RNA libraries were prepared using the NEB Ultra II ribodepletion kit (E6310) according to the manufacturer's instructions. Libraries were sequenced using an Illumina NovaSeq 6000 to a depth of approximately $2x10^7$ single-ended reads per sample with a read length of 75 nucleotides. Reads were aligned to the mm10 genome, and transcript abundance was quantified using salmon (version 1.3.0). Salmon quant command was executed with the following flags: --validateMappings --gcBias --seqBias. Read counts were normalized and differentially expressed genes were identified using the DESeq2 R package (version 1.28.1). The DESeq command was executed with default options. Genes with greater than 10 read counts were rank-listed by t-statistic, and GSEA analysis of the pre-ranked gene list was performed using the clusterProfiler R

package (version 3.16.1). The GSEA command was executed with the following options: eps = 0.0 and TERM2GENE = msigdb cancer hallmark gene set.

Statistics and reproducibility

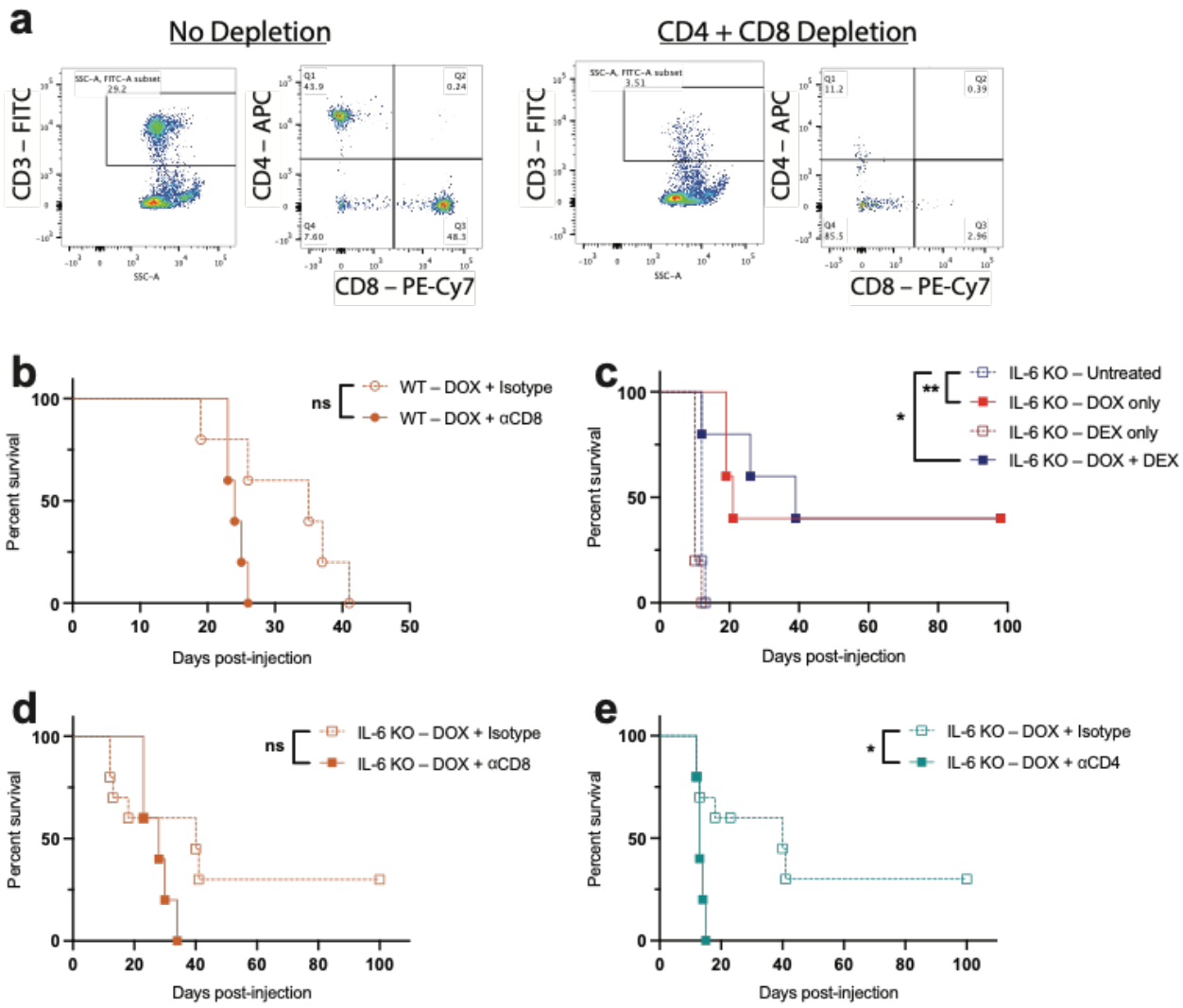
GraphPad Prism9 software (GraphPad Software, Inc.) or Microsoft Excel were used to perform statistical analysis. Respective tests are indicated in the figure legends. Error bars represent mean $\pm$ SEM, unless noted otherwise. Log-rank (Mantel-Cox) tests were used to compare Kaplan-Meier survival curves. Immune infiltration between WT and IL-6 KO samples were analyzed by a two-tailed Student's t-test. For all statistical tests, α was limited to 0.05 and $p<0.05$ was considered statistically significant. Boxplots show the median as the center lines, upper and lower quartiles as box limits, and whiskers represent maximum and minimum values. If present, outliers were included in the reported data.

Data availability

The gene expression datasets generated during the current study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) (Edgar et al., 2002), and are accessible through GEO Series with the following accession number: GSE184107 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184107].

