Mechanisms of Microenvironmental Paracrine Signaling in Cancer Chemoresistance

by

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ABSTRACT

Chemoresistance remains a major barrier to the effective treatment of cancer. Cancer therapy occurs in the context of a tissue environment, with cancer cells surrounded by many non-malignant cells that contribute to tumor growth and resistance to therapy. Yet much remains unknown about the tumor microenvironment and its impact on therapeutic response. The specific cell types and microenvironment factors that influence chemoresistance, how therapy induced damage of the microenvironment changes these factors and the mechanisms by which the microenvironment contributes to therapy failure remain incompletely understood.

To study microenvironment signaling and its impact on therapy response, I have used mouse models of B-cell leukemia and lymphoma. I find that the chemotherapeutic doxorubicin promotes endothelial IL-6 release through reactive oxygen species-mediated p38 signaling and use tissue specific knockout mice to demonstrate that endothelial-specific production of IL-6 promotes lymphoma chemoresistance in vivo. Doxorubicin induces endothelial-cell senescence, an irreversible growth arrest typically accompanied by a robust secretory response. I show that PI3K/AKT/mTOR signaling is repressed in senescent endothelial cells and serves as switch, turning off chronic senescence-associated IL-6 production. These data implicate endothelial paracrine signaling in the promotion of chemoresistance and elucidates the molecular control of acute vs. chronic therapy-induced cytokine secretion.

Conventional chemotherapeutics such as doxorubicin can induce anti-cancer immune responses through the promotion of an immunogenic form of cell death. However, these responses are normally repressed by the microenvironment, impairing therapeutic response. I demonstrate that microenvironment IL-6 inhibits the generation of anti-leukemia immunity after immunogenic chemotherapy and find that CD8+ T-cell responses cure the majority of IL-6-/- mice treated with immunogenic chemotherapy. IL-6 also inhibits the efficacy of immune checkpoint blocking agents, implicating it as a promising target to improve the generation of anti-cancer immunity. Thus, the work in this thesis identifies the molecular control of therapy induced endothelial paracrine signaling and identifies a key role for IL-6 in suppressing anti-cancer immune responses induced by therapy.

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Table of Contents:

Chapter 1: Introduction........................................................................................................7

Chapter 2: A senescence secretory switch mediated by PI3K/AKT/mTOR activation controls chemoprotective endothelial secretory responses...........43

Chapter 3: Microenvironmental IL-6 Inhibits the Efficacy of Immunogenic Cancer Therapy by Suppressing Anti-Cancer Immunity........................................81

Chapter 4: Discussion.......................................................................................................129
Chapter 1

Introduction
Cancer is a complex disease that ultimately arises from uncontrolled cell proliferation. The invasion and spread of abnormally proliferating cells can lead to morbidity and mortality by altering organ function and disrupting normal tissue homeostasis. Cancer arises from normal cells, which are embedded in tissues and surrounded by a diverse complement of non-malignant cell types(1), through the acquisition of a set of properties that have been termed the “hallmarks of cancer”(1, 2). These attributes, which cells must acquire to become malignant, include self sufficiency from growth signals, insensitivity to anti-growth signals, the ability to evade apoptosis and undergo a limitless number of cell divisions, and altered interaction with the surrounding tissue environment: the ability to promote angiogenesis, avoid immune destruction and ultimately spread through metastasis(1, 2). Traditionally, most attention has been paid to the genetic and cell-intrinsic changes that facilitate the acquisition of these properties. However, as we learn more about the context in which tumors develop, it is increasingly clear that interactions between cancer cells and the surrounding environment impact the majority of hallmarks of malignant progression(3). Better understanding of these essential traits, how they arise, and how they are influenced by the surrounding microenvironment has great promise to inform the development of novel cancer therapies.

The treatment of cancer remains a major clinical challenge. Roughly one fifth of Americans will ultimately die from cancer, and incidence rates for cancer remain stubbornly high(4). Cancer treatment rests primarily on 3 pillars: surgery,
radiation and chemotherapy. Surgery is the most frequently curative intervention, but only has this potential in patients with localized disease. Radiation is also only curative in the treatment of loco-regional disease but, like surgery, is also used in the palliative treatment of more advanced cancer. Unfortunately, many malignancies are not detected until after initial dissemination, when distant metastases have formed(5, 6). Systemic chemotherapy has the potential to cure patients with metastatic disease, however, chemotherapy is often not fully effective, creating a need for better approaches to treat disseminated cancer.

In this thesis, I use mouse models of cancer to study the impact of the tumor microenvironment on the treatment of cancer. I detail how microenvironment paracrine signals can be altered by therapy-induced damage and elucidate mechanisms by which these signals promote resistance to therapy, focusing on the role of the cytokine interleukin-6 (IL-6). IL-6 is a pleiotropic cytokine with significant growth-promoting and immunomodulatory effects and IL-6 has been implicated in both cancer development and chemoresistance(7).

Chemotherapy in cancer

Chemotherapy is a mainstay in the treatment of cancer and, for the significant numbers of patients with non-local cancer, systemic therapies such as chemotherapy are the only potentially curative treatment option. The era of modern chemotherapy began in the 1940’s with the discovery by Louis
Goodman, Alfred Gilman and Gustov Lindskog that nitrogen mustards, which were originally developed for chemical warfare during the second world war, could be effective in the treatment of non-Hodgkin’s lymphoma(8). Goodman and Gilman were aware that soldiers dying from exposure to these agents exhibited profound lymphoid hypoplasia and myelosuppression and used mice to test their hypothesis that these agents might be useful in the treatment of lymphoid malignancies before validating their findings in patients with lymphoma(9, 10). Early work in this vein was also underway in Boston at the time, with Sidney Farber and colleagues demonstrating that folate analogues could induce remissions in children with acute lymphoblastic leukemia shortly thereafter(11).

Over the following decades, extensive research identified additional chemotherapeutic agents, expanded the list of malignancies they could be used in, and established the first modern combinatorial chemotherapeutic regimens, some of which are still in use today(12, 13). Physicians deploying these combinatorial regimens leverage drug synergies, the non-overlapping toxicities of distinct agents, and improved knowledge of pharmacokinetics and pharmacodynamics to optimally deliver multiple drug regimens with manageable toxicity(13). Work in this vein has led to significant improvements in the treatment of many cancers over the past decades, with cures achievable in many hematologic malignancies and some solid tumors. It is in this context that doxorubicin, a glycoside antibiotic and widely used anti-cancer agent that is a
focus of my work in this thesis, was isolated from the Streptomyces fungus in the 1960's(14). Doxorubicin, a member of the anthracycline class of chemotherapeutics, exerts its anti-cancer effect through the specific poisoning of topoisomerase II, an enzyme essential for the relief of DNA-supercoiling. Doxorubicin treatment traps topoisomerase II on DNA, inducing double strand breaks that impair cancer-cell growth and promote apoptosis(15).

Conventional chemotherapeutic agents were typically identified empirically, through their ability to inhibit the growth of cancer cells in vitro or in mice, and the majority of these drugs exert their action through the induction of genotoxic stress. They cause this stress through the inhibition of diverse proteins that affect DNA synthesis and replication, such as dihydrofolate reductase, the target of the antifolate methotrexate, or through direct binding to DNA, as is the case for drugs such as the nitrogen mustards(16). Unfortunately, many of these agents have significant side effects due to damage caused in non-malignant cells that limits their maximal dose. In many malignancies, these side effects, as well as cancer resistance, limit the ability of chemotherapy to completely eradicate disease. Resistance to cytotoxic chemotherapy can arise through diverse cell-intrinsic and extrinsic mechanisms. Increased drug inactivation or efflux, changes in the processing of drug-induced lesions, mutations in drug targets, and resistance to apoptosis can all lead to the failure of chemotherapy(16). In addition to cancer-intrinsic mechanisms of resistance, the surrounding cells in the tumor microenvironment can have profound resistance-promoting effects,
producing growth factors or cytokines that modulate the sensitivity to apoptosis and altering the diffusion of drugs into the tumor, among other things(17, 18).

These challenges with conventional chemotherapy have led scientists to seek more targeted therapies that inhibit cancer-driving proteins or other cancer-specific dependencies(19, 20). Drugs in this class, targeting the Bcr-Abl, B-Raf, EGFR, Alk, and Met oncogenes, among others, have displayed clinical success in a subset of cancers(21). However, despite immense promise, the majority of patients do not have targetable lesions and those that do often relapse due to resistance that arises from the mutation of drug targets or the activation of bypass signaling, enabling growth despite inhibition of the targeted protein(22).

There has also been a recent explosion of interest in immune-stimulating therapies, which seek to activate the endogenous immune response to eliminate cancer. Immunotherapy was named Science Magazine’s breakthrough of the year in 2013(23) and the American Society of Clinical Oncology announced immune-stimulating therapy as their advance of the year in February of this year(24). The origins of these therapies date back many years and it has been recognized since at least 1979 that treatments such as hematopoietic stem-cell transplantation could generate anti-cancer immune responses that improve survival(25, 26). Multiple drugs that block immune-inhibitory checkpoint proteins have recently been approved by the FDA for the treatment of melanoma, and hundreds of trials with these and other immune-stimulating therapies are
currently ongoing(27-29). This class of drugs shows potential to transform the
treatment of cancer, but similar challenges to those faced by conventional and
targeted chemotherapy remain - including incomplete initial efficacy and the
evolution of resistance.

Resistance to conventional, targeted and immunotherapy remains a major
challenge. In addition to cell-intrinsic changes that mediate resistance to
therapy, the non-malignant cells that make up a significant portion of tumor mass
in many cancer types, play a major role in the emergence of resistance(17, 28,
30).

**The tumor microenvironment**

It is increasingly clear that tumors are complex tissues consisting of multiple
cellular and non-cellular components(3, 31). The non-malignant components of
tumors, which make up the tumor microenvironment, consist of the extracellular
matrix, innate and adaptive immune cells and non-immune cells such as
endothelial cells, pericytes and fibroblasts. These non-malignant components
are gaining increased attention as essential facilitators and drivers of tumor
growth and therapy-resistance(32). However, components of the tumor
microenvironment can also restrain tumorigenesis(1, 33, 34). The immune
system plays an especially complex role in tumor formation - restraining the
formation of tumors by recognizing and removing malignant cells(35) but also, in
some contexts, promoting tumorigenesis and cancer progression through the
induction of chronic inflammation(36). Indeed, inflammation resulting from immune-cell activity was arguably the first component of the tumor microenvironment suggested to influence tumorigenesis.

In 1863, Rudolf Virchow, the famed German pathologist, observed leukocytes in malignant tissue and posited that inflammation was an important contributor to tumor formation by enhancing cell proliferation(37, 38). This, and Stephen Paget’s 1889 ‘seed and soil’ hypothesis that crosstalk between cancer cells and their environment influences the location of cancer metastasis, formed the early conceptual framework for much of our modern work on the role of the tumor microenvironment in cancer development, progression and therapy resistance(39). Paget’s ‘seed and soil’ hypothesis, based on his observation that cancer was especially likely to form metastases in specific organs such as the liver, relies on the idea that signals produced by non-malignant cells are essential for supporting the growth of disseminated cancer. Paget also suggested that heterogeneity in these signals exists, making some tissues and tissue niches, more hospitable for cancer growth(39). This quite profound hypothesis forms the basis for later work that has elucidated the complex, and at times contradictory, ways in which the microenvironment can drive cancer progression. Below, I hope to touch on a few aspects of this biology most relevant to the work in this thesis, namely paracrine signaling in the tumor microenvironment and the impact of the immune system on cancer formation, with an emphasis on how both of these contribute to treatment failure.
Work on the tumor microenvironment initially focused on two major aspects of tumor biology: cancer immunology and angiogenesis. Early immunologists, carrying forward Virchow’s observations, established that diverse immune-cell subsets can infiltrate the tumor microenvironment and began to elucidate their function(39). Independently, Judah Folkman and colleagues began to study the tumor vasculature. Folkman and others demonstrated that tumors were abnormally vascularized and dependent on their blood supply for growth (40). They established that cancer cells promote vasculogenesis by secreting blood-vessel-stimulating factors and that cancer cells rely on the resultant oxygen and nutrient delivery(40). More recently, it has become clear that the vasculature, and the endothelial cells that line blood vessels, play more complex roles in cancer biology than simply serving as conduits for oxygen and nutrient delivery(41). Work in this area has found that blood vessels are active secretory organs that regulate tissue maintenance, regeneration, and tumor formation through their production of cytokines and growth factors(41). In recent years, an enormous and complex literature has emerged about many additional cell types and secreted and non-secreted factors produced in the microenvironment that play important roles in tumorigenesis.

Historically, little attention was paid to the role of the microenvironment in therapeutic response. While the identification of potential microenvironment dependencies of cancer, such as angiogenesis, has led to the development of
microenvironment-targeted therapies, relatively little attention has been paid to how the microenvironment can influence the response to conventional and targeted therapy. Yet, the difference in therapeutic response between cancer cells grown \textit{in vitro} and those transplanted into mice or grown in co-culture with cells from the surrounding environment clearly demonstrate the therapeutic impact of the tumor microenvironment(32). Additionally, gene-expression profiling of human cancers has identified stromal gene-expression signatures that associate with outcome after treatment, clearly implicating the tumor microenvironment as an important determinant of the response to therapy(42, 43).

**Secretory signaling in cancer and beyond: IL-6**

The cellular components of the tumor microenvironment exist in, and help create, an extracellular milieu of secreted factors and extracellular matrix proteins that impact cancer growth and resistance to therapy. Among the most important communicating signals sent between cells of the microenvironment are secreted proteins. Growth factors and cytokines released by non-malignant and malignant cells can act in autocrine, paracrine, and even endocrine fashions to influence the biology of surrounding cancer and other cell types(2, 3). Inflammatory cytokines such as IL-6 can regulate both cancer-intrinsic biology and can also alter the function of surrounding immune and non-immune cells in the microenvironment.
IL-6

A focus of this thesis is the role that IL-6 plays in cancer growth and chemoresistance. IL-6 is a pleiotropic cytokine produced by a number of cells in the tumor microenvironment, including fibroblasts, cells of the innate and adaptive immune system, endothelial cells, and cancer cells themselves(44). IL-6 has important roles in the control of immune responses(45), tissue regeneration(46), and cancer(47).

IL-6 Signaling

IL-6 activates downstream signaling through a hexameric receptor complex consisting of two members each of IL-6, the IL-6 receptor (IL-6R) and a signal transducing co-receptor, gp130, which is ubiquitously expressed(48). The IL-6R is the IL-6-binding subunit of this complex and can signal in two ways, as an 80kD trans-membrane protein, or as a 50-55kD shed or secreted soluble IL-6R (sIL6R)(44). Thus, IL-6 can activate downstream signaling even in cells that do not express the IL-6R through the interaction of sIL6R with ubiquitously expressed gp130. While the IL-6 receptor mediates the specific interaction between IL-6 and gp130, gp130 also serves as a co-receptor for other members of the IL-6 family of cytokine receptors. These include leukemia inhibitory factor (LIF), oncostatin M, cardiac neurotrophic factor, IL-11, cardiotrophin-1, IL-27 and IL-35, each of which have distinct but overlapping biological activities from IL-6(49). ‘Classical’ IL-6 signaling, in which IL-6 interacts with membrane bound
IL6R, and ‘trans’ signaling, through the sIL6R, are thought to mediate distinct biological function in vivo(50).

The formation of the IL-6 receptor complex causes a conformational change in the cytoplasmic tail of gp130, ultimately activating the JAK tyrosine kinases (JAK1, JAK2 and TYK2) which interact with a membrane-proximal, proline-rich sequence in the gp130 cytoplasmic domain(49). These kinases phosphorylate multiple tyrosine residues in the cytoplasmic tail of gp130, promoting the recruitment of additional SH2-domain-containing proteins and initiating a complex set of downstream signaling cascades. Among the key proteins recruited to gp130 are members of the STAT protein family, including STAT1, -3 and -5(49, 51). JAK-mediated phosphorylation of tyrosine residues on the STAT proteins enables their dimerization and subsequent trafficking to the nucleus, where they promote the transcription of IL-6 responsive genes. Whereas STAT proteins, and STAT3 in particular, are major effectors of IL-6 signaling, a number of other pathways are also activated by IL-6 exposure. The mitogen activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways are activated through the interaction of multiple adaptor proteins with proteins recruited to gp130(49). Stress-activated JNK signaling has also been observed to result downstream of IL-6(49, 50).

IL-6 signaling is also under control of significant feedback inhibition. The protein tyrosine phosphatase SHP-2 is recruited to phosphorylated-tyrosine residues on
the cytoplasmic tail of gp130 and proteins from the suppressors of cytokine signaling (SOCS), protein inhibitors of activated STATs (PIAS), and cytokine-inducible SH2-containing (CIS) families play an important role in restraining IL-6 signaling (49, 50). SOCS family members are induced in response to JAK/STAT signaling and suppress continued pathway activity. SOCS3 plays a major role in the suppression of IL-6R signaling in vivo and mice that carry gp130 mutations that abrogate SOCS3 binding display chronic inflammation and arthritis-like pathology (52).