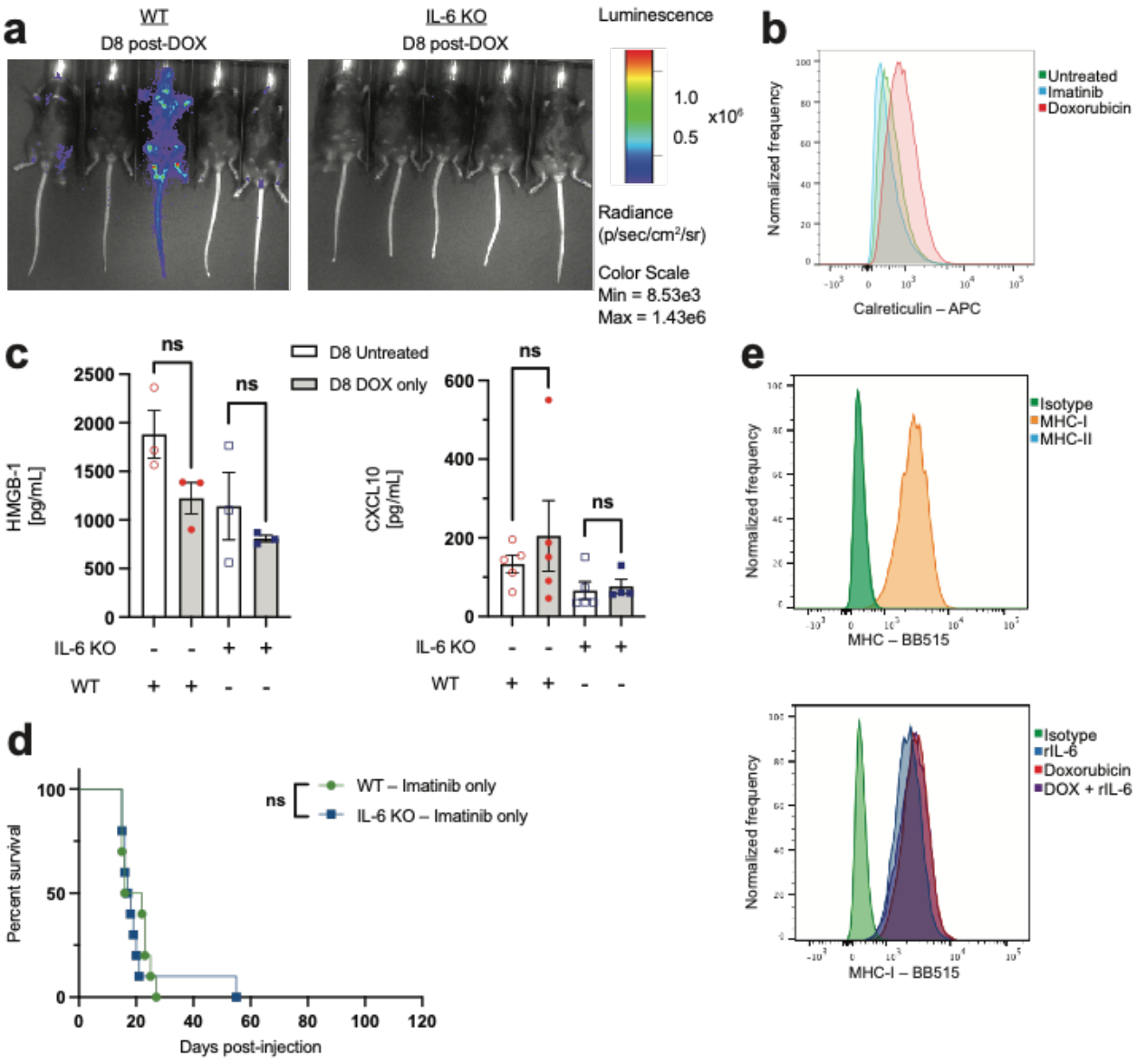
Supplementary information

Supplementary figure 2.1



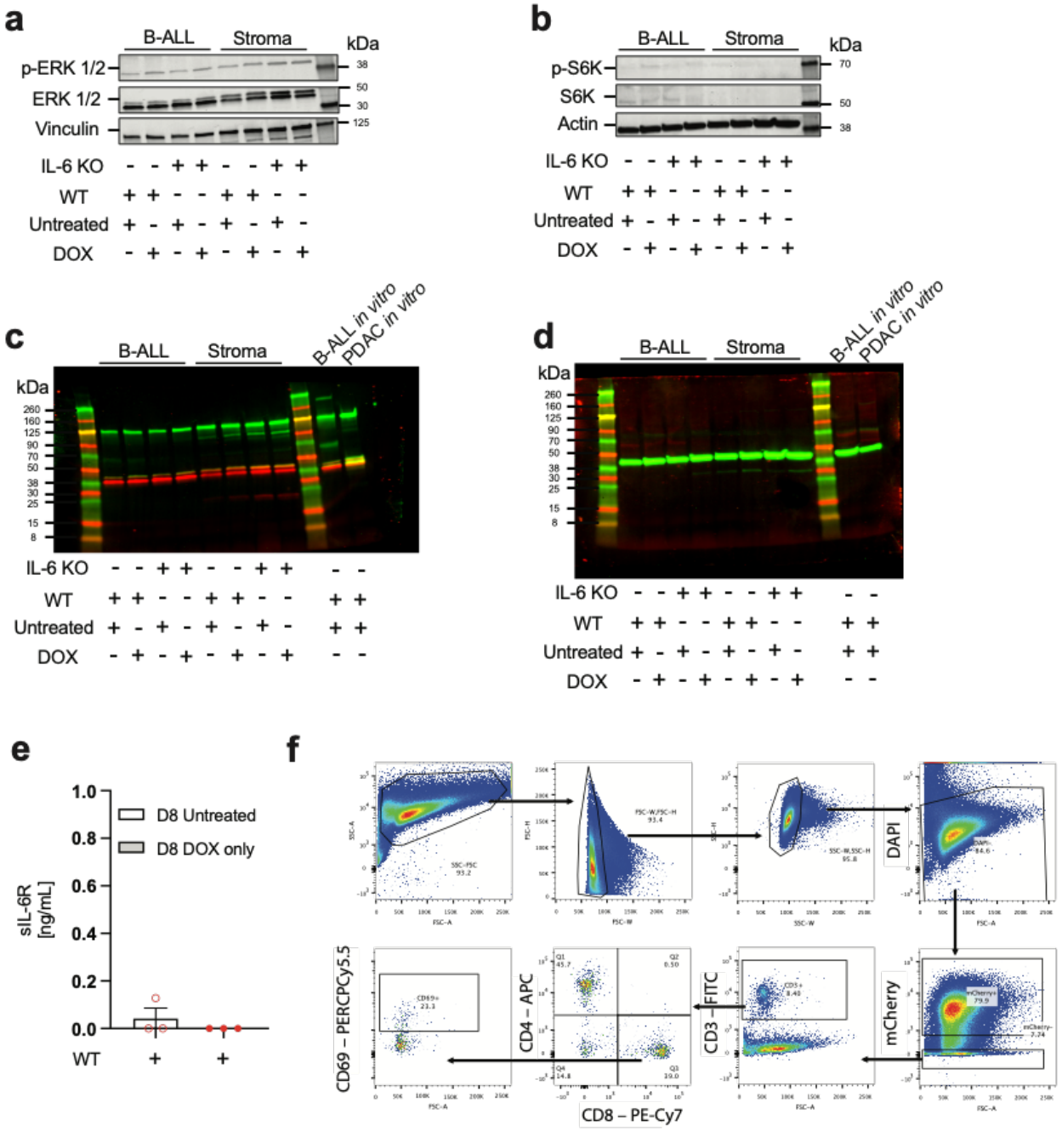
Supplementary Figure 2.1 | Doxorubicin and CD8/CD4 T-cell depletion treatments in WT and IL-6 KO mice. a, Flow cytometry analysis of peripheral blood samples from isotype control injected or T-cell depleted mice to confirm the efficacy of depletion. **b,** A Kaplan-Meier survival curve showing leukemic WT mice, treated with DOX and either $CD8^+$ T-cell depleted or not-depleted. n = 5 per cohort. **c,** A Kaplan-Meier survival curve showing leukemic IL-6 KO mice, treated with DOX and/or dexamethasone (DEX). n = 5 per cohort. *p = 0.0128 between IL-6 KO-untreated and IL-6 KO-DOX + DEX, **p = 0.0016 between IL-6 KO-untreated and IL-6 KO-DOX only. **d-e,** A Kaplan-Meier survival curve showing leukemic IL-6 KO mice, treated with DOX and either $CD8^+$ T-cell depleted, $CD4^+$ T-cell depleted, or not-depleted. n = 5 per cohort, except n = 10 for IL-6 KO-isotype. *p = 0.0249 in panel (**e**). Log-rank (Mantel-Cox) tests were used to compare Kaplan-Meier survival curves.

Supplementary figure 2.2



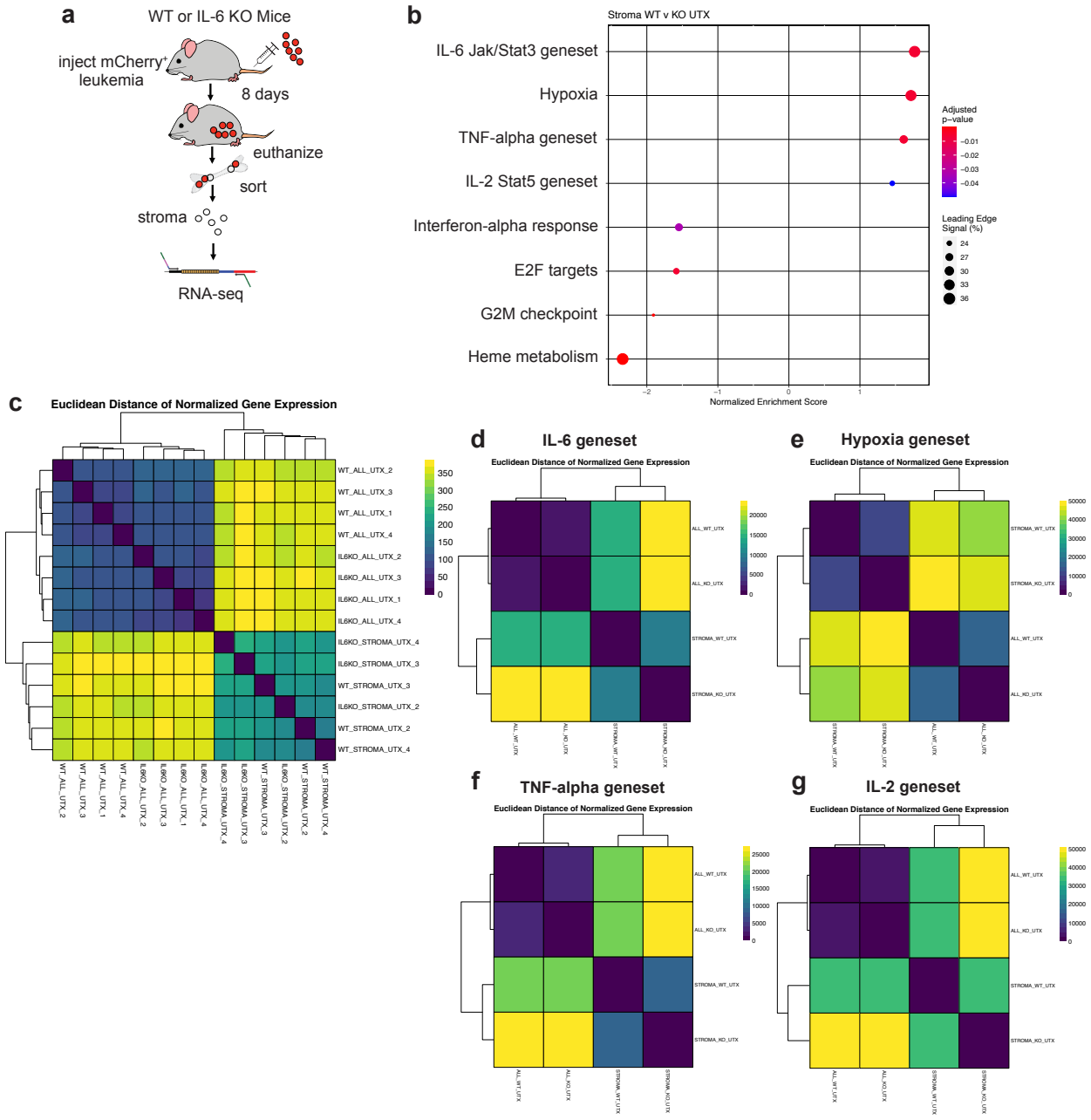
Supplementary Figure 2.2 | Doxorubicin treatments and characterization of immunogenic cell death markers in B-ALL bearing WT and IL-6 KO mice. a, IVIS images showing WT or IL-6 KO mice with leukemia burden, monitored by bioluminescent imaging. **b,** A flow cytometry plot showing calreticulin expression on the surface of leukemia cells cultured *in vitro*. Cells were treated with doses that induce similar killing between both drugs and stained 18 hours after treatment initiation. **c,** Left, a graph showing the concentration of HMGB-1 present in the bone marrow of B-ALL bearing mice. n = 3 per cohort. Right, a graph showing the concentration of CXCL10 in the bone marrow of B-ALL bearing mice. n = 5 per cohort, except n = 4 for IL-6 KO-DOX only. Data is represented as mean ± SEM. Shown Ns are biological replicates from 1 independent experiment. ns>0.05 by Ordinary one-way ANOVA test. **d,** A Kaplan-Meier survival curve showing leukemic WT or IL-6 KO mice, either treated with imatinib or untreated. n = 10 per cohort. Data from 2 independent experiments is shown. Log-rank (Mantel-Cox) test. **e,** Above, a flow cytometry plot showing surface expression of MHC class I and II on leukemia cells pre-treatment *in vitro*. Histograms for MHC-II and isotype control-stained cells are overlaid. Below, a flow cytometry plot showing surface expression of MHC class I on leukemia cells after treatment with doxorubicin and/or rIL-6, *in vitro*. Data is representative of 2 independent experiments with 1 replicate per group, per experiment. D8 = Day 8.