IL-6 is produced at sites of tissue damage and infection and is regulated both transcriptionally and post-transcriptionally by a number of transcription factors and RNA-binding proteins (44). Indeed, physiological IL-6 levels are quite low in the blood (1-5 pg/ml), but can be induced into the μg/ml range in response to infection, autoimmunity and cancer (53). IL-6 transcription is induced in response to the presence of pathogen and damage-associated molecular patterns, as well as other stress-inducing stimuli (44). The cis-regulatory region of the IL-6 gene contains binding sites for NF-kB, activator protein-1 (AP-1), and interferon regulatory factor-1 (IRF-1), among other transcription factors. IL-6 mRNA levels are further regulated by RNA-binding proteins that bind to AU-rich elements in the 3’ untranslated region of the mRNA to regulate its stability. Tristetraprolin (TTP), BRF1 and 2 and Arid5a, among others, interact with the IL-6 mRNA to promote or prevent its degradation under the control of stress-responsive signaling such as the MAPK, p38, and NF-kB pathways (44, 54).
Biologic activities of IL-6

IL-6 signaling has diverse and context-specific effects on tissue biology. The sIL6R is released from activated neutrophils, monocytes and T-cells(50) through either proteolytic cleavage from the cell surface or alternative splicing and is found both in the sera and in local tissue environments at sites of inflammation and tissue damage. Thus, theoretically IL-6 can act on and signal to nearly any cell in the body through the ubiquitously expressed gp130 co-receptor.

IL-6 was initially identified from the supernatant of stimulated peripheral blood mononuclear cells for its ability to induce immunoglobulin production from B-cells(55-57) and early work also demonstrated its role in the promotion of T-cell activation and the acute-phase response(45). It is now appreciated that IL-6 plays a role in diverse biological processes including vascular disease, lipid metabolism, neuropsychological behavior, wound healing, and cancer(50). A polymorphism near the IL-6 transcriptional start site (G>C, rs1800795), which leads to increased IL-6 production in humans, is associated with increased risk of coronary artery disease(58), idiopathic juvenile arthritis(59), and other inflammatory conditions(50), emphasizing the pro-inflammatory role of IL-6. Additionally, IL-6-blocking antibodies are used clinically for the treatment of inflammatory arthritis(50).
It is now clear that IL-6 classical and trans-signaling have distinct biologic outcomes. This, as well as the myriad downstream signaling pathways activated by IL-6, may explain some of its diverse biological effects. Classical signaling, which occurs downstream of membrane-embedded IL-6R, canonically activates the acute-phase response and controls homeostatic processes such as glucose metabolism and hematopoiesis\(^\text{(50)}\). Trans-signaling, through the sIL-6R, is active in inflammatory pathology and has been shown to regulate the apoptosis and recruitment of leukocytes, the effector function of T-cells, and inflammatory activity in stromal tissues\(^\text{(50)}\). IL-6 can regulate nearly every aspect of the innate immune system. It promotes initial neutrophil accumulation at sites of tissue damage and subsequent sIL6R shed from recruited neutrophils helps mature this acute inflammatory process. Shed sIL6R activates altered chemokine production and increased expression of cell adhesion molecules such as ICAM-1 and VCAM-1 to promote monocyte recruitment and the eventual progression to adaptive immunity while preventing neutrophil-mediated tissue damage\(^\text{(60)}\). The essential role of IL-6 in mounting an effective immune response is clear from children with auto-antibodies against IL-6 who show defective immunity and suffer from recurrent cellulitis and abscess formation\(^\text{(61)}\). Its role has been further demonstrated through the study of IL-6 deficient mice, which are more susceptible to multiple bacterial and viral infections\(^\text{(62, 63)}\).

IL-6 also regulates adaptive immunity. It is essential for the survival and maintenance of long-term plasma cells and IL6-/- mice have lower
immunoglobulin production(50). Mice with transgenic expression of IL-6 develop plasmacytomas(64) and the link between IL-6 and B-cell growth in humans has been demonstrated in Castleman’s disease, in which increased IL-6 production drives B cell hyperplasia that is treatable with IL-6 blocking therapy(65). In addition to its ability to regulate B-cell development and proliferation, IL-6 has T-cell stimulatory activity and can promote Th17 cell responses, especially when present in combination with TGF-β(66). IL-6 can also inhibit T-regulatory (T-reg) cell function(67) and development(68).

The ability of IL-6 to stimulate inflammatory T-cells may contribute to its role in promoting inflammatory pathology. Excessive IL-6 has been linked to the promotion of fibrosis in multiple systems(36, 50, 69). IL-6 promotes Th2 cell development and impairs Th1 commitment(70, 71), however, this may depend on surrounding tissue environment as IL-6 can also promote Th1 development in some contexts(69). While the predominant effects of IL-6 appear to be pro-inflammatory, IL-6 also has immunosuppressive functions. It can promote IL-10 production from T-cells(72, 73), impairing their activity, and inhibit dendritic cell maturation, preventing this important antigen-presenting cell from stimulating T-cell responses(74-76). Chronic IL-6 signaling, induced through SOCS3 loss, promotes gene expression resembling that induced by the immunosuppressive cytokine IL-10(77), suggesting that IL-6 can also restrict inflammatory processes when chronically present. Additionally, IL-6 can promote the release of IL1R-
antagonist, and TNRF antagonist p55(78) – promoting immune resolution after induction of an acute response.

In addition to these immune regulatory functions, IL-6 plays important roles in the promotion of tissue repair and barrier function integrity(79). Some of this occurs indirectly, through the ability of IL-6 to promote appropriate immune responses, but IL-6 also directly regulates cell division and the propensity of cells to undergo apoptosis through the activation of JAK/STAT signaling. JAK/STAT activity leads to increased cell-cycle and anti-apoptotic protein expression, spurring tissue regrowth and preventing excessive cell death(46). However, chronically elevated IL-6 is found at sites of tissue damage and is thought to contribute to tumorigenesis through its regulation of these processes(36).

**IL-6 in cancer**

IL-6 has been shown to promote cancer development, progression and resistance to therapy(7, 47). It is overexpressed in a number of malignancies and high IL-6 levels negatively correlate with prognosis in lung, liver, colon, breast, pancreatic, ovarian, kidney and in some hematopoietic malignancies, among others(47, 80). IL-6 has also been shown to promote the development of colon(81), lung(82, 83), liver(84) and pancreatic cancer(85) in mice. Sustained IL-6 production leads to chronic inflammation, in which damage generated by toxic intermediates such as reactive oxygen species contributes to cancer formation. IL-6 also directly promotes the survival of damaged cells in these
tissues, facilitating tumorigenesis. While the immunomodulatory effects of IL-6 in adaptive immunity may also contribute to its role in cancer progression, with IL-6-mediated Th17 development contributing to chronic inflammation and tumorigenesis(47), the role of IL-6 in the regulation of adaptive immunity has been less directly studied in the context of cancer biology(47).

IL-6 and downstream STAT3 and MAPK signaling up-regulate the expression of important cell-cycle regulators including cyclin D1, D2 and myc, which drive cell proliferation(47, 84) and IL-6 can promote the expression of the anti-apoptotic proteins Bcl-XL, Mcl-1, X-linked inhibitor of apoptosis and Bcl-2, among others. Thus, IL-6 can also regulate apoptosis and cell proliferation, hallmarks of cancer development, in developing tumors.

IL-6 also regulates the tumor microenvironment and can promote tumor dissemination. IL-6 induces matrix metalloproteinase expression, leading to extracellular matrix degradation and facilitating cell migration(47), and IL-6 can also spur angiogenesis. IL-6, through STAT3, also activates Hif-mediated VEGF transcription and elevated blood vessel growth(47). In combination with other inflammatory cytokines, IL-6 can promote the adoption of a stem-cell-like state(7) and it plays a role in driving the epithelial to mesenchymal transition, a developmental process that can be co-opted by cancer cells and that aids in metastasis formation(86).
For cancers that do not make their own IL-6, such as many hematopoietic malignancies, IL-6 produced by the microenvironment can affect tumorigenesis and promote chemoresistance (87, 88). However, whether IL-6-containing tissue niches that are more tumorigenic exist and which cell types produce local IL-6 is poorly understood. Additionally, the molecular control of IL-6 production in the tumor microenvironment, and how its production changes in response to cancer therapy, is incompletely understood.

Endothelial cells in the tumor microenvironment

Endothelial cells have been widely studied in cancer biology for decades and angiogenesis is a hallmark of tumor formation (1). However, in addition to serving as conduits for nutrient and oxygen delivery, endothelial cells have only more recently been recognized to produce cytokines and growth factors, termed ‘angiocrine’ factors, that play important roles in normal and tumor cell regeneration after damage (41). It has been known for many years that endothelial cells help instruct tissue patterning during embryogenesis (89, 90), guiding epithelial cell growth and development independently of their vascular function (91). More recently, endothelial paracrine signals have also been shown to create a bone-marrow niche that harbors long-term hematopoietic stem cells (92) and promote tissue regeneration after damage in the bone marrow (93), lung (94) and liver (95). In these contexts, damage sensed by the endothelium leads to the production of regenerative factors that stimulate the re-growth of neighboring cells. Emerging evidence indicates that these regenerative factors
can be co-opted by cancer cells, creating a perivascular niche that promotes cancer cell growth(96) and stemness(97, 98).

Angiocrine factors are thus potential targets for cancer therapy and targeting endothelial secretory responses may present a complementary approach to anti-angiogenic therapies, inhibiting tumor growth by a distinct mechanism(41). Recent work suggests that regenerative and pro-survival angiocrine signals sent by damaged microenvironments can also be co-opted by tumor cells to promote resistance to chemotherapy(41, 88, 97, 99, 100), however, which angiocrine factors are important and the extent to which endothelial production is essential remains unclear in almost all cases. Some of the work in Chapter 2 of thesis is driven by the hypothesis that understanding and targeting endothelial cell paracrine signals can improve the efficacy of cytotoxic chemotherapy.

Many other cell types in the tumor microenvironment, including fibroblasts and immune cells, can produce paracrine signals that impact normal tissue function and regeneration(101), as well as cancer growth and response to therapy(32, 102). These responses are controlled by the surrounding tissue environment and can be altered in response to damage or damage-mimicking signals.

**Tissue damage responses**

Chemotherapy, which is given systemically, acts not only on tumor cells but also damages non-malignant cells throughout the body. Chemotherapy-induced
damage of the tumor microenvironment can impact treatment response through the induction of pro-survival cytokines and growth factors from non-malignant cells. All mammals have evolved approaches to repair and restrain tissue injury, minimizing long-term impairment from acute damage. A network of chemical signals, released by injured cells, helps orchestrate a host response designed to heal the damaged tissue(38). This response involves the recruitment of innate and adaptive immune cells to damaged tissues as well as the production of growth factors and cytokines that can directly promote resistance to death in damaged cells and stimulate cell proliferation, orchestrating repair(103). Under normal circumstances, these responses are self-limiting due to the production of anti-inflammatory molecules that follow a pro-inflammatory stimulus(37). However, under conditions of chronic inflammation, reactive oxygen species released from responding immune cells can cause DNA-damage and growth factors and cytokines produced in the unhealed wound stimulate resistance to apoptosis, preventing the death of damaged cells and facilitating their transformation(7).

In many systems, chronic inflammation is associated with increased tumor development, likely through cell stresses induced by constant damage and repair signals. Chemotherapy-induced damage can activate these responses, and emerging data suggest that cancer cells co-opt these evolutionarily programmed reparative processes to survive the stress of therapy(104, 105). In addition to the activation of acute wound responses, chemotherapy can promote longer-term
changes in the tumor microenvironment that impact the efficacy of subsequent treatment cycles weeks later(106, 107). Relevant to these more chronic changes induced by therapy is the process of cellular senescence.

Cytotoxic chemotherapeutics can induce an irreversible cell-cycle arrest termed cellular senescence in malignant and non-malignant cells(108). Canonically, senescent cells produce pro-inflammatory cytokines and growth factors that remodel the surrounding environment(109-113). This inflammatory response has been termed the senescence-associated secretory phenotype (SASP) and in normal conditions is thought to promote tissue homeostasis by activating tissue repair and recruiting immune cells to clear damaged cells. However, the SASP can also lead to age-related pathology, including tumor formation, by creating an inflammatory tissue environment, and has been implicated in the promotion of cancer chemoresistance(114).

Cellular senescence was originally described in 1961 by Leonard Hayflick and colleagues, who observed that human fibroblasts fail to proliferate indefinitely in culture, arresting but staying metabolically active(115). In the years since this observation, cellular senescence has been shown to result from a number of cellular stressors including telomere shortening (the cause of the replicative senescence Hayflick described) as well as exogenous DNA damage. More recently, oncogene-activation has been found to induce senescence(116), at least in part also through the induction of DNA-damage responses(117). It is
thought that the cell-cycle arrest that results from oncogene-induced-senescence plays a major tumor-suppressive role, preventing growth and promoting clearance (in part through the SASP) of oncogene-activated pre-malignant cells. Senescence is thus an important tumor-suppressive mechanism but, paradoxically, can also promote cancer formation through the up-regulation of the SASP and resultant creation of a pro-inflammatory tissue milieu(118).

DNA-damaging chemotherapy can induce senescence in the tumor microenvironment and senescence-associated inflammation has been implicated in the promotion of chemoresistance *in vivo* (102). Interestingly, it has been appreciated for many years that regrowth of cancer cells between cycles of therapy accelerates over the course of treatment, contributing to treatment failure(106, 107). Senescence and the resultant SASP induced in the tumor microenvironment after chemotherapy may contribute to this accelerated regrowth. However, the extent to which therapy induces the SASP *in vivo* remains poorly studied. Some of the work in Chapter 2 of this thesis suggests that senescence-associated inflammation can contribute to chemoresistance, but is under tight molecular control *in vivo* to preserve tissue homeostasis.

**Cancer immunology**

At least 150 years ago, cancers were observed to have extensive immune infiltrates by the German pathologist Rudolf Virchow. Paul Ehrlich, the physician and Nobel Laureate who originated the idea that one could use specific
chemicals to treat human disease, proposed as early as 1909 that immune surveillance might limit tumor formation(119). It is now clear that the presence of immune cells in the tumor or surrounding tissue is a near-universal attribute of cancer and that immunity can restrain cancer progression(35, 120, 121). While immune cell infiltrates play complex roles in tumor formation, it is increasingly recognized that the immune system can limit cancer development through the recognition of mutational neo-antigens acquired during cellular transformation(33, 35, 121). The infiltration of CD8+ cytotoxic T-lymphocytes is associated with positive outcome in a number of cancer types(120) and, in mice, the absence of T or NK cells speeds the development of carcinogen-induced tumors(2). Individuals with immunosuppression or immunodeficiencies are much more likely to get certain types of cancer and numerous studies have associated immune-activating cell infiltrates with positive prognosis in human cancer(35).

The evasion of anti-tumor immune responses is thus an emerging hallmark of cancer progression(2). Indeed, it has been proposed that developing tumors advance through a classic progression from initial elimination by the immune system to equilibrium and, eventually, escape, as they evade immune surveillance, enabling the formation of tumors(33). This immune escape can occur through many mechanisms that are the source of considerable current investigation(35). Cancer cells can alter their ability to be recognized by immune cells through the mutation of T-cell neo-antigens or suppression of antigen-presentation machinery (one example of which is mutations in the TAP gene,
which helps facilitate peptide loading onto MHC class I(122)). Cancer cells also often directly express T-cell-inhibitory checkpoint proteins, such as PD-L1, on their surface. Much attention has recently been paid to the role these immune checkpoint proteins, which induce T-cell anergy, play in suppressing anti-tumor immunity, and drugs that target them show promising efficacy in many cancer types(123). Additional mechanisms by which cancer evades anti-tumor immunity include the secretion of immune-inhibitory cytokines, such as IL-10, and the up-regulation of 'don't eat me' proteins, like CD47, that inhibit phagocytosis of cancer cells, among others(121, 124).

In addition to these cancer-cell-intrinsic mechanisms of immune evasion, the immune context of the surrounding environment has a major impact on anti-cancer immunity(28, 121). Indeed, the presence of lymphoid aggregates in the tumor microenvironment is associated with responsiveness to immune stimulating therapies and the infiltration of tumors by specific immune cell subsets impacts prognosis(35, 120). The composition and activity of infiltrating immune cells can be controlled by both the tumor cells and cells in the microenvironment. IL10, prostaglandin E2, and TGF-β can induce multiple direct and indirect immune-suppressive effects on T-cells. Additionally, the production of chemokines by cancer cells can control the recruitment of T-regulatory cells and myeloid-derived suppressor cells, among others, suppressing anti-tumor immunity(124). Chronic inflammation is thought to contribute to tumorigenesis by creating an 'exhausted', immunosuppressive microenvironment in addition to the
damage it induces in non-malignant cells(38). Cancer-associated fibroblasts can also serve to restrain anti-cancer immune responses by production of CCL2 and CXCL12, which recruit immunosuppressive immune subsets, and have been shown to secrete TGF-β, directly impairing T-cell function(124). Thus, the immune cells found in the tumor are engaged in a complex crosstalk with neighboring malignant and stromal cells that has a major impact on cancer progression. The signals used and ultimate outcome of this crosstalk are only beginning to be elucidated. Further investigation of this aspect of cancer biology will be critical to identify which immune-suppressive mechanisms are essential across cancer types, informing our efforts to target these responses.

There is currently immense excitement about the prospect that we will be able to therapeutically exploit anti-tumor immunity to treat cancer. This is a rapidly progressing field, with drugs that inhibit microenvironment suppression of endogenous immunity as well as therapies in which new anti-tumor immune responses are generated, such as CAR-T cells, anti-tumor vaccines, and adoptive immunotherapy, under investigation. These agents show immense potential to transform the treatment of many types of cancer(29), but it is likely that combinations of multiple immune-stimulating therapies will be essential for the promotion of lasting anti-tumor immunity(28).