Supplementary figure 2.3



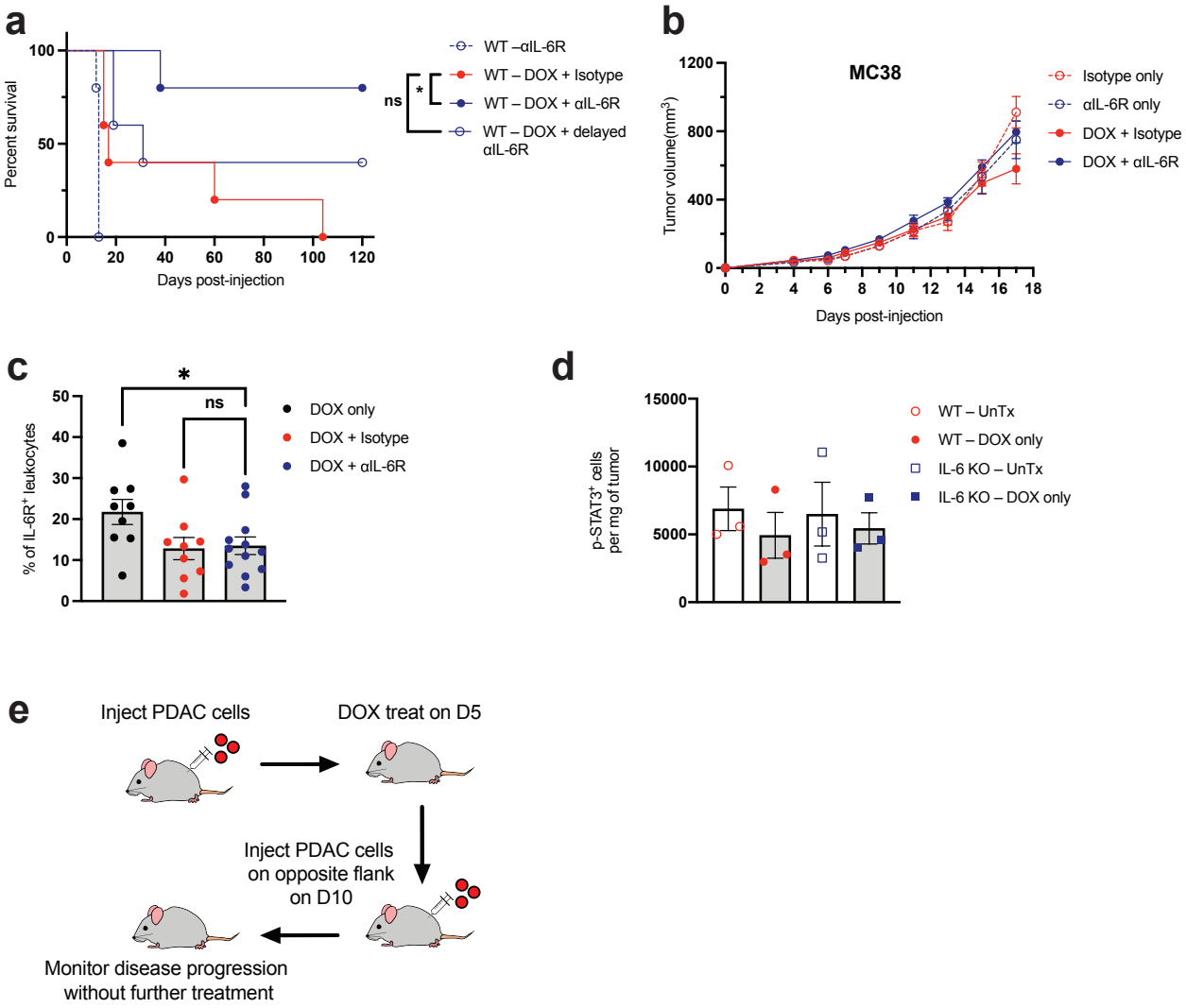
Supplementary Figure 2.3 | IL-6 downstream signaling characterization and identification of immune infiltrates. a, ERK 1/2 activity as assessed by Western blot analysis 24 hours after DOX treatment in B-ALL and stroma samples from WT and IL-6 KO mice. **b,** S6K activity as assessed by Western blot analysis 24 hours after DOX treatment in B-ALL and stroma samples from WT and IL-6 KO mice. Representative blots of 4 biological replicates per sample for both (**a**) and (**b**). **c-d,** Full overlayed Western blots with marks of protein size for blots shown in (**a**) and (**b**), respectively. **e,** A graph showing the concentration of sIL-6R present in the bone marrow of WT B-ALL bearing mice. Data is represented as mean ± SEM. Shown Ns are biological replicates from 1 independent experiment. **f,** Flow cytometry analysis for the identification of T-cell subsets.

Supplementary figure 2.4



Supplementary Figure 2.4 | RNA-sequencing from bone marrow. a, Schematic outline of experiment to isolate bone marrow stromal and leukemia cells from WT and IL-6 KO mice for RNA-sequencing. 500,000 cells per mouse were injected and mice sacked 8 days later, bone marrow collected and $mCherry^+$ leukemia cells, and $mCherry^-$ stromal cells, sorted and RNA isolated for RNA-sequencing. **b,** GSEA analysis of differential gene expression in stroma from IL-6 KO relative to wild-type samples using the MSigDB GO cancer Hallmarks collection. All significant results (adjusted p-value<0.05) are included and sorted by normalized enrichment score, and rank-listed by t-statistic. Dot size corresponds to the fraction of the gene set in the leading-edge signal. Color corresponds to adjusted p-value. **c,** Euclidean distance between global normalized gene expression. **d-g,** Euclidean distance between mean sample gene expression for enriched gene sets within the GSEA GO Hallmarks collection.

Supplementary figure 2.5



Supplementary Figure 2.5 | αIL-6R treatment optimization. a, A Kaplan-Meier survival curve showing WT mice that underwent treatment optimization with therapeutic antibodies against IL-6R. Treatment for all groups started on Day 3 after leukemia transplantation and continued for every other day. Mice on the 'DOX + delayed αIL-6R' group received inhibitor treatment starting on Day 7 after leukemia transplant and continued for every other day. n = 5 per cohort. *p = 0.0126 by Log-rank (Mantel-Cox) test. **b,** A graph showing MC38 tumor burden in WT mice treated with DOX and either an IL-6R blocking antibody or isotype control. n = 5 per cohort. Data is represented as mean ± SEM. **c,** A graph showing IL-6R expression percentages in the dissociated PDAC tumors from the indicated treatment groups. Data was quantified by flow cytometry, and is represented as mean ± SEM. n = 9 per cohort, except n = 12 for DOX + αIL-6R. *p = 0.0339 by two-tailed Student t-test. **d,** A graph showing the total number of p-STAT3 positive cells per mg of PDAC tumor in the indicated treatment groups. Data was quantified by flow cytometry, and is represented as mean ± SEM. n = 3 per cohort. **e,** Schematic outline of IL-6 KO mice previously treated with DOX, re-transplanted with PDAC cells, and then tumor burden progression monitored in the absence of further treatment. Data in panels (**b-d**) is represented as mean ± SEM. D5 = Day 5, D10 = Day 10.

Supplementary table 2.1

Table BM	Untreated		D2 post-DOX	
Immune Cell Population in BM (Percent of mCherry- cells)	**WT**	**IL-6 KO**	**WT**	**IL-6 KO**
T Cells				
1) CD3+	3.36	2.94	7.84[a]	8.10[b]
2) CD3+ CD4+	1.80	1.42	3.22[c]	3.40[d]
3) CD3+ CD4+ CD69+	0.41	0.28	0.67	0.58[e]
4) CD3+ CD4+ CD25+	0.15	0.11	0.13	0.19[f]
5) CD3+ CD8+	0.40	0.50	2.62[g]	3.18[h]
6) CD3+ CD8+ CD69+	0.30	0.33	0.87[i]	0.93[j]
Dendritic Cells				
7) CD11c+ MHCII+	1.80	1.70	3.43	3.21[k]
8) CD11c+ MHCII+ CD11b+	1.27	1.05	2.24[l]	1.88
9) CD11c+ MHCII+ CD11b+ CD86+	0.51	0.46	0.77	0.67
10) CD11c+ MHCII+ CD11b-	0.96	0.76	1.91	1.37
11) CD11c+ MHCII+ CD11b- CD86+	0.27	0.24	0.66	0.51
12) CD11c+ MHCII+ CD11b- CD103+	0.51	0.41	0.64	0.82
13) CD11c+ MHCII+ CD11b- CD103+ CD86+	0.22	0.17	0.26	0.38
Macrophages				
14) F480+ CD11b+ Gr-1-	1.60	1.65	1.19[m]	1.32
15) F480+ CD11b+ Gr-1- MHCII+	0.25	0.20	0.48[n]	0.42[o]
Neutrophils				
16) F480- CD11b+ Gr-1+	21.31	27.90	37.67[p]	39.00[q]

Supplementary Table 2.1 | Quantification of bone marrow immune infiltration before and after doxorubicin treatment. The average representation of immune cell subsets in the bone marrow of WT and IL-6 KO mice before and after treatment. Superscripts denote $p<0.05$ compared to untreated sample of the same genetic background. Analyzed by two-tailed Student t-test. Superscripts corresponding exact p-values: a = 0.0142, b<0.0001, c = 0.0044, d<0.0001, e = 0.0137, f = 0.0059, g = 0.0099, h<0.0001, i = 0.0032, j = 0.0018, k = 0.019, l = 0.0061, m = 0.0071, n = 0.0271, o = 0.0176, p = 0.0029, and q = 0.0189. Samples in group 4 have: n = 7 for WT-untreated, n = 8 for WT-D2 post-doxorubicin, n = 7 for IL-6 KO-untreated, n = 8 IL-6 KO-D2 post-doxorubicin mice, and show data from 3 independent experiments. Samples in groups 1, 2, 5, 7, 14, and 16 have: n = 7 for WT-untreated, n = 11 for WT-D2 post-doxorubicin, n = 7 for IL-6 KO-untreated, n = 10 IL-6 KO-D2 post-doxorubicin mice, and show data from 4 independent experiments. Samples in groups 3, 6, 8-13, and 15 have: n = 9 for WT-untreated, n = 10 for WT-D2 post-doxorubicin, n = 9 for IL-6 KO-untreated, n = 8 IL-6 KO-D2 post-doxorubicin mice, and show data from 3 independent experiments. There were no significant statistical comparisons between 'untreated' and 'DOX treated' samples of different genetic backgrounds.

Supplementary table 2.2

Table Spleen	Untreated		D2 post-DOX	
Immune Cell Population in Spleen (Percent of mCherry- cells)	**WT**	**IL-6 KO**	**WT**	**IL-6 KO**
T Cells				
1) CD3+	17.30	17.42	19.18	19.34
2) CD3+ CD4+	9.74	9.08	9.85	9.53
3) CD3+ CD4+ CD69+	0.61	0.53	1.16[a]	1.05[b]
4) CD3+ CD4+ CD25+	0.54	0.51	0.58	0.67
5) CD3+ CD8+	5.52	6.32	6.90	7.59
6) CD3+ CD8+ CD69+	0.21	0.24	0.43[c]	0.49[d]
Dendritic Cells				
7) CD11c+ MHCII+	2.26	1.86	5.13[e]	4.60[f]
8) CD11c+ MHCII+ CD11b+	0.96	0.81	1.34[g]	1.26[h]
9) CD11c+ MHCII+ CD11b+ CD86+	0.41	0.37	0.60[i]	0.61[j]
10) CD11c+ MHCII+ CD11b-	1.32	1.08	3.95[k]	3.09[l]
11) CD11c+ MHCII+ CD11b- CD86+	0.49	0.57	0.94[m]	1.09
12) CD11c+ MHCII+ CD11b- CD103+	0.58	0.52	1.24	1.55
13) CD11c+ MHCII+ CD11b- CD103+ CD86+	0.22	0.18	0.46	0.55
Macrophages				
14) F480+ CD11b+ Gr-1-	0.57	0.82	0.83[n]	0.85
15) F480+ CD11b+ Gr-1- MHCII+	0.29	0.33	0.41[o]	0.44
Neutrophils				
16) F480- CD11b+ Gr-1+	2.04	2.19	0.82[p]	0.81[q]

Supplementary Table 2.2 | Quantification of spleen immune infiltration before and after doxorubicin treatment. The average representation of immune subsets in the spleen of WT and IL-6 KO mice before and after treatment. Superscripts denote $p<0.05$ compared to untreated sample of the same genetic background. Analyzed by two-tailed Student t-test. Superscripts corresponding exact p-values: a = 0.0244, b = 0.0062, c = 0.0235, d = 0.0028, e = 0.0014, f = 0.0003, g = 0.0034, h<0.0001, i = 0.002, j = 0.001, k = 0.0232, l = 0.0182, m = 0.0208, n = 0.0289, o = 0.0132, p = 0.002, and q = 0.0118. Samples in group 1, 2, 5, and 7 have: n = 12 for WT-untreated, n = 14 for WT-D2 post-doxorubicin, n = 12 for IL-6 KO-untreated, n = 12 IL-6 KO-D2 post-doxorubicin mice, and show data from 4 independent experiments. Samples in groups 14, 15, and 16 have: n = 12 for WT-untreated, n = 13 for WT-D2 post-doxorubicin, n = 12 for IL-6 KO-untreated, n = 12 IL-6 KO-D2 post-doxorubicin mice, and show data from 4 independent experiments. Samples in groups 3, 4, 6, and 8-13 have: n = 9 for WT-untreated, n = 10 for WT-D2 post-doxorubicin, n = 9 for IL-6 KO-untreated, n = 9 IL-6 KO-D2 post-doxorubicin mice, and show data from 3 independent experiments. There were no significant statistical comparisons between 'untreated' and 'DOX treated' samples of different genetic backgrounds.

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Chapter 3

Discussion, limitations, and future directions

Chapter 3: Discussion, limitations, and future directions

Immunotherapy has emerged as the most promising new approach for managing cancer recurrence. Administration of immunotherapeutic agents can generate robust responses that stimulate the body's cytotoxic lymphocytes to eliminate tumor cells. During the initial phases of immune responses, antigens are captured by DCs, which mature and present antigen-derived peptides complexed to MHC molecules to T cells in lymph nodes. In turn, these T cells mature into effector antigen-specific T cells that migrate toward tumor microenvironments where they begin tumor elimination. However, normal immune regulation mechanisms that protect the host from immune damage are often hijacked by tumors to promote immunosuppression and promote escape from anticancer immune responses (Beatty & Gladney, 2015). Consequently, while numerous therapeutic strategies aimed at restoring the immune system's ability to eliminate malignant cells have been tested in preclinical and clinical settings, many of these interventions only produce limited clinical benefits in patients. Thus, there is an urgent need to develop better immunotherapeutic approaches.

Depending on the mechanisms of immune evasion exploited by cancers, distinct therapeutic strategies are required for restoring effective antitumor responses. In my thesis, using a physiologically relevant and immunocompetent mouse model of Philadelphia chromosome positive (Ph^+) B-cell acute lymphoblastic leukemia (B-ALL), we demonstrate that inhibiting IL-6 signaling in combination with doxorubicin treatment generates protective anticancer immune responses. Additionally, this combination approach overcomes one of the key obstacles for developing successful therapy regimens, as it prevents disease relapse of leukemia. Thus, we

provide preclinical evidence of combination immunotherapies that, if clinically evaluated, could improve the efficacy of already in-use powerful standard-of-care agents.

1.1 Conventional chemotherapy as immunotherapy: dose and schedule dependence

Certain chemotherapy agents, like doxorubicin, can kill tumor cells through immunogenic cell death (ICD) pathways, which operate by engaging robust innate and adaptive anticancer immune responses. However, tumor-independent toxicity from these agents can also be immunosuppressive, acting directly on cells from the immune system and limiting immune stimulation (Zitvogel et al., 2011). This immune suppression may result from doxorubicin and other conventional cytostatic chemotherapies being administered at maximum tolerated dose (MTD) schedules, which results in numerous co-morbidities. Therefore, metronomic treatment schedules, which deliver these chemotherapeutic agents at lower doses and more frequently, are an attractive approach to reduce toxicities, and strengthen and increase the duration of antitumor immune responses (Kareva et al., 2015).