Many of the immune-suppressive mechanisms described above show potential for therapeutic manipulation. Cancer cells often maintain neo-antigens that make
immune recognition and clearance possible, but T-cell responses are inhibited by immune-suppressive changes in the microenvironment. The recent clinical success of immune-checkpoint blockade, in which agents that inhibit proteins known to promote T-cell anergy are used, has spurred increased interest in the identification of additional mechanisms of immune suppression that can be targeted to improve therapeutic efficacy\(28, 123\). In Chapter 3 of this thesis, I discuss our efforts to better understand the mediators of immune suppression by the microenvironment with the aim of improving the efficacy of immune-stimulating therapy.

Conclusions
The tumor microenvironment plays an important role in cancer development and tumor progression. A significant proportion of the cells in a tumor are not themselves malignant, but support, facilitate, and in some cases, restrain tumor development. In this thesis, I present work on the mechanisms by which paracrine signals produced by non-malignant cells in the tumor microenvironment impact therapeutic response and discuss how these cells respond to therapy-induced damage.

References


Chapter 2

A senescence secretory switch mediated by PI3K/AKT/mTOR activation controls chemoprotective endothelial secretory responses

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Abstract
Cancer therapy targets cancer cells that are surrounded by a diverse complement of non-malignant stromal cells. How therapy-induced damage of normal cells changes the tumor microenvironment and how these changes influence therapeutic response remains unclear. Additionally, the specific cell types and microenvironment factors that influence chemotherapeutic efficacy are poorly understood. Here, we detail an acute therapy-induced secretory response that promotes chemoresistance. We show that the chemotherapeutic doxorubicin causes endothelial IL-6 release through reactive oxygen species-induced p38 signaling, and we demonstrate that IL-6 produced specifically in endothelial cells (ECs) promotes resistance to doxorubicin in vivo. Doxorubicin induces senescence, an irreversible cell cycle arrest typically accompanied by a robust secretory response, in ECs but interestingly without the typical senescence-associated inflammatory response. We find that down-regulation of PI3K/AKT/mTOR signaling represses cytokine secretion from senescent ECs and that its reactivation restores this response. Finally, we show that endothelial IL-6 release can be inhibited with histone deacetylase inhibitors, which synergize with doxorubicin in vivo. Thus, we describe a novel secretory response that is induced by chemotherapy, identify a way to inhibit this response therapeutically, and describe a mechanism by which damage-associated secretory responses can be restrained to preserve tissue homeostasis.

Contributions
The author performed the experiments described in this chapter except as follows. Luke Gilbert performed the in vivo senescence staining, in vivo IL-6 release experiment, the HUVEC and HCC cytokine arrays, and the p16 and p21 westerns. He also initiated some of the in vitro IL-6 experiments although all data shown was generated by the author other than as stated above.
Introduction

Cancer arises in the context of diverse non-malignant and malignant cell types that make up the tumor microenvironment. Despite recent advances, the treatment of patients with cancer remains a major challenge with the majority of patients who have non-local disease ultimately relapsing on current therapies (1). It is increasingly clear that non-malignant stromal cells not only play a role in cancer progression, but also contribute to therapeutic resistance (2-4). Cytokines and growth factors produced by cells in the tumor microenvironment can promote chemoresistance (5, 6) and gene-expression signatures predictive of survival indicate a strong stromal influence on therapeutic response in human cancer (7, 8), suggesting that the microenvironment represents a promising therapeutic target.

Systemic chemotherapy induces damage in both cancer and normal cells. While mammals have not evolved to survive damage from chemotherapy, all metazoans have evolved responses to tissue injury and infection. During inflammation associated with sterile injury, various cell types produce soluble factors that promote tissue repair (9). Chemotherapy-induced activation of these stress responses may promote pro-survival niches that can be co-opted by tumor cells, leading to relapse (2, 5, 6, 10, 11). However, it remains poorly understood how non-malignant cells in the tumor microenvironment respond to therapy-induced damage, whether these responses impact therapeutic response, and what cell types are involved in the promotion of chemoresistance.
Chemotherapy-induced DNA damage can lead to cellular senescence, an irreversible cell-cycle arrest, in both malignant and non-malignant cells(3, 12). Senescent cells up-regulate a number of pro-inflammatory cytokines and growth factors that remodel the surrounding environment, a process termed the senescence-associated secretory phenotype (SASP)(13, 14). The SASP is thought to promote tissue homeostasis by activating tissue repair programs and recruiting immune cells to clear damaged cells(15). However, the SASP can also promote age-related pathology and has been implicated in tumor formation and the promotion of chemoresistance as well(6, 16). Thus, there is active interest in understanding the mechanisms that control SASP factor production. While the biology of the SASP is beginning to be elucidated, it remains largely unclear what homeostatic mechanisms exist to restrain this potentially deleterious secretory response. Whether a SASP is consistently found in senescent cells in vivo is also unclear. Patients treated with DNA-damaging chemotherapy do not typically exhibit the systemic chronic inflammatory response that might be expected to result from the widespread induction of a SASP, suggesting that homeostatic mechanisms exist to restrain senescence-associated inflammation. Indeed, recent work indicates that senescence secretory responses are not universal in vivo. Mice accumulate senescent cells in many tissues as they age, but show increases in SASP component expression in only some of these tissues(17). Additionally, the deletion of senescent cells from aged mice does not alter SASP gene expression in all organs(17). This suggests that, in addition to the regulation of SASP-associated inflammation by immune-mediated clearance of
senescence cells, the SASP itself is repressed in some contexts(17). However, our understanding of how the SASP can be restrained, and how it relates to other physiological secretory responses(5), remains limited.

Here, we describe the molecular control of an acute therapy-induced secretory response that promotes chemoresistance. We provide direct genetic evidence that treatment-induced production of interleukin-6 (IL-6) specifically in endothelial cells promotes resistance to the chemotherapeutic doxorubicin. We further find that doxorubicin induces cellular senescence in endothelial cells, but surprisingly, that this is not associated with a canonical SASP. Instead, damaged endothelial cells activate an acute secretory response that is mechanistically distinct from the SASP and that depends on reactive oxygen species (ROS)-induced p38 signaling. Interestingly, this acute secretory response occurs in the context of down-regulation of the PI3K/AKT/mTOR pathway and we demonstrate that a SASP can be restored in senescent endothelial cells by activating this pathway, implicating PI3K/AKT/mTOR pathway activity as a molecular rheostat that determines senescent secretory responses. Finally, we find that endothelial IL-6 production can be inhibited with histone deacetylase (HDAC) inhibitors and that HDAC inhibition synergizes with doxorubicin to treat resistant lymphoma. We thus describe a novel senescence secretory response that promotes resistance to therapy but that is ultimately restrained. We provide insight into the control of this response, identifying a mechanism by which damage-induced secretory responses can be controlled to preserve tissue homeostasis.
Results

Endothelial IL-6 production promotes chemoresistance

Endothelial cell paracrine signaling has been implicated in the creation of tissue niches that promote stem cell homeostasis(18), tissue regeneration(19), and cancer cell growth and aggressiveness(20-23), however which secreted factors promote cancer chemoresistance in vivo is not well understood. We bred endothelial-specific IL-6 knockout mice which express tamoxifen-inducible Cre under the control of the endothelial-specific VE-Cadherin (CDH5) promoter(24) and also carry floxed alleles of IL-6(25), allowing its excision by Cre (Cdh5(PAC)-CreERT2; IL6f/f) (Fig 1a). Cre was induced by 5-day administration of tamoxifen, giving Cre activity in the vast majority of vessels as assessed by fluorescence imaging of mice with the mTmG(26) transgene, in which Cre-mediated recombination leads to the expression of GFP (Fig 1b). These mice were transplanted with Eu-myc p19^{myc} lymphoma cells, a well-established mouse model of Myc-driven B-cell lymphoma(27, 28), to monitor the effect of endothelial IL-6 production on the response to doxorubicin treatment (Fig 1a). Examination of tumor burden in the thymus, a key site of chemoresistance in this model(5), revealed decreased tumor burden in CDH5-CreERT2; IL6f/f mice (Fig 1c). This provides direct genetic evidence that endothelial cells promote a chemoresistant niche for B-cell lymphoma through the production of paracrine IL-6. Interestingly, dense vascularization and high expression of endothelial-specific gene signatures are predictive of poor survival in human B-cell lymphoma(7).
Additionally, high levels of IL-6 predict poor prognosis in human B-cell lymphoma(29). Our data suggest that endothelial cells specifically play a causative role in the promotion of chemoresistance through the secretion of paracrine IL-6.

Figure 1: Endothelial production of IL-6 promotes chemoresistance. (a) Schematic of experiment assessing the importance of endothelial IL-6 production in B-cell lymphoma chemoresistance. Mice were treated upon palpable tumor formation 12 days after transplantation. (b) Immunofluorescence showing endothelial-specific Cre activity. Cdh5-CreERT2; mTmG; IL6f/f mice were treated with a 5 day course of tamoxifen and stained for GFP and CD31 to identify endothelial specificity and activity of Cdh5-Cre. (c) B-cell lymphoma burden in the thymus, an important site of chemoresistance in this model, in endothelial IL-6 knockout mice (IL6ECKO) and littermate controls was assessed by measuring tissue weight. n=16 for control and n=13 for IL6ECKO mice. Data are shown as individual mice with mean +/- SEM. *= P<0.05

Doxorubicin induces an acute endothelial cell secretory response

The production of paracrine chemoresistance factors, such as IL-6 can be altered by chemotherapy-induced damage. How paracrine signals change over the course of treatment and the molecular control of paracrine damage responses
are not well understood. Doxorubicin treatment induces beta-galactosidase staining, a marker of cellular senescence, at sites of chemoresistance in this model (Supplementary Figure 1a) and IL-6 is a canonical SASP cytokine, leading us to ask what happens to IL-6 levels *in vivo* in the days following treatment. To do so, conditioned medium was made before treatment and at 18 hours, 4 days and 8 days following treatment. IL-6 levels are acutely induced by treatment but, surprisingly, return to baseline levels in the days following chemotherapy (Fig 2a), not typical of senescent cells undergoing a SASP(30). Similarly, human umbilical vein endothelial cells (ECs) treated with doxorubicin *in vitro* acutely induce IL-6 production before reducing IL-6 secretion to baseline levels by 5 days after treatment (Fig 2b). This discrepancy with the SASP led us to investigate whether ECs undergo senescence after treatment *in vitro*. Doxorubicin-treated ECs arrest and do not incorporate BrdU over a 24hr incubation period (Fig. 2c), stain positively for senescence-associated beta-galactosidase (SA-β-gal) (Fig 2d,e) and up-regulate senescence markers p16 and p21 (Fig 2f), suggesting that doxorubicin induces the hallmarks of cellular senescence in these cells, although without typical SASP IL-6 production. To confirm that doxorubicin treatment can induce a SASP in our hands, we treated hepatocellular carcinoma (HCC) cells with doxorubicin and observe canonical SASP IL-6 production as these cells senesce (Fig 2G and Supplementary Figure 1b). Thus, doxorubicin induces what we term an acute stress-associated phenotype (ASAP) in endothelial cells cultured *in vitro* as well as at sites of chemoresistance *in vivo*. The ASAP can
promote resistance to chemotherapy treatment, yet is not a canonical senescence secretory response.

Figure 2: Endothelial cells produce acute but not sustained IL-6 during doxorubicin-induced senescence. (a) Quantification of IL-6 levels in conditioned media from the thymus of untreated mice (n=10) or mice at various times after treatment with 10mg/kg doxorubicin (n≥3). Values were normalized by tissue weight. (b) Quantification of IL-6 in media from human umbilical vein
endothelial cells (ECs). ECs were treated with 225nM doxorubicin and IL-6 secretion at 1 and 5 days after treatment measured by ELISA. Measurements are of IL-6 secreted over a 24hr period preceding the collection time. n=9 (c) BrdU incorporation into doxorubicin treated or untreated ECs was measured by flow cytometry. Cells were pulsed with BrdU for 24hrs before measurement. n=3 (d) Light microscopy of ECs treated with doxorubicin to induce senescence and fixed and stained for senescence-associated-beta-galactosidase 5 days after treatment. (e) Quantification of cells treated as in (d). Data quantified from one of multiple representative experiments. (f) Western blots for senescence markers p16 and p21 in EC lysates created at various times after treatment with doxorubicin. (g) IL-6 levels in conditioned media from FOCUS cells, a hepatocellular carcinoma cell line. Cells were treated with 25nM doxorubicin and IL-6 secretion measured as in (b), n=4. For all panels, unless otherwise stated, data are shown as mean +/- SEM. ***p<0.0001

To further investigate the EC secretory response, the levels of 64 cytokines and growth factors secreted by senescent EC and HCC cells were measured at 24 hours and 5 days after treatment. Endothelial cells undergo a significantly dampened senescence secretory response with a number of cytokines and growth factors not changing in levels or being restrained during senescence (Fig 3a) compared with the more typical SASP observed in HCC cells (Fig 3b). The canonical SASP is controlled by the activity of the NF-kB transcription factor as well as DNA-damage kinases such as ataxia telangiectasia mutated (ATM)(31-33). Infection of endothelial and HCC cells with a dominant negative, non-phosphorylatable IkBα, which blocks NF-kB transcriptional activity, showed that EC IL-6 production was NF-kB independent (Fig 3c) while HCC SASP production requires NF-kB activity (Fig 3d), as expected. Thus, in addition to being temporally distinct from the SASP, the ASAP is controlled in a mechanistically distinct fashion. Additionally, the ASAP is not dependent on DNA-damage
response kinase activity, or mTOR signaling (Supplementary Fig. 2 a-c), further distinguishing it from the SASP.

Figure 3: The ASAP and SASP are temporally and mechanistically distinct. (a) A heat map displaying the relative concentration of 65 growth factors, cytokines and chemokines secreted by HUVECs untreated or treated with doxorubicin. The data are displayed as a log2 of the fold change comparing doxorubicin treated cells over untreated cells (n=3). (b) A heat map displaying the relative concentrations of 65 growth factors, cytokines and chemokines secreted by HCC cells untreated or treated with doxorubicin. The data are displayed as a log2 of the fold change comparing doxorubicin-treated cells over untreated cells (n=3). For (a) and (b) media were collected over a 24hr period preceding the timepoint listed and normalized to cell number. (c) IL-6 levels released by ECs infected with dominant-negative IkBa or vector control and either untreated or treated with 225nM doxorubicin. n≥6 (d) IL-6 levels in the conditioned media from HCC cells infected with a dominant-negative allele of IkBa or vector control and either untreated or treated with 25nM doxorubicin. n=4. ***p<0.001,  ns = p>0.1
ASAP IL-6 production is mediated by reactive oxygen species-induced p38-signaling

Since EC production of IL-6 promotes chemoresistance, we sought to understand how it is controlled mechanistically. The acute release of IL-6 could theoretically result from the dumping of pre-formed cytokine stores. To investigate whether transcription and translation are required for EC IL-6 production, ECs were treated with doxorubicin in the presence of the RNA polymerase II inhibitor actinomycin D (Fig 4a) or the translation elongation inhibitor cycloheximide (Fig 4b), both of which inhibit endothelial IL-6 production. Additionally, intracellular IL-6 levels in endothelial cells are extremely low (data not shown), suggesting that EC IL-6 secretion requires continuous protein production and is an active stress response enacted by damaged ECs.

Doxorubicin promotes DNA-double strand breaks through the inhibition of type II topoisomerases and is also known to induce ROS either through the induction of mitochondrial dysfunction or through direct redox cycling(34). ROS have previously been implicated in the control of acute inflammatory responses in multiple tissue types(35). To assess ROS levels after doxorubicin treatment, endothelial cells were stained with the ROS-sensitive dye H2-DCFDA and fluorescence was measured by flow cytometry. This demonstrated significant increases in ROS after treatment (Fig 4c). Treatment of ECs with doxorubicin in combination with the anti-oxidants N-acetyl cysteine (NAC) or glutathione (GSH)
inhibits IL-6 release (Fig 4d), suggesting that ROS production is essential for doxorubicin-induced IL-6 release. To further verify that ROS induction is sufficient to activate IL-6 secretion, we treated ECs with the oxidizing agents menadione or cumene hydroperoxide, which alone promotes IL-6 release at similar levels to doxorubicin (Fig 4e).

The stress-responsive mitogen-activated protein kinase p38 is a major downstream effector of ROS and also regulates acute inflammation through the control of cytokine translation and RNA stability (36, 37). How doxorubicin activates p38 signaling remains incompletely understood. Doxorubicin-induced EC IL-6 release is dependent upon p38 activity as shown by EC treatment with doxorubicin and a specific-p38 inhibitor (Fig 4f). p38 inhibition also blocks menadione and cumene hydroperoxide-induced IL-6 release (Fig 4g), suggesting that ROS-induced p38 activation is required for EC IL-6 production. Additionally, doxorubicin acutely induces p38 phosphorylation in ECs and the ROS inhibitor NAC blunts this doxorubicin-mediated activation of p38 (Fig 4h), implicating ROS production in the doxorubicin-induced activation of p38 signaling. p38 regulates cytokine production through the downstream kinase MAPKAP2 (MK2), which phosphorylates RNA-binding proteins, such as TTP, that control cytokine transcript stability and translation (38). We find that ROS inhibition does not reduce IL-6 transcript levels (Fig 4i), suggesting that ROS-induced p38 activation primarily regulates IL-6 translation or subsequent secretion. This defines a novel chemotherapy-induced secretory response in the tumor microenvironment.
mediated by ROS production and subsequent activation of p38-dependent IL-6 production in damaged cells.

Figure 4: The ASAP is dependent on ROS and p38. (a) Quantification of IL-6 release from endothelial cells (ECs) treated with doxorubicin in the presence of RNA polymerase II inhibitor actinomycin D. n=8. (b) IL-6 levels in media from doxorubicin and/or cycloheximide treated ECs. n=7. (c) Flow cytometry plot of ECs treated stained with the ROS-sensitive dye DCF-DA after treatment with 225nM doxorubicin. (d) EC IL-6 production. ECs were pre-treated for 2 hours with N-acetylcysteine (NAC) or glutathione (GSH), before the addition of doxorubicin. n\geq 7. (e) IL-6 release from ECs treated with doxorubicin or the ROS-inducers menadione or cumene hydroperoxide. n\geq 5. (f) IL-6 secretion by ECs treated with doxorubicin in the presence or absence of the p38 inhibitor SB203580. n=7. (g) IL-6 release by ECs treated with ROS inducers with or without the p38 inhibitor SB203580. Measured by ELISA. n=3. (h) Western blots of ECs treated with combinations of N-acetylcysteine (NAC) and doxorubicin. Whole cell lysates were collected 12 and 24 hrs after treatment. Intensity of phospho-p38 was quantified in ImageJ. Data representative of 2 independent experiments. (i) IL-6 transcript levels from ECs treated with NAC and/or
doxorubicin. RNA was extracted at 12 and 24 hrs after treatment. Transcript levels were measured by qPCR and normalized to levels of GAPDH. For all IL-6 secretion measurements IL-6 production over a 24hr period was determined by ELISA unless otherwise stated. Data are shown as mean +/- SEM. ***p<0.001 **p<0.01

**Lack of senescence-associated inflammation is determined by PI3K/AKT/mTOR pathway activity**

Senescent cells, and more recently the SASP, have been linked to the pathology of aging and the promotion of cancer cell growth as well as chemoresistance(6, 30, 39, 40). The absence of this response *in vivo* and in endothelial cells *in vitro* during doxorubicin-induced senescence led us to further investigate the mechanisms controlling ASAP resolution. To further understand the signaling that underlies this state, we created cell lysates from ECs at various times after doxorubicin treatment and profiled intracellular signaling pathway activity using a slide based antibody array. The PI3K/AKT/mTOR pathway was repressed as ECs became senescent (Fig 5a, b). Interestingly, mTOR has been hypothesized to regulate SASP-factor production as well as the senescence arrest, although its role in regulating the senescence arrest remains unclear(41, 42). We further investigated whether the SASP might be inhibited in ECs due to insufficient mTOR pathway activity during senescence. Activation of PI3K/AKT/mTOR signaling by expression of the adenoviral E4ORF1 protein (Supplemental Figure 2d), which has been used to model activation of the AKT pathway in ECs extensively(43), leads to sustained IL-6 secretion during senescence (Fig 5c) and increased secretion of a number of pro-inflammatory SASP components, including IL-8, GM-CSF, Gro and IFN-g (Fig 5d).
Senescent, doxorubicin-treated, ECs also do not exhibit the enlarged morphology classic to senescent cells (Fig 5e). Activation of PI3K/AKT/mTOR signaling restores this morphologic change, implicating this pathway in multiple aspects of cellular senescence. We do not observe substantial E4ORF1 mediated differences in either the proportion of arrested cells (Supplementary Fig. 3a) or in EC SA-β-Gal staining after doxorubicin treatment (Supplementary Fig. 3b).

These data demonstrate that the activation state of the PI3K/AKT/mTOR pathway influences both the morphologic changes associated with the senescent state as well as the ability of senescent cells to produce pro-inflammatory cytokine production and has implications for our understanding of cellular senescence and the SASP.
Figure 5: PI3K/AKT/mTOR pathway activity decreases after doxorubicin treatment and controls SASP cytokine production. (a) A heat map displaying results of PathScan antibody array analysis of ECs lysates treated with doxorubicin for 8, 24, or 120 hrs. Data are displayed as log2 fold change over untreated cells with protein and specific phosphorylation site recognized by antibodies listed. (b) Western blots for p-S6 in lysates from doxorubicin-treated ECs various times after treatment. (c) IL-6 secretion from ECs infected with MSCVpuro vector control or the adenoviral E4ORF1 protein, which activates PI3K/AKT/mTOR pathway activity. IL-6 secretion 24 hrs and 5 days after doxorubicin treatment was measured by ELISA. (d) Heatmap displaying the levels of 6 different cytokines and growth factors in endothelial cell conditioned media from vector control (MSCVp) or E4ORF1 infected endothelial cells. (e)
Light microscopy of senescence-associated-beta-galactosidase stained ECs infected with vector control (MSCVp) or E4ORF1 5 days after treatment with doxorubicin to induce senescence. Cell size and Beta-gal positivity were assessed.

**Activation of PI3K/AKT/mTOR signaling increases IL-6 production in an NF-kB-independent but mTORC1-dependent fashion**

PI3K/AKT signaling can activate multiple downstream regulators implicated in the SASP including mTOR and NF-kB. To distinguish which of these are essential for IL-6 production, ECs were infected with the E4ORF1 vector, and co-cultured with the NF-kB inhibitor caffeic acid phenethyl ester (CAPE) or the mTORC1 inhibitor rapamycin (Fig 6a). Rapamycin treatment blocks sustained IL-6 production, which is unaffected by CAPE or by co-infection of endothelial cells with E4ORF1 and a dominant negative-NF-kB inhibitor (Fig 6b). mTOR has recently been suggested to regulate the SASP through the promotion of IL-1a translation, NF-kB activation and a subsequent feed-forward loop that reinforces the SASP(44) or by regulating MK2 translation and subsequently SASP transcript stability(45). Our data indicate that, in ECs, PI3K/Akt/mTOR pathway activation can induce a SASP in an NF-kB-independent manner. Additionally, IL-1a is produced at near undetectable levels (<1 pg/ml) in ECs and IL1a levels are identical between E4ORF1 and vector control infected ECs (Supplementary Fig. 3c). This differentiates the mechanism by which mTOR activity can promote SASP-factor production in ECs from that recently observed in fibroblasts.

Activation of PI3K/AKT/mTOR pathway activity by infection of ECs with a constitutively active myristoylated form of AKT (myr-AKT) or mutant H-RAS (G12V) also leads to sustained IL-6 secretion after doxorubicin treatment that is
dependent on mTORC1 activity and mTOR activation regulates the secretion of additional SASP factors in an mTORC1-dependent fashion (Fig 6d). We have thus identified the activity of the PI3K/AKT/mTOR signaling pathway as a switch that determines SASP-factor production and can be regulated to control the duration of inflammation associated with senescence.

Figure 6: PI3K/AKT/mTOR activation switches ASAP to SASP-like cytokine production which is inhibited by rapamycin. (a) IL-6 secreted by endothelial cells (ECs) infected with PI3K/AKT/mTOR pathway activating E4ORF1 construct or vector control (MSCVp). ECs were treated with doxorubicin in combination with the NF-κB inhibitor caffeic acid phenylethyl ester (CAPE) or the mTORC1 inhibitor rapamycin. IL-6 secretion is measured for the 24hrs preceding the time-point listed and normalized to cell number. (b) IL-6 secretion from ECs infected
with both E4ORF1 and a dominant-negative allele of IkBa or vector control following doxorubicin treatment. (c) IL-6 produced by ECs infected with HRasV12, myristoylated-AKT or vector control and left untreated or treated with doxorubicin in the presence or absence of rapamycin. (d) Heat map displaying the levels of 6 different cytokines and growth factors in EC-conditioned media from vector control (MSCVp) or E4ORF1-infected ECs treated with or without the mTORC1 inhibitor rapamycin.

**ASAP IL-6 production is blocked by HDAC inhibition, which synergizes with doxorubicin to treat B-cell lymphoma**

EC acute IL-6 production is sufficient to promote lymphoma resistance to doxorubicin treatment in vivo. Inhibition of IL-6 production or activity thus represents a potential microenvironment-targeted therapeutic strategy. We performed an ELISA-based small-molecule screen to detect compounds that inhibit doxorubicin-induced IL-6 production. Multiple histone deacetylase (HDAC) inhibitors blocked IL-6 release in this screen and also inhibit endothelial IL-6 secretion in validation experiments (Fig 7a). HDAC inhibitors lead to a rapid decline in IL-6 transcript levels, suggesting that HDAC inhibitors prevent EC IL-6 production in a manner mechanistically distinct from its regulation by ROS, inhibition of which does not affect IL-6 transcript levels. Many histone deacetylases target cytoplasmic as well as nuclear proteins and HDAC inhibitors can regulate RNA stability(46, 47). To determine whether HDAC inhibitors affect IL-6 transcript stability, ECs were treated with Act. D in the presence or absence of doxorubicin and the HDAC inhibitor Vorinostat (SAHA) and IL-6 transcript levels determined by qPCR at various times thereafter to monitor IL-6 transcript decay (Fig 7c). The lack of differences between SAHA-treated and untreated samples suggests that HDAC-inhibition regulates IL-6 levels though the
modulation of IL-6 transcription, not through the regulation of IL-6 transcript stability. When tumor-bearing mice were treated with doxorubicin in combination with SAHA, a synergistic extension in survival is observed (Fig, 7d), suggesting that SAHA might be useful in combination with doxorubicin for the treatment of lymphoma.

Figure 7: HDAC inhibitors block endothelial IL-6 production and synergize with doxorubicin. (a) Quantification of IL-6 release from endothelial cells (ECs) treated with the HDAC inhibitors SAHA and scriptaid with or without doxorubicin. IL-6 secreted into the media was measured by ELISA and normalized to cell number. (b) IL-6 transcript levels in ECs treated with or without SAHA and doxorubicin at the indicated times. IL-6 mRNA levels were measured by qPCR and normalized to GAPDH. Data for each independent time point is from one experiment. (c) The effect of SAHA on IL-6 mRNA half-life was determined by qPCR. Endothelial cells were treated with or without doxorubicin and/or SAHA and 2 hours later (time 0) the RNA-polymerase inhibitor actinomycin D (Act. D) was added to stop transcription. IL-6 mRNA levels were measured by qPCR at various times after treatment. (d) Survival of mice transplanted with Eu-Myc B-
cell lymphomas that were treated with doxorubicin and/or SAHA 12 days after transplantation. Length of survival of the mice-post treatment is shown. Data are shown as mean +/- SEM. * = P<0.05

Discussion

Efforts to improve the treatment of cancer have traditionally focused on cancer-cell-intrinsic susceptibilities. However, therapies that target the tumor microenvironment represent a promising new approach to improve the efficacy of currently used chemotherapy(2-4). Tumors are complex tissues made up of malignant and non-malignant cell types with local nutrient availability, cytokine and growth factor production and many additional factors playing a role in tumor growth. A better understanding of the tumor microenvironment will be essential to intelligently target it therapeutically. Additionally, the microenvironment changes during both tumor progression and over the course of therapy(3, 10), but the changes that occur during therapy are poorly understood. Here, we show that the chemotherapeutic doxorubicin induces acute production of the cytokine IL-6 from endothelial cells in the tumor microenvironment, that this release promotes chemoresistance in B-cell lymphoma and that it can be targeted with histone deacetylase inhibitors to improve lymphoma survival after treatment.
Figure 8: Schematic of EC secretory control. Doxorubicin-induced ROS lead to increased p38 phosphorylation and increased ASAP cytokine production. In the setting of sustained PI3K/Akt/mTOR signaling ECs undergo a SASP with sustained cytokine production, however, when signaling through this pathway is restrained at baseline ECs resolve the ASAP and enter a less secretory senescent state.

Surprisingly, we find that while doxorubicin treatment induces cellular senescence in endothelial cells, it does not lead to a canonical SASP with sustained high-level cytokine production. The connection between cellular senescence and tumor progression is complex, with senescence of damaged or oncogene-activated cells leading to cell-cycle arrest and preventing malignant transformation but also potentially creating a SASP-fueled pro-tumorigenic and chemoresistant microenvironment(30). Senescent cells are found in pre-malignant and malignant tumors and cellular senescence can be induced in both malignant and non-malignant cells by chemotherapy and irradiation(12). How senescence-related secretory responses vary across genetic backgrounds and cell types remains unclear and the molecular control of the SASP is only beginning to be fully elucidated(12, 15, 48). Our observation that senescent...
endothelial cells do not undergo a canonical SASP after chemotherapy-induced damage suggests that these responses can be quite diverse in different cellular contexts. The ASAP may represent a mechanism by which pro-survival paracrine signaling can be activated acutely without the chronic inflammation that might result from a full-blown SASP, thus preserving tissue homeostasis(49). Intriguingly, rapamycin, which inhibits mTOR and which we show inhibits the SASP, has been shown to extend lifespan from yeast to mammals, yet the mechanism by which it does so is not clear(50). These data suggest that rapamycin may extend lifespan, at least in part, through its ability to regulate the SASP.

While SASP suppression may be one mechanism by which tissue homeostasis is preserved, it remains possible that the absence of a SASP could be deleterious and contribute to tissue dysfunction by impairing the clearance of damaged cells whose accumulation might promote organ dysfunction. Further work will be necessary to elucidate the stimuli, cell types and extent to which SASP-factor production occurs during senescence in vivo in humans. Patients treated with radiation and chemotherapy have an increased risk of vascular pathology and tissue dysfunction years after treatment(51) and senescent endothelial are found in atherosclerotic lesions(52). Thus, the regulation of chemotherapy-induced senescence phenotypes is likely relevant beyond its direct impact on tumor biology.
mTOR has recently been shown to be essential for the SASP in fibroblasts, although it remains unclear whether it regulates the SASP by the same mechanism in senescence induced by different stimuli(41, 44, 45). Our data further support a role for mTOR in the regulation of the SASP. We have shown that depending on the activity of the PI3K/AKT/mTOR pathway DNA damage can induce two distinct secretory responses that differ in their timing and molecular mechanisms. mTOR repression may serve as a useful brake on inflammatory cytokine production, preserving homeostasis in the context of significant tissue damage and senescence - including that occurring in the context of chemotherapy treatment or irradiation. The resolution of inflammation is essential for proper wound healing(49) and molecular control over ASAP vs. SASP secretory responses may be important for the preservation of tissue homeostasis by acutely protecting damaged cells while preventing prolonged inflammation.
Supplementary Figure 1: Doxorubicin induces cellular senescence in vivo and in vitro. (a) Mice treated with doxorubicin show a significant number of senescent cells in the thymus, a site of chemoresistance, as determined by SA-\(\beta\)-gal staining. Few senescent cells are present in the thymus of untreated mice. (b) Hepatocellular carcinoma cells were treated with doxorubicin and 5 days after treatment, senescence measured by SA-\(\beta\)-gal staining.
**Supplementary Figure 2:** (a) IL-6 secretion from endothelial cells treated with doxorubicin in the presence of NVP-BEZ235 which inhibits the DNA-damage responsive kinases ATM, ATR and DNA-PK. (b) IL-6 secretion from ECs treated with doxorubicin in the presence of a DNA-PK inhibitor. (c) IL-6 secretion from endothelial cells acutely after doxorubicin and/or rapamycin treatment. For all ELISA experiments IL-6 levels were collected over 24hrs and normalized to cell number. (d) A heat map displaying results of PathScan antibody array analysis of E4ORF1 infected EC lysates at various times after treatment. Data are displayed as log2 fold change over untreated cells.
Supplementary Figure 3: (a) BrdU incorporation into doxorubicin treated or untreated endothelial cells infected with E4ORF1 or vector control (MSCV-puro) was measured by flow cytometry. Cells were pulsed with BrDU for 24hrs before measurement. (b) Percentage of B-galactosidase positive cells. E4ORF1 or vector control endothelial cells were treated with doxorubicin to induce senescence and fixed and stained for senescence-associated-beta-galactosidase 5 days after treatment. (c) IL-1α levels secreted by endothelial cells infected with MSCVpuro vector control or E4ORF1 construct and treated with doxorubicin for the indicated length of time. Media is collected over the 24hrs prior to the time indicated and normalized to cell number. (d) BrdU incorporation into doxorubicin and/or rapamycin treated or untreated endothelial cells was measured by flow cytometry. Cells were pulsed with BrDU for 24hrs before measurement.
Methods

Cell culture and chemicals

HUVEC cells were purchased from Lonza and cultured in Endothelial Cell Growth Medium 2 (Lonza). Hepatocellular carcinoma (FOCUS) cells were grown in 45%DMEM/45% IMDM/10% FBS, supplemented with 2mM L-glutamine and 5μM β-mercaptoethanol. Cells were treated with doxorubicin at the specified concentrations. N-Acetylcysteine (Sigma) and Glutathione (Sigma) were resuspended in HUVEC media at final concentration of 10mM and pH adjusted to 7.0 with NaOH. Menadione (5-10uM), Cumene Hydroperoxide (50-100uM) were purchased at Sigma and used as described. SB203580 (LC labs) was used at 10uM. Rapamycin (8nM) (LC Labs), CAPE (Tocris), DNA-PK inhibitor II (Calbiochem). Actinomycin D, cycloheximide were purchased from Sigma. SAHA was purchased from LC labs.