Coupled with other immunomodulatory therapies, like agents that inhibit suppressive cytokines, metronomic dosing of chemotherapies could result in even more pronounced anticancer immune responses. This approach could prove useful because inclusion and combination of different immunotherapies may stimulate diverse antitumor immune responses. Our results support this approach, as we report enhanced efficacy of doxorubicin when administered in the absence of the cytokine IL-6. In our system, doxorubicin inhibits tumor growth both by its intrinsic tumor cell cytotoxicity and by converting immunologically “cold” bone marrow

microenvironments into “hot” niches that are abundantly infiltrated by many types of immune cells. In turn, as shown by our RNA sequencing data, IL-6 absence from these microenvironments results in engagement of multiple immune stimulatory pathways that potentiate doxorubicin’s activity.

Notably, as shown in Chapter 2, changes in the treatment schedule for IL-6 inhibitor dosing did not result in significant enhancement of our phenotype. However, changes in the timing and dosing administration of doxorubicin could potentially translate into decreased overall drug toxicity and improve immune stimulation. Empirical testing to validate enhanced anticancer effects induced by these proposed metronomic doxorubicin-dosing schedules remains to be performed.

Toxic effects of ALL standard of care treatment

About 1-2% of children with ALL perish from therapy-induced toxic effects (Blanco et al., 2012). An increased risk of death is also seen for older patients and for those receiving more intensive therapy regimens. Additionally, the type of therapy and genetic factors that influence drug metabolism and activity can dictate toxic effects, which include lympho- and myelodysplasia, osteonecrosis, metabolic syndromes and obesity, central and peripheral nervous system toxicities, and cardiovascular impairments (Hunger & Mullighan, 2015). In particular, clinical use of anthracyclines like doxorubicin induces chronic cardiomyopathy and congestive heart failure in patients from all demographics (Minotti et al., 2004). Therefore, tailoring of drug exposure in treatment regimens is largely influenced by the specific toxic effects these agents can cause. In

many occasions, these decisions are made at the expense of drug efficacy. Thus, as suggested from our results, therapy administration schedules may benefit from modified regimens centered on immune-modulating effects of immunotherapy rather than maximal direct cytotoxicity.

Toxic effects of immune checkpoint blockade

Among many new immunotherapies, immune checkpoint inhibitors (ICIs) have seized the spotlight by showing remarkable clinical activity against numerous cancer types. However, increased activity of immune responses generated by ICI therapies often come at a cost, as they also stimulate inflammatory side effects or immune-related adverse events. These inflammatory adverse events most commonly involve the gastrointestinal tract, endocrine glands, skin, and liver, among other tissues and organs (Weber et al., 2017). The precise pathophysiology causing these events, and why do some patients develop them and some do not, is currently unclear (Postow et al., 2018). Regardless of these underlying mechanisms, immune-related adverse events are effectively treated by delaying administration of ICIs or by administering immunosuppressants, like oral glucocorticoids. Still, clinical practice remains variable as there are no consensus guidelines for appropriately managing these specific immune-related adverse events in patients.

Overall, in light of these observations, our data reveals new strategies for combinations of chemo- and immuno-therapies to treat cancer patients. Our results suggest that timing of administration of immunotherapies matters. Rather than waiting for adverse toxic events to

dictate drug exposure, concomitant administration of reduced-dose immunogenic-inducing therapies and immunosuppressive inhibitors might result in more effective tumor control and toxicity management in patients.

1.2 Immunogenic cell death induction and characterization

Doxorubicin and several other chemotherapeutics have the capacity to promote ICD. Key events in this programmed cell death pathway include tumor cell surface exposure of “eat-me” signals, like calreticulin (CRT), for dendritic cell (DC) engulfment and tumor antigen uptake; and post-apoptotic release of the nuclear chromatin binding protein HMGB1, which helps in priming innate and adaptive immune responses by activating toll-like receptors (TLRs). Using our B-ALL mouse model, we detected upregulation of CRT by tumor cells after doxorubicin treatment.

Yet, not all ICD markers are seen in this setting. For example, HMGB1 concentrations in the tumor microenvironment (TME) did not change after doxorubicin treatment. Nonetheless, this agent clearly has ICD capacity in our system, given that doxorubicin treatment promotes immunity against live-tumor rechallenge. Thus, the extent to which all chemo-induced ICDs are accompanied by the established hallmarks of ICD remains to be addressed.

Key experiments remain to be performed to fully characterize the ICD roles of doxorubicin in our system. For example, a more extensive characterization of cell surface markers that present “do not eat-me” signals to antigen presenting cells (APCs) could be implemented. Surface markers like CD31, CD46, and CD47, have been shown to be downregulated during ICD induction, allowing

phagocytosis to occur due to prevailing “eat-me” signals (Martins et al., 2010). Particularly, CD47 surface molecules counter phagocytic signals like CRT exposure. Thus, further characterization of these molecules, as well as the biomarkers that underlie their exposure, could unveil more mechanistic information of the role of doxorubicin in our system. Additionally, release of HMGB1 into the extracellular matrix of dying tumor cells should specifically trigger TLR4 expressed on DCs (Apetoh et al., 2007). TLR4 engagement controls tumor antigen processing and presentation. The role of this ICD pathway in our system, if any, could potentially be studied using *Tlr4*$^{-/-}$ mouse strains, and model antigens – discussed next.

1.3 Model antigens and immune responses: T cell and dendritic cell dynamics

Adaptive immune responses rely on interactions between T cell receptors (TCRs) and antigen peptides bound to major histocompatibility complex (MHC) molecules. However, these interactions are highly variable and complex due to polymorphisms on MHC encoding genes, copious antigenic peptides or epitopes, and heterogenous gene recombination events involved in the generation of TCRs. These complexities have made it very difficult to meticulously study TCR repertoires and to identify and track antigen-specific T cells in mice or humans. To circumvent these issues, mouse studies have heavily relied on model antigens and TCR transgenic mice to interrogate the interface of tumor-specific antigens (TSAs) and T cell adaptive responses (DuPage et al., 2012).

Contrived experimental setups with transgenic TCRs and model antigens allow for finer articulation of the particularities of T cell mediated immune responses (although this

experimental resolution comes at the expense of identifying naturally occurring antigen-TCR complexes). In most immune responses, activated and responsive T cells with antigen-specificities of interest are often scarce and represent a very small minority of total lymphocytes. Thus, the value of implementing these contrived approaches and restricting analysis to relevant antigen-specific T cells becomes clearer. For example, limiting analysis to precise antigen-TCR dynamics permits the interrogation of clone-specific proliferation, surface marker expression, and cytokine production; all without the interference of other activated cells from the same samples (Andersen et al., 2012; Bettini et al., 2012; Newell & Davis, 2014).

In my thesis, since we report T cells as the central effectors of immune responses after treatment with doxorubicin in the absence of IL-6, future experiments would benefit from contrived systems as described above. Using B-ALL cells that express model antigens would more feasibly allow for developing a deeper understanding of T cells dynamics in our system. This approach would allow for characterization of T cell mechanisms like activation, differentiation, tolerance, cytokine production, and cytotoxicity. For example, tumor antigen-specific endogenous $CD8^+$ and $CD4^+$ T cells from different tissues, like draining lymph nodes, spleens, and bone marrows, could be tracked and characterized with respect to their cytokine release profiles and activation state. Importantly, these tools can also be employed for identifying and characterizing properties from $CD4^+$ and $CD8^+$ circulating and tissue resident memory T cells that are presumably generated after treatment with doxorubicin in our system (Renkema et al., 2014; Kok et al., 2021).