Mice

C57Bl/6J (wild type) mice were purchased from Jackson Labs. Cdh5(PAC)-CreERT2(24) and mTmG(26) mice back-crossed onto C57Bl/6J mice were provided by Patrick Murphy and Richard Hynes. IL-6 floxed mice were obtained from the European Mutant Mouse Archive, re-derived from embryos and back-crossed an additional 2 generations onto C57Bl/6J mice before use. For lymphoma experiments 2x10^6 Eu-Myc; p19Arf/- lymphoma cells were injected by tail vein. Disease progression was followed by palpation of lymphoma burden in the axillary and brachial lymph nodes and at earliest palpable disease, ~12 days
post-injection, mice were treated with doxorubicin (10 mg/kg, IP). For SAHA treatment experiments mice were treated ~1hr before doxorubicin injection (300mg/kg, IP). Mice were sacrificed when moribund. For all experiments with transgenic mice littermate controls were used. All mice are housed in the Koch Institute Animal Facility at MIT.

**Immunofluorescence**

Mouse tissues were embedded and frozen in OCT compound (Tissue-Tek) and frozen tissue sections were fixed in 3% PFA 1xPBS for 20 minutes prior to blocking in 2% BSA .1% tritonX-100. Primary antibody stains were performed overnight at 4C and secondary stains for 1 hr, both in .2%BSA .01%tritonX-100 in a humid chamber to prevent drying. DAPI (Invitrogen) was used at 1ug/ml. Antibodies used were: 1:500 Rabbit Anti-GFP (Invitrogen), 1:100 Anti-CD31 MEC13.3 (BD), goat-anti rat Alexa Fluor 647 (Invitrogen), goat anti-rabbit- Alexa 488 (Invitrogen). Images were acquired on a Nikon A1R Confocal Microscope.

**Flow cytometry**

For flow cytometry, HUVEC or FOCUS cells were trypsinized for ≥5 minutes, trypsin quenched with media and cells run on a FACScan, FACS Calibur, LSRII HTS, or LSR-Fortessa HTS (BD) flow cytometer with or without the membrane exclusion dye propidium iodide. FlowJo was used for post-acquisition analysis. DCFDA ROS Detection Kit (Abcam) was used by the manufacturers instructions.
Protein Analysis

For western blots, cell lysates were collected in RIPA buffer with protease and phosphatase inhibitor cocktails (Roche). Debris was removed by centrifugation and protein concentration in the supernatant determined by BCA (Thermo). Lysates were reconstituted to equal concentrations, mixed with SDS-Sample buffer (Boston BioProducts) and boiled for 5 minutes prior to running. Samples were run on a polyacrylamide gel non-gradient (BioRAD) or NOVEX 4-20% Tris-Glycine (Invitrogen), transferred to PVDF (Millipore) and blotted with the following antibodies: p16 (Santa Cruz #759, 1:500), p21 (Santa Cruz #397, 1:500), HSP90 (BD #610418, 1:10,000), Actin, p38 (CST #9212, 1:1000), p-p38 (CST, #9211, 1:1000), p-S6 (D57.2.2E CST #4858, 1:1000). PathScan Intracellular Signaling Array Kit (CST) was used to profile endothelial signaling according to manufacturers instructions. Gels and antibody array slides were imaged with an ImageQuant LAS 4000 (GE) or exposed to film.

RT-PCR

RNA was extracted using the QIAshredder and RNeasy kits (Qiagen). cDNA was reverse transcribed from RNA using random hexamers. Transcript levels were determined by qPCR using an Applied Biosystems thermal cycler with SYBR Green MasterMix. Samples were normalized to GAPDH and relative transcript levels quantified by the delta delta Ct method. For RNA-half life experiment actinomycin D was added at time 0 and samples extracted as described above to assess transcript levels.
ELISA and Luminex Cytokine Measurements

All secreted factor concentrations determined by ELISA and cytokine arrays are normalized to the weight of the dissected tissue or the number cells at the end of the assay. Mouse and human IL-6 Ready-SET-GO ELISA kits (eBioscience) were used according to manufacturers instructions. IL-6 standard was either re-suspended in ELISA diluate (per manufacturers instructions) or cell culture media with compounds used in the corresponding conditioned media. Concentrations were determined with a 4 parameter logistic regression using a standard curve on readerfit.com or elisaanalysis.com. Multiplexed luminex assays for growth factor, chemokine and cytokine levels were preformed as described by the manufacturer by Eve Technologies. Unless otherwise stated ELISA experiments are n=4 from at least two independent thaws of cells.

Conditioned media and drug treatments

Conditioned medias for HUVEC and FOCUS cell experiments were made by collecting the media on cells for 24hrs prior to each timepoint from 50,000 or 150,000 cells seeded into 24 well or 6 well plates respectively. Endothelial cells were untreated or treated with 200 or 225nM doxorubicin. FOCUS cells were untreated or treated with 200nM doxorubicin for 24 hour time points or 25-100nM for 5 day time points. For 5 day timepoint for FOCUS cells the media was changed and drug removed 24hrs after addition, for HUVEC day 5 timepoint drug was left on cells for 4 days. Conditioned media was made from the thymus of
individual mice the indicated times after doxorubicin treatment. All tissues were
dissociated manually in FOCUS cell media. Soluble factors in the thymus were
allowed to condition media for 6 hours at 37°C. Conditioned media was cleared
of tissue and cells by centrifugation.

cDNA Constructs
The E4-Orf1 allele was cloned from Human Adenovirus Type C Strain 5 genomic
DNA into a retroviral MSCV-puro vector. Each cDNA retroviral vector was
transfected into Phoenix cells to produce amphotropic retrovirus. pBABEpuro-
IkBa-DN was a gift from Tyler Jacks. pBABE puro H-RAS V12 Addgene plasmid
#9051) and pBABE puro myrAKT were a gift from William Hahn.

β-galactosidase activity staining
Cells were fixed with 0.5% gluteraldehyde in PBS for 15 min, washed with PBS
supplemented with 1 mM MgCl₂, and stained for 6 hours in pH 5.5 PBS (for
mouse samples) or pH 6.0 PBS (for human) containing 1 mM MgCl₂, 1mg/ml X-
Gal, and 5 mM each of potassium ferricyanide II and potassium ferrocyanide III.
Images were collected on an Eclipse TE2000-U (Nikon) equipped with Spot
software or Axioplan2 (Zeiss) microscope equipped with Openlab software from
Improvision.

BrdU Staining
Cells untreated or treated for the specified amount of time were stained using the
APC-BrdU Flow Kit (BD Bioscience) according to manufacturers instructions and cells analyzed by flow cytometry as described.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism4 software. Two-tailed Student’s t tests were used, as indicated. Error bars represent mean ± SEM or as noted.
References


Chapter 3

Microenvironmental IL-6 Inhibits the Efficacy of Immunogenic Cancer Therapy by Suppressing Anti-Cancer Immunity

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Abstract:
Immunotherapy represents an exciting new approach to treating cancer, with the potential to promote durable remissions in diverse cancer types. Yet current immune-stimulating drugs only work in a subset of patients and the mechanisms by which tumors evade anti-cancer immune responses remain incompletely understood. Combination therapy with multiple immune-stimulating agents to circumvent this evasion is a promising approach to induce more effective and widespread anti-tumor immune responses. Recent work indicates that certain commonly used chemotherapeutics induce immunogenic cell death (ICD), which promotes the generation of anti-cancer immune responses in animal models of cancer. However, these immunogenic chemotherapeutics are widely used clinically but rarely induce sustained anti-tumor immune responses. It is unclear what immunosuppressive mechanisms prevent durable responses after immunogenic chemotherapy. We have found that the tumor microenvironment suppresses anti-cancer immunity induced by ICD through the production of the cytokine interleukin-6 (IL-6). Using a mouse model of Bcr-Abl+ B-cell acute lymphoblastic leukemia (B-ALL) we show that the immunogenic chemotherapeutic doxorubicin promotes CD8+ T-cell-mediated leukemia clearance and cure in the majority of IL-6-deficient animals but that these responses are suppressed in WT mice. We demonstrate that IL-6-deficient mice have lasting anti-cancer immunologic memory and reject re-transplanted B-ALL and that IL-6 does not promote chemoresistance directly by signaling to leukemia cells. Microenvironmental IL-6 also suppresses the effectiveness of immune-checkpoint inhibition with anti-PD-L1 blockade, suggesting that the inhibition of IL-6 represents a promising approach to enhance and maintain anti-tumor immune responses induced by immune-stimulating therapy.

Contributions
This chapter describes experiments I performed in the Hemann lab. Iris Zhuang, an undergraduate I supervise, assisted with the immune profiling experiments, many of the experiments requiring mouse dissection, and the in vitro dose-response experiments. Boyang Zhao, Vincent Butty and Yunpeng Liu assisted with RNA-sequencing analysis.
Introduction

The treatment of disseminated cancer remains a major challenge. Most non-local cancers are treated primarily with conventional chemotherapy, but complete eradication of disease is difficult to achieve with either conventional or targeted agents. Immune-stimulating therapy represents a promising approach to treat cancer, with durable responses seen for a number of novel agents(1, 2). However, many patients treated with these drugs still fail to respond(3), and additional ways to stimulate anti-tumor immunity are needed.

The ability of anti-cancer immune responses to influence the development and growth of cancer is increasingly clear(4). Immune evasion is viewed as an emerging hallmark of cancer development(5) and extensive clinical data indicate that immune infiltrates have significant, but complex, prognostic value(4). While the mechanisms by which cancer cells evade anti-tumor immunity remain incompletely understood, the identification of novel ways to inhibit cancer immune evasion promises to inform the development of more effective immune-stimulating therapy and to improve the treatment of cancer.

Some of the most widely used chemotherapeutics, such as doxorubicin and oxaliplatin among others, have been proposed to induce anti-tumor immunity in mouse models of cancer through the stimulation of immunogenic cell death (ICD)(6). Immune-stimulating, or ‘immunogenic’, chemotherapy promotes increased antigen presentation on cancer cells and provokes the release of
inflammatory damage-signals such as HMGB1 and ATP from cancer cells, increasing the recruitment and activity of antigen-presenting dendritic cells(6). Calreticulin (CRT) exposure on the surface of damaged cancer cells additionally promotes their engulfment by innate antigen-presenting cells, ultimately increasing immune recognition(7). However, immunogenic chemotherapeutics rarely, if ever, cure patients through the generation of anti-cancer immune responses, suggesting that any responses they induce are ultimately suppressed, preventing optimal efficacy. Therapies that increase the immunogenicity of these widely used drugs thus have promise to improve the treatment of cancer.

Cancer cells arise in the context of a tissue microenvironment composed of diverse immune and non-immune cell types that influence tumor growth as well as the generation of anti-cancer immunity(8, 9). These cells provide essential growth factors and nutrients to support the growth of early neoplastic lesions(8), but immune cells in the microenvironment can also inhibit tumor formation and progression through the clearance of malignant cells recognized as foreign(2, 10). It is now widely accepted that T-cell responses can restrain tumor development. The infiltration of CD8+ cytotoxic T-lymphocytes (CTL) into the tumor microenvironment is associated with positive outcome in a number of cancer types(4) and the absence of either CD8+ or CD4+ T-cells speeds the development of carcinogen-induced tumors in mice(11). Additionally, at least some malignancies appear to develop more frequently in immunocompromised
patients, for example in transplant recipients(2, 12). For tumors arising in the context of an intact immune system, the evolution of approaches to escape immune recognition appears to be essential for progression(13, 14).

Immune evasion can occur through cancer-intrinsic changes as well as through alterations in the tumor microenvironment. Mechanisms of intrinsic immune evasion include the mutation of T-cell antigens, the down-regulation of cancer antigen-presentation machinery and up-regulation of T-cell inhibitory immune-checkpoint ligands(14, 15). Cancer cells can also promote changes in the microenvironment including the exclusion of T-cells, production of immune-inhibitory cytokines or recruitment of immunosuppressive cell subsets(14, 16, 17). The recent clinical success of immune-checkpoint inhibitors, with drugs blocking T-cell inhibitory signals downstream of CTLA-4 and PD-1 showing promising clinical results in more than 15 cancer types(1), has generated significant excitement about therapeutically targeting these evasive mechanisms. Yet CTLA-4 and PD-1 blocking agents still fail in many patients, demonstrating that multiple layers of immune suppression exist and that checkpoint blockade can be subverted by additional immune-suppressive mechanisms.

One major barrier to the generation of anti-tumor immune responses is the exclusion of cytotoxic immune cells from the tumor microenvironment(6, 18). Immunogenic chemotherapy shows potential to reverse this by stimulating the production of damage signals that prime both innate and adaptive responses.
These agents can alter the repertoire of MHCI peptides presented on cancer cells and induce the activity of antigen-presenting dendritic cells, which promote tumor immune recognition. However, these and other immunostimulatory agents do not circumvent all of the immune-evasive approaches present in the tumor microenvironment. Paracrine production of immunosuppressive cytokines and metabolites such as IL-10, IDO and other chemokines that influence recruitment or activity of immune cells is a promising therapeutic strategy(19). Tumors are complex tissues with many cell types communicating through diverse secreted and membrane-bound proteins. Given this complexity, it is likely that redundancy in the immune-suppressive approaches employed by cancer cells will necessitate combinations of immune-activating agents to achieve durable responses in the majority of patients(19). The identification of additional approaches to inhibit anti-cancer immunity is thus of great value.

We have found that production of the canonically pro-inflammatory cytokine interleukin-6 (IL-6) by the tumor microenvironment suppresses anti-tumor immune responses induced by immunogenic chemotherapy. Using, a mouse model of Bcr-Abl+ pre-B-cell acute lymphoblastic leukemia (B-ALL), we show that the leukemia microenvironment is normally immunosuppressive, and that the immunogenic chemotherapeutic doxorubicin extends survival, but fails to promote durable immunity, similar to its impact in patients with this type of B-ALL(20). Interestingly, we find that IL6KO mice treated with doxorubicin rapidly clear their leukemia, with the majority of these mice undergoing T-cell-dependent
anti-leukemia immune responses and developing lasting immunologic memory. Microenvironment IL-6 also suppresses the effectiveness of immune-checkpoint inhibition with anti-PDL1 blocking therapy, suggesting that the inhibition of IL-6 may be a broadly effective therapeutic strategy to enhance anti-tumor immune responses that deserves further pre-clinical investigation.

**Results**

**WT B-ALL mice are resistant to doxorubicin treatment**

Bcr-Abl+ B-ALL represents a treatment-refractory subtype of B-ALL with a median survival of less than 2 years despite the use of cytotoxic chemotherapeutics in combination with targeted Bcr-Abl kinase inhibitors (20). Most chemotherapy regimens for ALL include the anthracycline doxorubicin, which can promote ICD and induce anti-tumor immunity in some contexts(6, 20). However, patients with Bcr-Abl+ B-ALL rarely, if ever, experience immune-mediated cures, suggesting that anti-tumor immunity induced by doxorubicin is normally suppressed in this disease. To investigate the mediators of immunosuppression and resistance to immunogenic chemotherapy, we used a transplantable mouse model of BCR-Abl+ B-ALL that closely recapitulates the human disease(21). Transplanted leukemia cells are found primarily in the bone marrow, blood and spleen, recapitulating the relevant tissue microenvironments in the human disease, and the model recapitulates therapeutic responses seen in humans(22, 23).
To investigate the response of this leukemia to immunogenic chemotherapy, we treated wild-type leukemic mice with doxorubicin and monitored their disease burden and survival (Fig 1a). Doxorubicin-treated mice survive longer than untreated mice, but ultimately relapse from their disease (Fig 1b), similar to the responses seen in humans. Interestingly, disease latency and response to doxorubicin is not significantly altered in Rag2KO mice, which lack functional T and B-cells, demonstrating that the increase in survival occurs largely independently of anti-leukemia immune responses and that adaptive immunity induced by doxorubicin treatment is suppressed in WT mice (Fig 1C). CD8+ T-cell depletion leads to moderate, although not statistically significant, impairment in doxorubicin response (Supplementary Figure 1).
Transplant B-ALL Monitor disease progression and survival
Treat with doxorubicin

Figure 1: Doxorubicin does not elicit long-term anti-tumor immunity in WT leukemic mice. (a) Schematic of the experiments shown below. (b) Kaplan-Meier survival curve of mice transplanted with B-ALL cells and either treated with doxorubicin or untreated. (c) Survival of WT or immunodeficient, Rag2−/−, mice transplanted with B-ALL and untreated or treated with doxorubicin as in (b). ** p<.001, n.s. = p>.1 for doxorubicin treated WT vs Rag2KO.
**IL-6 promotes resistance to immunogenic cell death induction**

IL-6 is a pleiotropic cytokine initially discovered for its pro-inflammatory properties that has been implicated in tumor development and resistance to therapy in diverse cancer types (24-26). IL-6 is essential for the initiation of innate and adaptive immune responses in many contexts (27) and its over-expression can promote chronic inflammatory conditions such as inflammatory arthritis (26), but it has also been suggested to have pro-resolving and anti-inflammatory properties (26, 28). The ultimate impact of IL-6 on the generation of anti-tumor immune responses in vivo is not clear (25, 29).