Additionally, using B-ALL cells expressing model antigens and performing DC and tumor cell co-culture assays, allows for the study of DC involvement and antigen presentation in our system. Briefly, these assays utilize tissue derived DCs fed with model-antigen expressing dying tumor cells. “Eat-me” signals from these dying tumor cells prompt DCs to engulf them or their cellular debris and prepare the known model antigens for presentation. Once presented on the surface of DCs, these antigens can be detected with fluorescently-conjugated antibodies that specifically bind model-antigen-MHC complexes. These assays would be useful to interrogate if ICD inducers allow for better model-antigen production, and for determining the role of genetic backgrounds (e.g., *Il6*$^{-/-}$ vs WT conditions) in DC antigen processing and presentation. Other markers of DC activation could also be characterized, like production and secretion of IL-2. Lastly, adoptive cell transfer of DCs loaded with tumor cell-derived model antigens can be performed to analyze their potential to elicit endogenous $CD4^+$ and $CD8^+$ T cell responses *in vivo* (Wculek et al., 2019). However, this last approach is unable to assess and confirm the DCs abilities to physically reach sites of tumor cell death and cross-prime tumor derived antigens *in vivo*.

1.4 Transplantable versus autochthonous mouse models

Immunocompetent *in vivo* mouse cancer models have shifted the experimental paradigm of cancer as a cell-autonomous driven disease, and have enhanced our appreciation and investigation on how malignant and stromal cells interact to promote cancer progression. Particularly, there has been increasing interest in how cancer can be treated by targeting components of the TME, e.g., specifically targeting immune elements of the TME (Leach et al., 1996; Iwai et al., 2005). Additionally, accumulating evidence indicates that numerous types of

cancer therapies achieve long-term benefits by mobilizing host immune responses against malignant cells (Galluzzi et al., 2015).

Indeed, increased use of immunocompetent mouse models of cancer should allow for better-quality examination of immunosurveillance mechanisms during tumor progression (Zitvogel et al., 2016). The most common type of mouse models used in oncoimmunology are transplantable mouse models – like the B-ALL model used for this thesis work. Generally, these models consist of histocompatible cancer cell lines that are transplanted into immunocompetent inbred mice. In most cases, tumor cells are subcutaneously injected into the flanks of mice, as we performed for the pancreatic ductal adenocarcinoma (PDAC) model used for some of the experiments presented on Chapter 2. Occasionally, tumor cells are injected either orthotopically – which mimics tumor growth in their native TME – or systematically by intraperitoneal or intravenous injections. Arguably, intravenous systemic injections of hematologic models, like our B-ALL model, could also be considered as orthotopic injections since the disease homes-to and populates its normal environments within mice (e.g., bone marrow, spleen, and circulation).

Transplantable mouse models offer multiple advantages, like reduced experimental cost, technical simplicity, shortened tumor-growth kinetics, and synchronous growth of tumors. However, in general, mouse cancer models are affected by many limitations, like not being able to study human-derived molecules and cell populations, which may, in turn, limit translational potential to human settings. Limitations of transplantable mouse models also include: (1) reliance in cancer cell lines that are largely homogenous populations of cells with low mutational

burden compared to human cancers; (2) the use of cells that have been immunoedited and selected to evade immunosurveillance in their original host, which could make them intrinsically immune-tolerant cells; (3) tumor cell rapid growth kinetics that may exempt them from the chronic inflammatory microenvironment progression seen in human tumors, and many other features of natural carcinogenesis; (4) indirect effects of injections that may mediate some degree of immune activation regardless of tumor cells (Bonnotte et al., 2003). Nevertheless, transplantable mouse models have been instrumental for important discoveries in oncoimmunology and antitumor therapy designs.

Orthotopically transplanted tumors have key differences compared to other types of transplantation – particularly to those transplanted via subcutaneous injections. For example, orthotopically transplanted tumors more precisely resemble normal sites of tumor growth, their vascularization, and sensitivity to therapies. However, many of these features make orthotopically transplanted tumors more immunosuppressive, as in most cases they have less functional blood vessels that impair drug delivery and immune cell infiltration (Devaud et al., 2014).

Autochthonous tumors, generated in genetically engineered mouse models (GEMMs), arise from transgenic expression of oncogenes and/or the inactivation of tumor suppressor genes by genetic recombination in developing mice. These mouse models provide important insights into the interactions between tumorigenesis and the host. For example, seminal studies using KP mouse models (mice with conditional expression of oncogenic *Kras* and concurrent loss of the tumor

suppressor *p53*) have fundamentally supported the immunosurveillance and immunoediting theories of cancer biology in the context of whole *in vivo* organisms (DuPage et al., 2011; DuPage et al., 2012). Broadly, and compared to other mouse models, GEMMs tend to more accurately reflect human tumors as they are highly heterogenous with respect to their onset, progression, and antigenic makeup.

Therefore, having discussed some of the advantages and limitations of transplantable models versus autochthonous models, ideal follow-up experiments for our observations presented in Chapter 2 should include the study of autochthonally-derived B-ALL tumorigenesis. This approach should allow for better characterization of immune effector roles during initiation, progression, and treatment of tumors. In fact, Claudia Huettner and colleagues, have already established the feasibility of this approach using autochthonous B-ALL mouse models induced by oncogenic BCR-ABL activity (Huettner et al., 2000). Others have also shown how tumor regression of autochthonous B-ALL mouse models is dependent on $CD4^+$ lymphocyte activity (Rakhra et al., 2010). Additionally, use of GEMMs can also be combined with other tools, like expression of model antigens to further study and differentiate antigen-dependent and -independent mechanisms of immune responses.

1.5 Final remarks

Accumulating evidence demonstrates that ICD is critical for eliminating malignant tumors, and its induction stands out as a major therapeutic opportunity for successful cancer therapy in the clinic. Additionally, numerous studies substantiate how the efficacies of several ICD-inducing

agents are limited by robust immunosuppressive dynamics established in tumor microenvironments. Therefore, attaining greater knowledge of the immune system and regulation of its cellular and humoral components will allow for rational development of new therapeutics and therapeutic combinations to circumvent these immunosuppressive forces. In that regard, the results presented in this thesis demonstrate that loss of IL-6 enhances anticancer immune responses to multiple immune-stimulating therapies. Thus, we establish that IL-6 inhibition with doxorubicin treatment may potentially achieve long-term anti-leukemia immune responses in clinical patients. Ultimately, I am excited to witness how researchers and clinicians alike continue to harness the power of combining immune activating therapies to induce permanent cancer remission in patients.