We sought to understand the effect that loss of IL-6 in the tumor microenvironment has on leukemia response to immunogenic chemotherapy like doxorubicin. To do so, we transplanted leukemia cells into syngeneic IL6-/− mice (27) and treated these mice with doxorubicin. Surprisingly, we found that doxorubicin-treated mice lacking IL-6 in the tumor microenvironment live significantly longer than wild-type (WT) treated mice, with a majority of mice appearing to be cured of their disease (Fig. 2a). IL-6 does not appear to have any impact on leukemia cell proliferation in the absence of treatment (Fig 2a, b) and the difference in survival between WT and IL6KO mice was not seen in mice treated with the Bcr-Abl inhibitor imatinib (Fig. 2c). Imatinib is not known to induce immunogenic cell death, suggesting that the effect of microenvironmental IL-6 may depend on the chemotherapeutic agent employed and its ability to induce ICD (Fig 2c). Indeed, analysis of calreticulin (CRT), a pro-engulfment
signal and hallmark of ICD(7), on the surface of treated leukemia cells shows that doxorubicin, but not imatinib, induces CRT surface exposure (Fig 2d). These data suggest that the presence of IL-6 impacts the efficacy of immunogenic chemotherapy, possibly through the regulation of anti-leukemia immune responses.

**Figure 2: IL-6 promotes chemoresistance to doxorubicin but not imatinib treatment.** (a) Survival of leukemic WT or IL6-/- (IL6KO) mice either untreated or treated with doxorubicin 8 days after disease initiation. n=10 for treated mice, n=6 and n=5 for untreated WT and IL6KO mice respectively. Shown is data from 2 independent experiments. ***p<0.0001 by Mantel Cox test. (b) Leukemia burden in WT and IL6KO mice at various times after treatment. Data is shown as a Tukey plot. n.s. = p>0.05, **p<0.05 by Mann Whitney test. (c) Survival of imatinib treated B-ALL bearing mice. Mice were treated for 7 days with 50mg/kg imatinib by oral gavage and sacrificed when moribund, n=10 per cohort, 2 independent experiments. (d) Calreticulin levels on the surface of leukemia cells cultured in vitro. Cells were treated at a dose that induces similar killing and stained for surface Calreticulin exposure 18hrs after treatment initiation.
Calreticulin levels were determined by flow cytometry and are displayed along the x axis.

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

We next sought to understand whether IL-6 could directly promote resistance to therapy either through activation of anti-apoptotic signaling or through the regulation of other cytokines and growth factors that do so(25). 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, which can be shed from neighboring cells(24). Thus, cells do not have to express the IL-6 receptor to engage in IL-6-mediated signaling, but can activate signaling from binding of soluble IL-6 receptor (sIL6r)-IL-6 complexes to gp130. To test whether IL-6 can directly mediate resistance to doxorubicin, we cultured leukemia cells in the presence of IL-6, the sIL6R, or both IL-6 and sIL6R to simulate signaling through sIL6R-IL-6 complexes. None of these conditions altered the sensitivity of leukemic cells to doxorubicin (Fig 3a), suggesting that IL-6 does not directly promote resistance to doxorubicin in this system.

IL-6 regulates the production of a number of other cytokines and growth factors in the bone marrow(30), including, IL-10, IL12, IL-15 and GM-CSF, leading us to test whether any of these could directly promote doxorubicin resistance in leukemia. 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* (Fig 3b), nor did co-culture of leukemia cells with bone-marrow stromal cells from WT or
IL6KO mice (Fig. 3c), suggesting that the resistance conferred by IL-6 does not result from direct regulation of leukemia cell sensitivity to treatment. Similar to what is observed in the majority of B-ALL patients(31), we do not find expression of the IL-6 receptor on leukemia cells either in vitro (Fig. 3d) or in vivo (Fig. 3e), although the IL-6 receptor is expressed on many non-leukemic cells in the bone-marrow microenvironment and is up-regulated on these cells after doxorubicin treatment (Fig. 3e). 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 (Fig. 3f). Interestingly, while stromal p-STAT3 levels increase in response to doxorubicin treatment, there are no differences in the levels of p-STAT3 between WT and IL6KO mice (Fig. 3f), suggesting that the effects of IL-6 occur independently of changes in STAT3 activity. Other groups have also reported similarly unaffected or increased levels of STAT3 in IL6KO mice in other tissues(32). These data suggest that the inhibition of IL-6 can have therapeutic benefit in vivo in contexts in which cancer cells do not express the IL-6R or directly respond to IL-6 treatment, thus expanding the situations in which the therapeutic benefit of IL-6 inhibition should be investigated.
Figure 3: IL-6 does not promote chemoresistance through direct IL-6R signaling. (a) Leukemic cell viability in response to doxorubicin treatment in the presence or absence of IL-6 and/or the sIL-6R. Viable cells counted by flow cytometry 48hrs after the addition of doxorubicin. Data shown from 4 independent experiments. (b) B-ALL cells treated as in (a) but in the presence or absence of a number of cytokines previously observed to be regulated by IL-6. (c) Viability of leukemia cells co-cultured with bone marrow stromal cells (BMSC) from WT or IL6KO mice in response to doxorubicin treatment as in (a). Data are from one experiment. (d) IL-6R expression on leukemia cell surface in vitro. Cells were stained with an APC-conjugated IL-6R or isotype-control antibody and fluorescence intensity determined by flow cytometry. Histograms for IL-6R and isotype-control stained cells are shown overlain. (e) IL-6 receptor expression in
the bone marrow of leukemia-bearing mice pre- and post-doxorubicin treatment was quantified by flow cytometry. mCherry+ leukemia cells were used to distinguish stromal and leukemia cells and median APC intensity from IL-6R-stained cells minus the signal from isotype-control stained cells was calculated. Each dot represents data from one mouse. Data from 2 independent experiments of 3 mice each. (f) phospho-STAT3 levels in the bone marrow of leukemia-bearing WT and IL6KO mice. Cells were fixed and stained for intracellular phospho-STAT3 and data shown as in (e).

**Leukemia clearance in IL6KO mice is dependent on T-cell mediated anti-tumor immune responses**

The inability of IL-6 to directly promote doxorubicin resistance as well as the efficacy of immunogenic chemotherapy in IL6KO mice led us to investigate whether IL-6 might affect therapeutic response through modulation of the immune system. To study the role that anti-tumor T-cell responses have in the durable responses seen in IL6KO mice, we depleted T-cells through the injection of anti-CD4 and CD8 antibodies (Supplementary Fig. 2a). While, T-cell-depleted IL6KO mice exhibit similar initial responses to doxorubicin 2 days after treatment, these mice fail to fully clear their leukemic burden and rapidly relapse (Fig 4a). T-cell-depleted IL6KO mice rapidly succumb to their disease and do not exhibit the long-term survival typically seen after treatment in this background (Fig. 4b), suggesting that anti-tumor T-cell responses are essential for the response to doxorubicin in IL6KO mice. Depletion of CD8+ cells alone also replicated the effect seen with CD4- and CD8-depletion (Supplementary Figure 2b), suggesting that long-term survival of doxorubicin-treated IL6KO mice is dependent on CD8+ cytotoxic T-lymphocyte (CTL) responses. These data indicate that doxorubicin promotes an anti-tumor immune response that is suppressed in WT mice through
the production of IL-6. In the absence of IL-6, that suppression is relieved, allowing effective tumor clearance and long-term remission. To verify that treated IL6KO mice develop anti-leukemia immunity, we re-transplanted leukemia cells into previously “cured” IL6KO mice or naive controls (Fig 4c) and monitored leukemia progression (Fig 4d). Previously cured mice are completely resistant to disease re-transplantation, demonstrating the presence of lasting anti-cancer immunity and suggesting that IL-6 serves as an important immunosuppressive cytokine in B-ALL.

**Figure 4: T-cell dependent anti-tumor immunity develops after treatment of IL6KO mice.** (a) Leukemia burden in IL6-/- mice following doxorubicin treatment. CD4 and CD8 cells were depleted by injection of depleting antibodies and response to doxorubicin treatment monitored by bioluminescent imaging. (b) Survival of IL6KO mice treated as in (a). (c) IL6KO mice previously 'cured' (living >100 days) by doxorubicin treatment were re-transplanted with leukemia cells and disease progression monitored by bioluminescent imaging. (d) Leukemia burden in control and re-transplanted leukemia bearing mice was determined by bioluminescence imaging.
IL-6 regulates diverse immunomodulatory pathways

To further investigate the differences between WT and IL6KO mice, leukemic and stromal cells were sorted out of the bone marrow and RNA-sequencing performed to look for differences in gene expression between WT and IL-6 knockout mice (Supplementary Figure 4a). Principal component analysis was able to isolate components that explain the majority of the difference between ALL and stromal cells (PC1) as well as differences between WT and IL6KO samples (PC2) (Supplementary Figure 4b). Ingenuity pathway analysis applied to top不同ially regulated genes between WT and IL6KO stromal cells returned many immune-related pathways as top hits (Supplementary Table 1), clearly demonstrating a difference in immune activation state between WT and IL6KO mice. Analysis of gene ontology terms enriched in the genes that contribute positive loadings to PC2 (which incorporates much of the difference between WT and IL6KO samples) also suggest changes in the regulation of the immune system in IL6KO mice (Supplementary Figure 4c). Paradoxically, however, the directionality of gene expression changes in these samples indicates that IL6KO mice should have an impaired immune response. Indeed, this is consistent with the published role of IL-6 as a pro-inflammatory cytokine, essential for the initiation of immune responses (24, 27, 33, 34). However, this broad pathway analysis does not preclude the presence of gene signatures that explain the immunosuppressive properties of IL-6 in this context, which may be obscured by the more prevalent immune-activating proteins lost in the context of decreased IL-6.
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 (35). It is thought that these proteins exist, at least in part, to restore homeostasis after an immune stimulus, preventing potentially hyperactive immune responses and autoimmunity (29). Cancer cells often express high levels of inhibitory checkpoint ligands and exploit the presence of these proteins on T-cells to inhibit their activity. This raises the question of whether a less basally activated immune system might, paradoxically, be more efficacious at responding to an acute stimulus like ICD in the immunosuppressive tumor microenvironment due to lower baseline checkpoint-protein expression and therefore resistance to inhibition by checkpoint ligands overexpressed on leukemia cells. Interestingly, the expression of many inhibitory immune checkpoint proteins were subtly decreased in IL-6 knockout mice, along with activating proteins (Supplementary Figure 4d), suggesting that decreased baseline immune activation through IL-6 loss may allow the cells to respond more effectively to an acute stimulus provided by immunogenic chemotherapy.

IL-6 is also known to regulate the activity of the immunosuppressive cytokine IL-10 (30, 36), and elevated levels of IL-10 release by T-cells have been proposed to mediate potential anti-inflammatory effects of elevated IL-6 production. IL-10 has also been implicated in mediating tumor immunosuppression in many contexts (2, 37, 38), prompting us to ask whether its inhibition might further activate anti-
tumor immunity in our system. We thus attempted to replicate the immune activation observed with IL-6 loss through the inhibition of IL-10. Leukemia-bearing WT mice injected with IL-10R-blocking antibody at doses previously shown to inhibit IL-10 signaling(37) and treated with doxorubicin show minimal differences in tumor burden (Supplementary Figure 5a), and no differences in survival (Supplementary Figure 5b), suggesting that either IL-10 plays no role in the immune suppression mediated by IL-6 or that redundancy prevents the efficacy of IL-10 inhibition alone with immunogenic chemotherapy. Collectively, these data indicate that IL-6 has complex and potentially conflicting impacts on immune activity and it is likely that the primacy of the pro- or anti-inflammatory functions of IL-6 depend on the tissue context and specific immune stimulus.

**Doxorubicin induces immune cell infiltration into the leukemia bone marrow**

Immunogenic cell death involves the release of immune-activating factors into the surrounding environment, stimulating immune activity(6). Among these are ATP and HMGB1, which serve to recruit immune cells to sites of damage and activate downstream inflammatory signaling that can recruit additional immune-cell subsets to the inflamed tissue, spurring anti-cancer immunity(7). To investigate the role that doxorubicin has on immune-cell composition in the bone marrow and spleen, major sites of leukemia burden, we profiled immune-cell composition in leukemia-bearing mice before and after treatment (Fig 5 a-e, Table 1 and Table 2). Before treatment, T-cells make up a small portion of cells
in the bone marrow (Fig 5 a-b, Table 1), but are much more prevalent in the spleen (Fig 5 c, Table 2), suggesting that the bone marrow may be a T-cell exclusionary microenvironment (18).

**Figure 5: Doxorubicin treatment induces T-cell influx into the bone marrow.**
(a) CD3+ T-cell numbers in the bone marrow of WT and IL6KO mice before and after treatment were determined by flow cytometry. For (a-f) data are collected from mice transplanted with mCherry+ leukemia cells and shown as percentage of mCherry- stromal cells staining positive for the markers in the graph title. Each
dot represents data from one mouse. n>=8. (b) CD8+ cytotoxic T-cell numbers in the bone marrow. (c) CD3+ T-cell numbers in the spleen. (d) CD11c+, MHCII+ dendritic cell infiltration into the bone marrow (e) CD11b+ Gr-1+ myeloid cells in the bone marrow. **p<.05, n.s>.1

Interestingly, doxorubicin selectively promotes T cell influx into the bone marrow, with increased cytotoxic and helper T-cell subsets observed after treatment in both WT and IL6KO mice (Fig 5a-b, Table 1, Supplementary Figure 3). Doxorubicin also promotes increased CD11c+, MHCII+ dendritic cell infiltration into the bone marrow (Fig. 5d), although it does not lead to major increases in CD103+ Batf3-dependent dendritic cell numbers, which have previously been implicated in the generation of anti-cancer immunity(39) (Table 1). We observe relatively low levels of CD3+, CD4+ CD25+ cells in the leukemic bone marrow (Table 1), a subset that includes T-regulatory cells, and these numbers are only marginally changed after doxorubicin treatment (Table 1), suggesting that the T-cell recruitment promoted by doxorubicin treatment is cell-type specific and that doxorubicin increases the CTL/T-reg ratio in the bone marrow, a ratio that is positively associated with survival in multiple cancer types(4, 40).

Doxorubicin does promote recruitment of F480-, CD11b+, Gr-1+ neutrophils to the bone marrow, but does not lead to major changes in overall CD11b+, Gr-1+ cells, a population which includes multiple mature and immature myeloid cell subsets and which make up a major portion of the cells in the bone marrow (Figure 5e, Table 1). As mentioned above, the spleen, the second major site of leukemia burden, has much higher baseline T-cell infiltration than the bone
marrow and T-cell numbers do not change significantly after treatment (Fig. 5c, Table 2), although doxorubicin does lead to small elevations in active CD69+ T-helper and CTL subsets (Table 2).

Collectively, these data suggest that the bone marrow is initially a more exclusionary environment for leukemia-reactive T-cells, but that doxorubicin-mediated ICD leads to increased dendritic and T-cell infiltration, thus promoting leukemia recognition and clearance in the absence of immunosuppressive IL-6. Interestingly, we have previously shown that the bone marrow is a site of resistance to antibody-based therapy in double-hit lymphoma, due to the creation of an immunosuppressive microenvironment that impairs innate immune-mediated clearance of antibody bound cells (41). Here, we show that the immunogenic chemotherapeutic doxorubicin can promote the generation of anti-cancer immune responses, at least in part, through its ability to induce influx of adaptive and innate immune cells into previously exclusionary tissue microenvironments.
<table>
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<tr>
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<tr>
<td>CD3+</td>
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Table 1: Quantification of bone marrow immune infiltration before and after doxorubcin treatment. The average representation of immune cell subsets in the bone marrow of WT and IL6KO mice before and after treatment. Squares colored in blue denote p<.05 compared to pre-treatment sample of the same genetic background. n>6 mice for all samples.
Table 2: Quantification of spleen immune infiltration before and after doxorubicin treatment. The average representation of immune subsets in the spleen of WT and IL6KO mice before and after treatment. Squares colored in blue denote p<.05 compared to pre-treatment sample of the same genetic background. n>6 mice for all samples.

Immunogenic cell death induction and IL-6 deficiency synergize with anti-PDL1 therapy to treat leukemia

In order to investigate the broader implications of our observation that IL-6 suppresses anti-leukemia immunity, we decided to examine the efficacy of immunogenic chemotherapy or IL6 loss in the context of immune-checkpoint blockade. Our leukemia cells express high levels of the checkpoint ligand PD-L1 (Fig. 6a) and PD-L1 expression has previously been implicated in B-ALL.
resistance to immune-stimulating therapy(42), suggesting this is an appropriate system in which to study the determinants of response to PD-L1 inhibition. Additionally, a significant proportion of the T-cells in the bone marrow of leukemia-bearing mice express PD-1 (Fig. 6b), leading us to investigate 1. whether PD-1 inhibitory signals might contribute to the failure of immunogenic chemotherapy and 2. whether IL-6 can enhance the efficacy of PD-L1 blockade.

Treatment of mice with PD-L1 blocking therapy demonstrated that suppression of this immune checkpoint can promote anti-leukemia immune responses and a subset of mice exhibited reduction in leukemia burden (Fig 6c), although with modest, not statistically significant, extension in survival observed in responding mice (Fig. 6d), similar to the efficacy of this drug in many human malignancies(3). Combination of PD-L1 inhibition with doxorubicin, to promote T- and dendritic cell influx into the bone marrow leads to a synergistic reduction in disease burden with a subset of mice appearing to show durable responses (Fig. 6d). This suggests that stimulation of immune-cell influx into previously exclusionary microenvironments through ICD induction can improve the efficacy of checkpoint-inhibition and that PD-1-mediated suppression of T-cell responses contributes to the failure of immunogenic chemotherapy. Combination therapy of these drug classes is thus a promising approach to improve response to each agent, in agreement with very recent work in lung cancer(43). Interestingly, IL-6 can promote PD-1 expression on T-cells(44) (Fig. 6b), suggesting that part of the
mechanism by which IL-6 suppresses anti-cancer immunity may result from the induction of PD-1 expression by T-cells.

To investigate whether IL-6 also suppresses anti-leukemia immune responses induced by immune-checkpoint blockade, we treated WT and IL6KO 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 (as discussed above), IL6KO mice undergo nearly complete leukemia eradication by 9 days after the initiation of PD-L1 blockade (Fig 6e). Almost all of the PD-L1-treated IL6KO mice appear to have undergone durable remissions and 80% remain alive without apparent disease (Fig. 6f). This further confirms that microenvironment production of IL-6 is a major barrier to the efficacy of immune-stimulating therapy in leukemia. IL-6 impairs the efficacy of both immunogenic chemotherapy and checkpoint blockade and its inhibition is thus a promising approach to improve the treatment of cancer. Preliminary work suggests that inhibition of IL-6 signaling with therapeutic antibodies against the IL-6R can extend survival in doxorubicin-treated mice (Supplementary Figure 6 a,b), although with modest changes in survival with IL6R-antibody regimens used here. Optimization of the dosage and timing of administration will be necessary to improve the efficacy of this approach. The suppression of anti-cancer immunity remains a major barrier to the effective treatment of cancer(16) and the inhibition of IL-6 in combination with immune-stimulating therapies thus represents a promising approach to circumvent this barrier and improve the treatment of cancer.
Figure 6: IL-6 inhibits the efficacy of PD-L1 inhibition, which is synergistic with the ICD-drug doxorubicin. (a) PD-L1 levels were measured by flow cytometry. Leukemia cells were stained with PE-Cy7 conjugated PD-L1 or a control PE-Cy7-CD11b antibody (which ALL cells do not express). (b) PD-1 levels in bone marrow CD3+ T-cells from WT and IL6KO mice were determined by flow cytometry. (c) Leukemic burden in mice treated with doxorubicin, PD-L1 or PD-L1 and doxorubicin measured by bioluminescence imaging 8 days after doxorubicin treatment. (d) Survival of mice treated with PD-L1 and/or doxorubicin. (e) Leukemic burden in WT mice treated with control nanobody or PD-L1 nanobody, or IL6KO mice treated with PD-L1 nanobody. (f) Survival of mice shown in (e). **<.05, ***< .001

Discussion

The extent to which immune responses contribute to the efficacy of cytotoxic chemotherapy remains controversial. Work from multiple labs has demonstrated
that a subset of commonly used chemotherapeutics stimulate immunity in some mouse models of cancer, but the broader relevance of these findings is not clear given that, in both mice and humans, immunogenic chemotherapy rarely, if ever, promotes lasting anti-tumor immune responses. We have used a mouse model of acute lymphoblastic leukemia that closely recapitulates the leukemia microenvironment and therapy responsiveness of the human disease to investigate the mechanisms of immune suppression after treatment with immunogenic chemotherapy. We find that while doxorubicin extends animal survival, WT mice ultimately fail to clear their leukemia burden and fail to generate lasting anti-leukemia immunity. In contrast, the majority of leukemic IL6KO mice are cured after doxorubicin treatment through the generation of anti-leukemia T-cell responses that are induced by therapy. We extend the relevance of this observation to immune-checkpoint blockade and demonstrate that the efficacy of PD-L1 inhibition is also suppressed by microenvironmental IL-6. We also show that doxorubicin promotes T- and dendritic cell influx into the bone marrow, transforming it into a pro-immunogenic microenvironment more conducive to the clearance of leukemia cells and that doxorubicin-induced ICD increases the efficacy of PD-L1 blockade. Taken together, these data suggest that combination therapy with IL-6 inhibition, immunogenic chemotherapy and checkpoint blockade may be a promising therapeutic approach in human cancer and that IL-6 secretion into the tumor microenvironment may, in part, explain the limited anti-cancer immunity induced by immunogenic chemotherapy in humans.
Indeed, ICD does appear to contribute to the efficacy of anthracyclines, such as doxorubicin, in some human cancers, although it typically fails to induce lasting anti-cancer immune responses in most cases. The release of HMGB1, which signals through TLR4 on neighboring cells, is a characteristic of ICD(45). Interestingly, a significant minority of Western Europeans have a TLR4 polymorphism (896A/G), which prevents HMGB1 from binding in a dominant-negative fashion(12, 45), impairing the sensing of ICD. Breast-cancer patients with this polymorphism show decreased survival after anthracycline treatment, despite a lack of differences in other major tumor characteristics. This suggests that immunogenic cell death may enhance the efficacy of conventional chemotherapy in human cancers, but only in limited fashion(45). IL-6 inhibition may be useful to sustain the limited anti-cancer immune responses normally induced by ICD and expanding our findings to additional cancer types will be of future interest.

The mechanisms by which cancer cells evade immunosurveillance are only now beginning to be fully elucidated. It is clear that these varied responses include tumor-intrinsic changes such as the mutation of tumor neo-antigens(46, 47) and alterations in antigen-processing machinery, such as the down-regulation of MHCI on the surface of cancer cells(15). Cancer cells can also exploit immunosuppressive programs that normally serve to prevent autoimmunity, such as immune-checkpoint engagement(2). Tumor co-option of the PD-1 and CTLA4 T-cell checkpoints, has been exploited clinically with inhibitors of these proteins.
and their ligands, and the success of checkpoint inhibitors suggests that the stimulation of anti-cancer immunity is a viable therapeutic proposition in humans, although one that is not always effective due to the diversity of immunosuppressive signals employed in cancer.

Paracrine signals produced in the tumor microenvironment play a major role in defining the immune context of tumors and show great potential for therapeutic manipulation(48). For example, the production of CSF-1 in the tumor microenvironment can recruit immune-suppressive macrophages that inhibit the efficacy of anti-cancer immune responses(37), microenvironmental CXCL13 production can recruit IgA-expressing plasma cells to suppress CTL responses in prostate cancer(38) and both of these recruited cell types act, at least in part, through the secretion of IL-10. Immunosuppressive cytokines, such as IL-10 can also be produced directly by cancer cells, thus suppressing T- and dendritic cell responses(2). Our data suggest that paracrine IL-6 production by cells in the microenvironment can also suppress anti-cancer immune responses and is a promising target.

IL-6 is a pleiotropic cytokine involved in the regulation of tissue repair, immune responses and the acute-phase response(25). IL-6 secretion is essential for mounting a productive immune response against certain bacterial and viral insults, but chronic IL-6 activity can also impair the generation of a robust immune response(26, 27). Many conflicting reports exist as to whether the
primary effect of IL-6 is pro- or anti-inflammatory and its ultimate impact likely depends on the context in which it is expressed(26).

IL-6 promotes T-cell proliferation, B-cell maturation, inhibits T-regulatory cell development and function and can contribute to chronic inflammation associated with inflammatory diseases such as arthritis, for which IL-6 targeting antibodies are used clinically(24). However, it also appears as if IL-6 can promote potentially immunosuppressive effects when expressed at high levels. IL-6 impairs the maturation of dendritic cells, promotes myeloid derived suppressor cell development and can activate PD-1 expression on T-cells(28, 44).

In our system, IL-6 impairs the generation of anti-leukemia immune responses. Interestingly, expression analysis of the leukemic microenvironment in untreated WT and IL6KO mice, indicates less active baseline immune activation in IL6KO mice. This conflicts with our experimental results showing elevated anti-leukemia immunity after doxorubicin treatment, although is consistent with past work demonstrating the importance of IL-6 in mounting initial immune responses(24, 27, 33, 34). One explanation for this apparent contradiction might be the inhibitory effect of a chronically active immune environment. Chronic STAT3 activity, which can occur downstream of IL-6 as well as immunosuppressive cytokines like IL-10, can impair the generation of new adaptive immune responses(49). Interestingly, we see no differences in pSTAT3 levels in IL6KO mice suggesting that differences in STAT3 activity are not a major contributor to
the increased immunity seen in our system. IL-6 may act by up-regulating immune-checkpoint proteins, impairing effective responses in settings, such as the leukemic bone marrow, where T-cell inhibitory proteins are expressed on the surface of cancer cells. We also see no major differences in dendritic cell levels or in the presence of DC activation markers between WT and IL6KO mice, but more extensive investigation of dendritic cell activation is a promising avenue for future investigation. One possibility that remains to be investigated is the potential ability of IL-6 to skew towards IL-4 producing Th2 cell development(28). While the presence of Th2 cells has a variable association with tumor outcome depending on tumor type(4), one mechanism by which cancer cells may evade anti-tumor immunity is to skew the immune response away from Th1-cell and towards Th2 cell development(28, 50). However, the exact role of IL-6 in promoting Th1 vs Th2 development remains controversial(26, 51). While IL-6 may execute its immune-suppressive properties through the multifactorial regulation of multiple processes such as T-cell exhaustion and dendritic cell activation, our data clearly demonstrate that IL-6 impairs the generation of anti-cancer immunity in response to immune-stimulating therapy and thus represents an exciting therapeutic approach to investigate in combination with other immunotherapies.

Given the many mechanisms by which cancer can evade immune surveillance(2, 14), combination therapies that block multiple immunosuppressive mechanisms will likely be essential to promote responses in the majority of tumors. An
increased repertoire of immune-stimulating agents as well as prognostic indicators of efficacy will be essential for the application and combination of these drugs. Here, we demonstrate that three immune-modulating interventions, IL-6 inhibition, doxorubicin treatment, and PDL1 blockade, each of which alone fails to promote lasting anti-leukemia immunity, achieve much more durable responses in combination. These agents and others have immense potential if combined intelligently to improve the treatment of cancer.
Supplementary Figure 1: Survival of WT CD8+ T-cell depleted or un-depleted doxorubicin treated mice. n.s, p=.0588
Supplementary Figure 2: a) Flow cytometry on peripheral blood samples from isotype control injected or T-cell depleted mice to confirm the efficacy of depletion. b) Survival of control and CD8-depleted IL6KO mice treated with doxorubicin. ** p<.05
Supplementary Figure 3: Gating strategy for the identification of T-cell subsets.
Supplementary Figure 4: RNA-sequencing bone marrow and spleen. a) Schematic outline of experiment to isolate bone marrow stromal and leukemia cells from WT and IL6KO mice for RNA-sequencing. 50,000 cells per mouse were injected and mice sacked 12 days later, bone marrow collected and mCherry+ leukemia cells sorted from the bone marrow and RNA isolated for RNA-sequencing. b) PCA on these samples can distinguish ALL and stromal cells (PC1) and WT and IL6KO conditions (PC2). One WT sample was excluded due to variability as an outlier from all other samples. c) Top 10 GO terms enriched in the set of genes contributing to PC2. d) Expression of inhibitory checkpoint proteins and T-cell co-stimulatory proteins in WT and IL6KO stromal compartments.
Supplementary Figure 5: IL10R inhibition does not improve response to doxorubicin treatment. (a) Leukemia burden 8 days after doxorubicin treatment in mice treated with IL10-receptor blocking antibody. Burden quantified by bioluminescence imaging. (b) Survival of untreated or doxorubicin-treated mice with or without IL10R-blocking antibody.
Supplementary Figure 6: Therapeutic IL-6R blockade trends towards decreased tumor burden and increased survival. (a) Leukemia burden 8 days after doxorubicin treatment in mice treated with IL-6R-blocking antibody. Burden quantified by bioluminescence imaging. (b) Survival of mice treated with doxorubicin with or without IL-6R-blocking antibody.
### Ingenuity Pathway Analysis Canonical Pathway

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**Supplementary Table 1:** Pathways enriched among top differentially regulated genes between WT and IL6KO stromal cell RNA-sequencing samples. All pathways listed are underrepresented in IL6KO samples, suggesting down-regulation.
Methods

Cell culture and transplantation

Cells were grown at 37°C, 5% CO₂, in 500mL RPMI, 50mL FBS, 10mL glutamine, 5.5mL 5mM β-ME, 5mL Pen Strep. Luciferase+ BCR-Abl+ B-ALL cells were a gift of Richard Williams(21). To make mCherry+ B-ALL cells the MSCV-mCherry retroviral vector was transfected into Phoenix cells to produce retrovirus and B-ALL cells infected in the presence of polybrene and sorted twice on an Aria3 (Beckton Dickenson) to get a pure mCherry+ population. 500,000 BCR-Abl+ B-ALL cells (mCherry+ or negative depending on the experiment) were injected via tail vein into C57B6/J mice of the appropriate genotype. Transgenic mice were obtained from Jackson Labs and bred in the SPF-animal facility in the Koch Institute at MIT. On day 8 post-injection, mice were treated with 10mg/kg doxorubicin (LC Labs) dissolved in normal saline solution via IP injection. Mice were sacrificed when moribund. For re-transplantation experiments IL6KO 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 was monitored.

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 ~15 minutes prior to imaging and mice were anaesthetized using isofluorane prior to imaging on the IVIS.
Spectrum-bioluminescence and fluorescence imaging system (Xenogen Corporation).

**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 IL6KO mice were extracted by crushing both femurs and tibias with mortar and pestle in RBC Lysing Buffer (Sigma) 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 CD3-FITC (17A2, BioLegend), CD4-APC (RM4-5, BD Biosciences), CD4-APC-Cy7 (GK1.5, BioLegend), CD8-PE-Cy7 (53-6.7, BD Biosciences), CD25-APC-Cy7 (PC61, BioLegend), CD69-PerCP-Cy5.5 (H1.2F3, BioLegend), CD11c-FITC (HL3, BD Biosciences), CD103-PerCP-Cy5.5 (2E7, BioLegend), CD86-APC (GL-1, BioLegend), MHCII-APC-Cy7 (M5/114.15.2, BioLegend), MHCII-PerCP-Cy5.5 (M5/114.15.2, BioLegend), CD11b-PE-Cy7 (M1/70, BioLegend), F4/80-APC (BM8, BioLegend), Gr-1-FITC (RB6-8C5, eBioscience), IL6R-APC (D7715A7, BioLegend), Rat IgG2b, κ isotype control (RTK4530, BioLegend), PD1-BV421 (29F.1A12, Sirigen), and PDL1-PE-Cy7 (10F.9G2, BioLegend) for one hour, washed in most experiments 3uM DAPI was added to the last wash to determine live cells and samples were analyzed.
on LSRII HTS flow cytometer (Beckton 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 ≥10ng/mL IL10, GM-CSF, IL12, IL15, VEGF, IL6, sIL6R, or IL6 + sIL6R (PeproTech) and doxorubicin (LC Labs) at 100nM, 50nM, 25nM, 15nM, 10nM, 7.5 nM, 5nM, 2.5nM, 1nM, 0.5nM, and 0nM concentrations. Cell count was obtained via flow cytometry FACS Calibur HTS (Beckton Dickinson) with propidium iodide used to exclude dead cells.

**Bone Marrow Co-culture**

Bone-marrow cells from WT and IL6KO mice were extracted as described above but without RBC lysis and extracted cells plated in leukemia cell medium and grown, with washes 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.

**pSTAT3 Stain**

Bone-marrow and splenic cells from WT and IL6KO mice were extracted as described above, fixed in 3 or 4% paraformaldehyde, stained with primary pSTAT3 (Tyr705, Cell Signaling, 1:200) or IgG isotype control (CST) at the same concentration. Donkey anti-rabbit Alexa Fluor 488 was used for secondary
antibody staining (Invitrogen, 1:1000). Cells were analyzed by flow cytometry using a LSRII HTS-II (Beckton Dickinson) and pSTAT3 levels were measured. Median FITC channel of isotype controls were subtracted from pSTAT3-stained samples to get pSTAT3 levels in a given cell population.

In Vivo T-Cell Depletion

WT and IL6KO leukemic mice were IP-injected with 200μg CD4 (GK 1.5) and 200μg CD8 (GK 1.5) depletion antibodies (gift from Tyler Jacks) dissolved in sterile PBS on days 3 and 4 post B-ALL transplantation and then every 3 days thereafter. Rat IgG2b (LTF-2, BioXCell) was used as an isotype control.

Mouse Antibody Treatment

IL6R Ab (15A7, BioXCell) – starting 5 days after leukemia transplantation, every other day, 500μg/mouse. IL10R Ab (1B1.3A, BioXCell) – every 3 days, 300μg/mouse. PDL1 nanobody (gift of Hidde Ploegh) – twice weekly, 100μg/mouse

References


Chapter 4

Discussion
The treatment of disseminated cancer remains a challenge. Resistance to current therapeutic regimens frequently arises through both cancer-intrinsic and extrinsic mechanisms. Intrinsic resistance can occur through mutation of drug targets, up-regulation of drug export pumps or DNA-repair machinery, and acquisition of apoptotic defects, among other mechanisms(1). The regulation of drug resistance by extrinsic factors, such as the tumor microenvironment, is less well understood. In this thesis, I have used syngeneic immunocompetent mouse models of cancer to investigate the microenvironmental determinants of chemotherapeutic efficacy. In these animal models, transplanted cancer cells grow in a relevant tissue microenvironment, enabling the investigation of microenvironmental paracrine signals and their role in therapy resistance.