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Appendix A

Modeling inflammatory bowel disorders after treatment with IL-6R inhibitors and doxorubicin

Appendix A: Modeling inflammatory bowel disorders after treatment with IL-6R inhibitors and doxorubicin

Doxorubicin treatment generates long lasting anti-tumor immunity in mice treated with IL-6 receptor inhibitors

After discovering that T-cells are the ultimate mediators of B-ALL elimination and long-lasting anti-cancer immunity in IL-6 KO mice (**Figure 2.4**), we wanted to test if cured-WT mice treated with IL-6R inhibitors and doxorubicin also experienced the generation of immunologic memory. To address if WT mice treated with doxorubicin and IL-6R inhibitors developed lasting immunologic memory after treatment, we re-transplanted leukemia cells into previously cured WT mice (**Figure ApA.1a**). As similarly seen with the previously cured IL-6 KO mice, previously cured WT mice were completely resistant to leukemia initiation upon tumor re-transplantation, as monitored by bioluminescent imaging (**Figure ApA1.b**). However, B-ALL re-transplanted WT mice did succumb short after re-transplantation, without any indication of tumor progression or tumor related symptoms like hind-leg paralysis (**Figure ApA1.c**). Nevertheless, mice did present symptoms of lethargy soon before becoming moribund.

Figure Appendix A.1

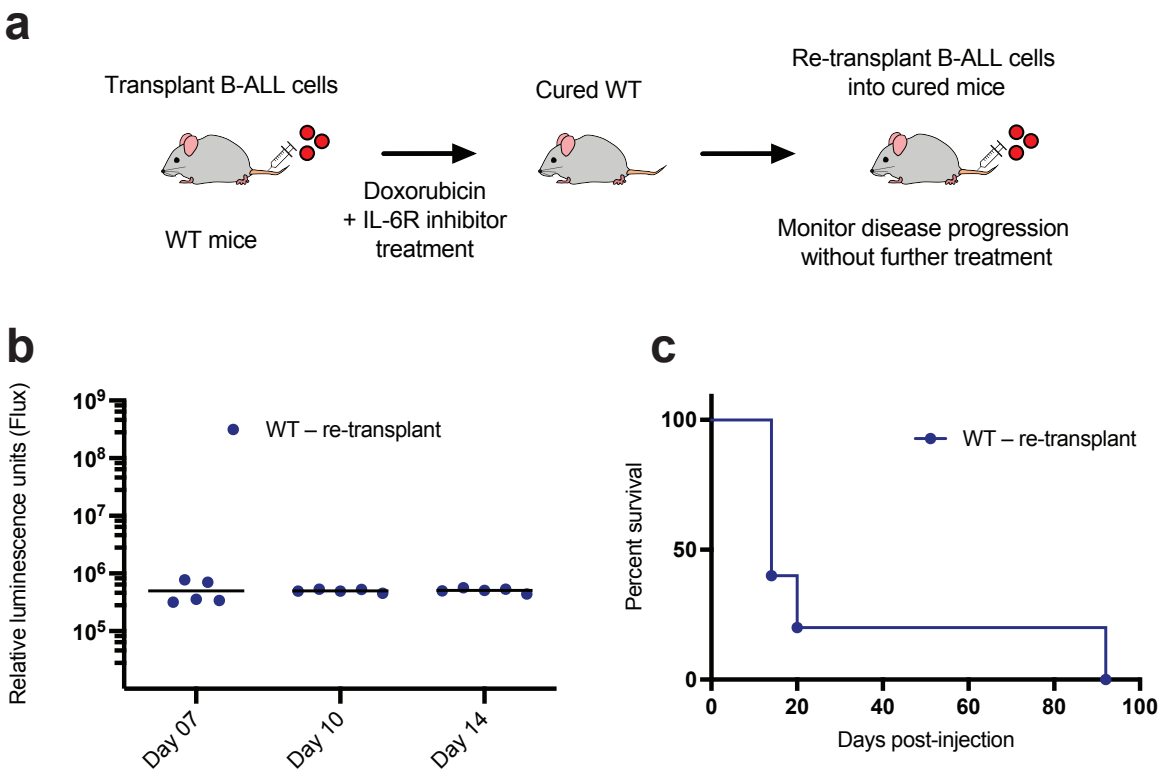


Figure Appendix A.1 | WT mice develop anti-tumor immunity after combination treatment with doxorubicin and IL-6R inhibitors. a, WT mice previously cured (living >80 days) by IL-6R inhibitor and doxorubicin treatment were re-transplanted with leukemia cells and disease progression monitored by bioluminescence imaging in the absence of further treatment. **b,** A graph showing leukemia burden *in vivo* monitored by bioluminescence imaging. n = 5. **c,** A Kaplan-Meier survival curve showing untreated leukemic WT mice, same animals as in (**b**). n = 5.

Cured WT mice show signs of destructive autoimmunity upon tumor re-transplant

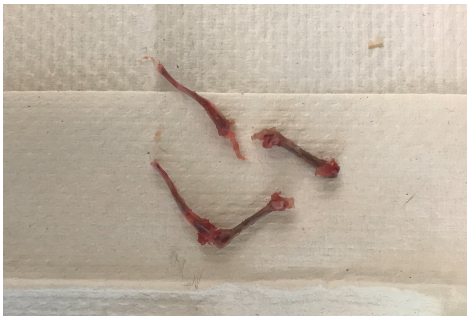
When mice became moribund, they were sacrificed and processed for tumor burden evaluation. As confirmed by flow cytometry, there was no detectable tumor burden in the bone marrow and spleen from these animals (data not shown). Additionally, we did not observe any discoloration of the bone marrow within the femurs and tibia of sacrificed animals, an indication of lack of disease (**Figure ApA.2a**). However, we did observe enlargement of the small intestine, resembling a solid growth or swelling (**Figure ApA.2b**). Upon further inspection, these animals presented with spleen sizes smaller than usual, a symptom of lymphopenia or lymphocytopenia (**Figure ApA.2c**). Lymphopenia is described as a state where there are reduced levels of lymphocytes in a living system, it is a common hematologic finding in sick animals, and is commonly associated with diverse autoimmune diseases (Schulze-Koops, 2004; Boes & Durham, 2017).

Crohn's disease (CrD) is found among the many autoimmune conditions that are associated with lymphopenia, as patients with CrD frequently present with low peripheral lymphocyte counts (Heimann et al., 1986). CrD is described as a chronic inflammatory disease that affects the

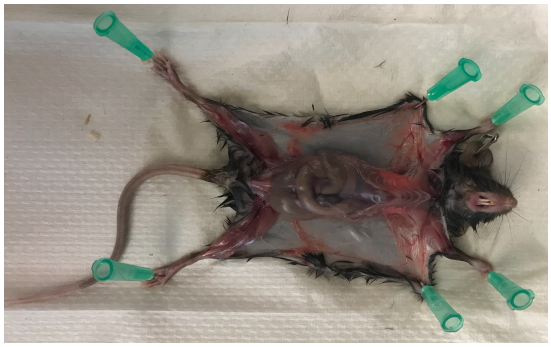
gastrointestinal tract, and is characterized by focal or segmental transmural inflammation (Taxonera et al., 2012). Animal models to study disease pathogenesis of both acute and chronic intestinal inflammation have already been developed (Pizarro et al., 2003; DeVoss & Diehl, 2014; Cominelli et al., 2017). However, many of these models are characterized by colitis – inflammation of the colon – rather than directly involving the small intestine, as seen here with our results. Whether our observed phenotype could be deployed to model CrD or inflammatory bowel disease in mice remains to be empirically tested, especially since we would need proper and consistent diagnosis of both lymphopenia and CrD in our system.

Figure Appendix A.2

a



b



c

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