I show that in B-cell lymphoma, endothelial cells create a chemoresistant niche through the secretion of IL-6, the secretion of which is acutely induced by therapy before being restrained to preserve tissue homeostasis. IL-6 similarly promotes resistance in pre-B-cell acute lymphoblastic leukemia, although through distinct mechanisms involving the inhibition of anti-leukemia immunity. The B-cell lymphomas studied in this thesis express the IL-6 receptor (data not shown) and IL-6 directly promotes resistance to therapy in these cells through the up-regulation of anti-apoptotic proteins such as Bcl-XL(2). In contrast, and like most human B-ALL, the leukemia cells studied here do not express the IL-6R. In this context, IL-6 inhibits the therapy-induced generation of anti-leukemia immunity.
This research emphasizes the considerable contextual diversity by which the microenvironment can control therapeutic response and these findings suggest that the inhibition of IL-6 in combination with chemotherapy is a promising treatment approach in diverse contexts. Below, I will further discuss some of the implications of this work and examine additional areas in which these findings may find utility.

**Damage-induced paracrine signaling in chemoresistance**

While it is widely appreciated that tumor development and progression involve interactions between cancer cells and their associated stroma(3), it has only become clear more recently that these interactions also play a key role in therapeutic response. Systemic therapy causes damage throughout the body and this damage in non-malignant cells can activate paracrine stress responses that preserve tissue homeostasis by promoting tissue repair and regeneration(4, 5). Our work indicates that the release of IL-6 from endothelial cells as part of an acute damage response is co-opted by tumor cells to promote resistance to therapy. Here, IL-6 is produced acutely downstream of reactive oxygen species-mediated stress kinase signaling, a response mechanism to diverse stressful stimuli. We also find that chemotherapy-induced damage activates cellular senescence in endothelial cells, linking our observations to a body of literature that implicates cellular damage responses in diverse pathology(6).
Cellular senescence is induced in response to multiple genotoxic stresses, including telomere shortening, oncogene activation and the induction of DNA-damage by external stimuli, such as cancer therapy(7). Senescence leads to a robust, chronic secretory response, the senescence-associated secretory phenotype (SASP), in damaged cells that has been implicated in the control of tissue homeostasis(8), but also the promotion of chronic inflammation, cancer- and age-related pathology(6). Secretory responses are normally tightly controlled across biology as uncontrolled inflammation can lead to tissue destruction(9). However, it has remained largely unclear how the existence of a senescence secretory response can be reconciled with the requirement for tight control of cytokine secretion to avoid tissue damage and the relative absence of chronic inflammation following systemic cancer therapy. One mechanism by which senescence-associated inflammation is restrained is the clearance of senescent cells by immune surveillance(10). In this context, the SASP promotes the restoration of homeostasis by activating an immune response that clears damaged cells(11, 12). However, senescent cells accumulate with aging and the clearance of senescent cells is not fully efficient. Interestingly, this accumulation of senescent cells does not appear to be uniformly associated with a secretory response *in vivo*(13), suggesting that senescence-associated inflammation is restrained by additional, but unclear, mechanisms.

We have found that the SASP can be restrained by the suppression of PI3K/Akt/mTOR signaling, which serves as a switch that can toggle senescent
cells between non-secretory and pro-inflammatory states. It will be interesting to further investigate the relevance of this pathway to the SASP in additional cell types and senescence stimuli in vivo. Encouragingly, recent work by multiple labs indicates that mTOR is essential for the regulation of the SASP, although through conflicting mechanisms (14, 15). Thus, mTOR activity may be a broadly relevant evolutionary approach to regulate chronic tissue inflammation in response to damage. However, relatively little is known about senescence secretory responses in human tissues, how widespread they are, and how they are regulated to restore homeostasis. It will be important to confirm these findings and others in the senescence literature in the context of human tissue.

Intriguingly, the lifespan-extending drug rapamycin, which inhibits mTOR activity, prevents senescence-associated cytokine production. Senescent cells accumulate during aging and have been suggested to contribute to age-associated tissue dysfunction (6). Thus, the ability of rapamycin to inhibit the SASP may be one mechanism by which it promotes more healthy aging.

Survivors of cancer suffer from increased risk of diverse vascular pathology in the years following treatment: they have elevated risk of atherosclerosis, coronary artery disease, and cerebrovascular events, suggesting that endothelial damage contributes to the increased mortality seen in cancer survivors (16, 17). This treatment-related pathology is a growing clinical concern as more and more cancer patients live for many years after treatment. However, it remains unclear
whether the absence of a SASP in endothelial cells would be expected to contribute to or restrain this pathology. The clearance of senescent cells can promote tissue regeneration, and may be impaired in the absence of a SASP. However, the presence of endothelial inflammation can also promote tissue dysfunction and the endothelium is an important gatekeeper of tissue infiltration by immune cells, suggesting that endothelial inflammation in particular may need to be under tight control(18). Investigation of the basic biology of these long-term therapeutic toxicities will be important in the application of approaches, such as senescent cell clearance(19), in the prevention of disease.

**Targeting IL-6**

The work in this thesis and work from many others labs has demonstrated a role for IL-6 in tumor progression and chemoresistance(20). The inhibition of IL-6 is thus a promising approach to improve the treatment of cancer. We believe that 3 broad strategies to inhibit paracrine factor production are most promising for therapeutic intervention: 1. Direct inhibition of paracrine proteins or their receptors. 2. Blockade of downstream signaling activated by these receptors. 3. Inhibition of paracrine protein release from cells. The FDA-approved drugs siltuximab and tocilizumab inhibit IL-6 and the IL-6R respectively and ruxolitinib, which inhibits JAK/STAT signaling downstream of IL-6, is approved for use in myelofibrosis. However, less effort has gone into the identification of inhibitors of paracrine IL-6 release. Here, we show that the histone deacetylase inhibitor SAHA can inhibit IL-6 release and that it improves the efficacy of doxorubicin in
IL-6 responsive B-cell lymphoma. The identification of additional drugs that block cytokine release may be a useful, but understudied therapeutic approach, circumventing the compensatory up-regulation of paracrine proteins that can result from their direct inhibition(21).

In humans, IL-6 inhibitors are used to treat inflammatory arthritis and Castleman’s syndrome (a B-cell lymphoproliferative disorder), but these agents have had limited efficacy as monotherapy for cancer(20). Our data indicate that the full efficacy of IL-6 blockade will only be achieved when it is inhibited in combination with other agents and we propose further investigation of IL-6-blocking therapies in combination with chemo- and immuno-therapy. Improved IL-6 inhibitors are currently in clinical development and the application of existing agents as part of combination regimens as well as use of novel IL-6 inhibitors warrants further pre-clinical and clinical investigation.

**IL-6 regulation of anti-cancer immunity**

The generation of anti-cancer immune responses requires the interaction of multiple cell types in a context that potentiates both the initiation and maintenance of immune recognition. Monotherapy with immune-stimulating agents is unlikely to promote lasting immunity in most contexts as the generation of productive immune responses requires both the initial induction and subsequent persistence of an immune response, which often involve distinct cell types and regulation(22). Combination immunotherapy with agents that stimulate
the initial generation of anti-cancer immunity and those that enable its persistence is thus a promising approach to overcome the frequent clinical failure of immune-stimulating agents. Immunogenic chemotherapy promotes the induction of an initial anti-tumor immune response (23). It stimulates the release of damage signals from cancer cells leading to increased dendritic cell activity and influx into the tumor microenvironment (24). However, immunogenic chemotherapy typically fails to sustain anti-tumor immunity and this failure may relate to its inability to promote the persistence of pro-inflammatory anti-cancer signals.

We show that the immunogenic chemotherapeutic doxorubicin can promote robust and persistent anti-leukemia immunity when used in the absence of microenvironmental IL-6. Typically studied more for its pro-inflammatory properties, IL-6 promotes T-cell activation, immunoglobulin production by B-cells and the acute-phase response (25), but has also been suggested to restrain the immune responses in some contexts through a number of mechanisms. However, while IL-6 can elicit some immune-suppressive effects, relatively little work has demonstrated inhibition of full immune responses by IL-6 in vivo. We find that IL-6 restrains anti-cancer immunity induced by immunogenic chemotherapy in B-ALL, impairing doxorubicin efficacy. This suggests that the inhibition of IL-6 is a promising approach to improve cancer immunotherapy. Below I will discuss some of the mechanisms by which IL-6 may impair immunity.
as evidenced by work we have done in this system, although this work is still ongoing.

IL-6 has been shown to promote T-cell IL-10 production and to induce antagonists of IL-1 and TNF-receptors during the acute phase of inflammation, promoting its resolution(21). In our system, IL-10R blockade does not increase survival of leukemic mice after doxorubicin treatment and initial expression analysis does not show changes in IL-1 or TNF-antagonist expression between WT and IL6KO mice. In contrast to our experimental evidence, pathway analysis of expression data from WT and IL6KO bone marrow stromal cells indicated decreased, not increased, immune activation in IL6KO mice. Interestingly, however, this baseline suppression of pro-inflammatory genes is also associated with lower expression of immune-inhibitory checkpoint proteins, high levels of which impair the generation of anti-cancer immunity(22). Checkpoint protein expression is often induced with immune stimulation and we hypothesize that IL-6 may inhibit anti-leukemia immunity by inducing baseline immune activation, with concomitant increases in immune-inhibitory protein expression. In the tumor microenvironment, which has high expression of immune-checkpoint ligands and other immune-suppressive proteins by cancer cells, these differences in checkpoint and other inhibitory protein expression may impair the generation of lasting immune responses to the chemotherapy-induced acute immunogenic stimulus, preventing therapy-induced immunity.
Our understanding of the mechanisms by which IL-6 mediates its anti-cancer effects in this system are not complete. IL-6 can impair the generation of an initial immunologic stimulus by inhibiting antigen-presenting dendritic cell activation in some contexts (26) and has also been suggested to increase myeloid-derived suppressor cells, which also inhibit anti-cancer immunity (27). However, we see no major differences in myeloid cell numbers or in dendritic cell activation between WT and IL6KO mice in this system, although further characterization will be necessary to rule out an effect. Additionally, IL-6 may prevent anti-cancer immunity by skewing T cell development towards a cancer-promoting, Th2 phenotype (28), although the ability of IL-6 to skew T-cells towards a Th2 state and the role of Th2 cells in cancer immune responses remains controversial (21, 29). We do observe lower IL-4, IL-5, and IL-13 expression in IL6KO mice (data not shown), cytokines that are produced by Th2 cells, suggesting that Th2 responses may be impaired in IL6KO mice, warranting further investigation. Interestingly, many of these immunosuppressive effects of IL-6 have been shown to be mediated through STAT3, and we see no major differences in p-STAT3 between WT and IL6KO mice in the bone marrow, suggesting that IL-6 can also mediate immune-suppression through other signaling pathways.

Immunogenic cell death is thought to promote anti-cancer immunity in large part due to its induction of dendritic cells and their recruitment to sites of damage. Interestingly, we observe both dendritic and T-cell influx into the bone marrow
after treatment. This response appears to be tissue specific as we do not see changes in T-cell numbers in the spleen, despite the presence of cancer cells in that tissue. T-cells move into the bone marrow within 48hrs of treatment, but it is unclear whether this is a direct result of doxorubicin-mediated damage or occurs indirectly through the recruitment of other immune cell subsets, such as dendritic cells, that produce T-cell chemoattractants(30).

We have previously shown that the bone marrow creates an immunosuppressive microenvironment and that this can impair the response to antibody-based therapy(31). In those experiments, which were performed using NSG mice, which lack an adaptive immune system, to study resistance to therapeutic-antibody treatment, cyclophosphamide induces macrophage influx into the bone marrow through the activation of a secretory response in cancer cells thus improving the response to antibody-based therapy. Here, we show that doxorubicin induces T- and dendritic-cell influx into the bone marrow, transforming a previously immune exclusionary microenvironment and promoting effective anti-cancer immunity in IL6KO mice.

IL-6 has complex and varied effects on cancer biology. While widely characterized as a key pro-inflammatory cytokine that promotes innate and adaptive immune responses, we demonstrate that IL-6 can also inhibit immune activity. Excitingly, we are able to extend these findings to immune-checkpoint blockade with Fc-domain conjugated nanobodies inhibiting PD-L1. PD-L1
blockade has relatively limited impact in leukemic WT mice as monotherapy, however, in IL6KO mice, PD-L1 blockade cures the majority of mice, suggesting that the inhibition of IL-6 could be a broadly relevant approach to improve immune responses to cancer. Additional investigation of the impact IL-6 has on specific immune cell subsets will be necessary to more completely understand the mechanisms by which IL-6 regulates the responses we observe in this system. *In vitro* co-culture experiments in combination with *in vivo* profiling of immune activation and exhaustion promises to further inform the biology underlying this response.

**Endothelial paracrine signaling in metastatic-cell chemoresistance**

The treatment of disseminated cancer remains a major challenge and as many as 90% of patients with metastatic or micrometastatic disease fail treatment due to chemoresistance(1). Significant work has gone into the study of the metastatic cascade and the elucidation of the molecular determinants of the metastatic process(32). Some of this research is predicated on the idea that the identification of metastatic mediators might enable the inhibition of metastasis and aid in the treatment of cancer. However, the majority of patients with incurable cancer have metastases at the time of diagnosis and current chemotherapies do not effectively treat metastatic disease. Relatively little is known about metastasis-specific resistance to chemotherapy. The identification of the mechanisms of metastatic-cell resistance to therapy may represent a more widely relevant approach to improve the treatment of disseminated disease.
Of relevance to the work presented in this thesis is the metastatic microenvironment. Metastasis occurs in diverse tissue environments and it has long been suggested that these tissues must provide a fertile 'soil' for metastasis to form(33). This fertile metastatic 'soil' may also facilitate some of the chemoresistance seen in these lesions. For malignancies that spread hematogenously, micrometastases initially form in close proximity to the vasculature. In some cases, early metastases have been observed to grow along the vasculature, suggesting that they may receive nourishment from neighboring vascular or perivascular cells(34).

Our findings indicate that endothelial cells promote the formation of a chemoprotective niche through the secretion of paracrine signals sensed by neighboring cells. It will be interesting to further investigate whether chemoresistance in micrometastases also derives from supportive signals produced by the endothelial cells and whether the inhibition of endothelial paracrine signaling can improve the treatment of micrometastatic disease. Indeed, preliminary work I have performed in the lab suggests that endothelial paracrine signals can promote resistance to therapy in mouse models of pancreatic cancer micrometastasis (data not shown), suggesting that this is a promising avenue for further investigation. Indeed, recent work has suggested that endothelial TNF can promote chemoresistance in metastatic cells through the induction of cancer-cell CXCL1 release and the recruitment of
chemoresistance-promoting myeloid cells(35). Endothelial cell paracrine signaling has also been implicated in the promotion of metastatic dormancy(36), cancer stemness(37), and chemoresistance(38) by other labs and the impact that therapy-induced changes in endothelial paracrine signaling has on this biology will be of interest to study further.

**Future approaches to understand the tumor microenvironment**

The tumor microenvironment is enormously complex, consisting of 10’s if not 100’s of distinct cell types that produce an enormous number of soluble, cell surface and matrix proteins that impact the fitness of surrounding cancer cells. Investigation of the role that specific cell types and proteins play in cancer on a gene-by-gene basis is an important, but laborious, way to investigate the impact of the microenvironment on cancer biology. How then to better understand this enormous complexity? The development of microarray and then RNA-sequencing approaches to study mRNA transcript levels have enabled some of the first unbiased profiles of gene expression in bulk tumor tissue. This work has identified microenvironment-derived gene expression signatures that correlate with survival and suggested genes that warrant further investigation for a causal role in cancer biology(39, 40). The development of techniques that enable transcriptome profiling in single-cells has immense potential to more fully characterize the individual cell types seen in the tumor microenvironment in an unbiased manner(41, 42). New technologies enable gene-expression profiling in thousands of individual cells from the same tissue and have already led to the
identification of novel cell types in some tissues (42). The application of this approach to the tumor microenvironment promises to provide an unbiased map of all of the cell types residing in the tumor microenvironment and the genes expressed in those cells. The application of this type of profiling also shows promise to reveal changes that occur in the microenvironment during the course of treatment and to identify microenvironmental cell types and gene expression profiles that have prognostic relevance. This promises to provide, for the first time, a more comprehensive look at the cell types, and genes they express, in the tumor microenvironment.

The combination of unbiased profiling approaches such as this with pooled in vivo Crispr and shRNA-based screening approaches to interrogate the functional role of genes implicated in tumor progression and chemoresistance should enable more rapid identification of genes with direct influence on cancer. Genes not amenable to these pooled approaches, such as secreted factors or proteins that act indirectly on other microenvironment cell types, will require more labor-intensive approaches more similar to those employed in this thesis.

The presence and role of acute secretory responses like those we describe here, remains undetermined in human disease in part due to the difficulty studying acute processes in human tissue samples. It is rare for biopsies to be taken in the immediate aftermath of chemotherapy treatment and many studies of the tumor microenvironment examine the state of the microenvironment in untreated
samples. Indeed, relatively little work has closely examined changes that occur in the tumor microenvironment before and after treatment in the same patient. Given, the major impact that cancer therapy has on non-malignant cells, further investigation of these changes in human cancers will be essential to further understand this important aspect of cancer biology.

Conclusions
The treatment of cancer remains a major challenge due to the difficulty of managing disseminated disease. The work presented in this thesis demonstrates the impact that the tumor microenvironment has on the efficacy of cancer therapy and reveals some potential settings in which manipulation of IL-6 promises to improve the treatment of cancer, highlighting the potential to target paracrine signaling for improved therapeutic outcome in cancer. Understanding the cell types, signals and mechanisms by which the microenvironment facilitates the emergence of resistance to therapy will be essential for the development of therapies that exploit these responses to better treat cancer.

References


